Activation of angiotensin II type I receptor promotes protein kinase C translocation and cell proliferation in human cultured breast epithelial cells

S Greco, A Muscella, M G Elia, P Salvatore¹, C Storelli
and S Marsigliante

Laboratorio di Fisiologia, Dipartimento di Scienze e Tecnologie Biologiche e Ambientali, Università di Lecce, Via Provinciale per Monteroni,
73100 Lecce, Italy

¹Reperto di Chirurgia Generale, AO 'Vito Fazzi', Via F Muratore, 73100 Lecce, Italy

(Requests for offprints should be addressed to S Marsigliante; Email: smarsi@ilenic.unile.it)

Abstract

The effect of angiotensin II (Ang II) on Ca^{2+} signalling in human primary cultured breast epithelial cells was investigated by using fura-2 as the Ca^{2+} probe. Ang II (0.1–1000 nM) induced an intracellular free calcium ($[\text{Ca}^{2+}]_i$) transient peak which was unchanged by external Ca^{2+} removal. In Ca^{2+} -free medium pretreatment with thapsigargin abolished Ang II-induced Ca^{2+} release. Suppression of 1,4,5-inositol trisphosphate formation by U73122, a phospholipase C inhibitor, blocked the Ang II-induced Ca^{2+} response.

Losartan (DuP753), an inhibitor of Ang II type I receptor (AT1), decreased the $[\text{Ca}^{2+}]_i$ increase evoked by Ang II, while CGP4221A, an inhibitor of Ang II type II receptor (AT2) did not. AT1 desensitisation was demonstrated with respect to the Ca^{2+} response after subsequent exposure of cells to Ang II and also after pretreatment for

25 min with 1000 nM phorbol 12-myristate 13-acetate. Staurosporine, an inhibitor of protein kinases C (PKC), inhibited the AT1 desensitisation.

Epithelial breast cells expressed PKC- α , - β 1, - δ and - ζ isoforms, and Ang II provoked translocation from the cytosol to the membranes of PKC- α , - β 1, and - δ (but not - ζ). Ang II was also able to stimulate cell proliferation in a dose-dependent manner; this effect was blocked by Gö 6976, a specific inhibitor of PKC- α and - β 1, the Ca^{2+} -dependent isoforms.

The main conclusion of this study is that the Ang II signalling mechanism in breast epithelial cells is based on the elevation of $[\text{Ca}^{2+}]_i$ released from intracellular stores through AT1 activation. In addition, Ang II stimulates cell proliferation by the activation of PKC isoforms.

Journal of Endocrinology (2002) **174**, 205–214

Introduction

Angiotensin II (Ang II) has a wide spectrum of human and animal target tissues, the human breast epithelial cells included (Vinson *et al.* 1995, Inwang *et al.* 1997, Tahmasebi *et al.* 1998). In most tissues, the effects ascribed to Ang II are mediated via two different subtypes of its receptor, the AT1 and the AT2. Several lines of evidence suggest that the major physiological functions of AT1 are expressed through Gq-induced activation of phospholipase C (PLC) followed by Ca^{2+} signalling. Moreover, an increase in the intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) activates numerous intracellular proteins, several of which belong to the family of Ca^{2+} - and phospholipid-dependent protein kinases (PKC) (Liu & Heckman 1998).

In several tissue types Ang II has a growth factor-like effect linked to the phosphoinositide/ Ca^{2+} signalling system (Lundergan *et al.* 1999, Bataller *et al.* 2000, Kuwahara *et al.* 2000, Hou *et al.* 2000, Shen *et al.* 2001)

and to the increased expression of several early response genes including *c-fos*, *c-jun*, and *c-myc* (Griendling *et al.* 1997).

Since the transduction pathways of Ang II in human breast epithelial cells have not yet been studied, the aim of this work was to test the possible involvement of the $[\text{Ca}^{2+}]_i$ transient peak in the AT1 signalling pathways in human primary cultured epithelial breast cells. We also investigated the translocation to the membrane of some of the PKC isoforms after Ang II treatment and the effects of the Ang II signalling system on cellular proliferation.

Materials and Methods

Materials

RPMI 1640 medium, antibiotics, glutamine and foetal bovine serum (FBS) were purchased from Euroclone (Paignton, Devon, UK). Fura 2-AM, thapsigargin (TG)

Table 1 Ang II-induced $\Delta[Ca^{2+}]_i$ and oestrogen and progesterone receptor (ER and PgR) concentrations at different passages. The cells used in these experiments were obtained from four different individuals and grown separately

	$\Delta[Ca^{2+}]_i$ (nM)	ER (fmol/mg protein)	PgR (fmol/mg protein)
Number of culture passages			
Passage 1	108 ± 5 ^a	48 ± 3 ^a	67 ± 3 ^a
Passage 2	106 ± 6 ^a	43 ± 6 ^a	64 ± 5 ^a
Passage 3	101 ± 5 ^a	45 ± 2 ^a	66 ± 3 ^a
Passage 4	100 ± 3 ^a	44 ± 3 ^a	63 ± 2 ^a
Passage 5	93 ± 3 ^b	46 ± 4 ^a	70 ± 4 ^a
Passage 6	83 ± 2 ^c	53 ± 6 ^b	72 ± 5 ^b

Different letter indicate statistical significance by Fisher's PLSD test.

and Pluronic F-127 were purchased from Molecular Probes (Leiden, The Netherlands); losartan (DuP 753) from Du Pont Merck (Wilmington, DE, USA). SV Total RNA Isolation System, Moloney murine leukaemia virus (M-MLV) RT and Taq polymerase were purchased from Promega (Madison, WI, USA); AT1 primers were purchased from Celbio (Pero, Milano, Italy). Mouse monoclonal antibody for cytocheratin 19 was purchased from Chemicon International (Temecula, CA, USA) and all other reagents were from Sigma (Milano, Italy). PKC antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Gö 6976 was obtained from Calbiochem (La Jolla, CA, USA).

Primary cell culture of breast tissues

Four breast tissues were obtained from reductive mammo-plasty and portions of tissue were placed into transport medium and disaggregated immediately or after storage at 4 °C for less than 6 h. Transport medium contained RPMI 1640 medium, 0.05 mg/ml nystatin, 0.0025 mg/ml amphotericin B, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine, 10% FBS and 0.010 mg/ml insulin.

Adipose material was removed and the tissue minced using a BP 10 blade scalpel in PBS pH 7.4 under sterile conditions and digested in transport medium containing 1 mg/ml collagenase type I and 100 U/ml hyaluronidase overnight at 37 °C on a rotary platform (200 r.p.m.). When digestion was completed, tissue suspension was centrifuged at 100 g for 5 min and the pellets were resuspended in sterile PBS pH 7.4 and pushed through three stainless steel screens (100, 60, and 50 µm mesh size respectively). The dispersed cell suspensions were centri-fuged at 100 g for 5 min and the pellets were resuspended in 20% FBS growth medium (RPMI 1640 medium, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine, 0.005 mg/ml insulin, 5 ng/ml epidermal

growth factor, 0.5 µg/ml hydrocortisone, 5 µg/ml trans-ferrin, 0.1 µM isoproterenol, 0.01 µM ethanolamine, 0.01 µM *o*-phosphoethanolamine) and seeded into culture flasks.

After the first passage, cell cultures were maintained at 37 °C in a humidified environment containing 5% CO₂ for up to 7 passages in 10% FBS growth medium to avoid fibroblast contamination. The cultured cells exhibited the characteristic features of epithelial cells: a positive immunocytochemical staining for cytocheratin 19 (data not shown) and expression of oestrogen and progesterone receptors (ER and PgR respectively) (Table 1).

Enzyme immunoassay (EIA) of ER and PgR

ER-EIA and PgR-EIA (Abbott, Chicago, IL, USA) were carried out in accordance with the manufacturer's instructions.

Measurement of $[Ca^{2+}]_i$

Growth medium was changed to fresh FBS-free culture medium for 18 h and then the cells were harvested by gentle trypsinisation. Cells were loaded with 5 µM Fura 2-AM for 45 min at 37 °C in HEPES-buffered Krebs-Ringer solution (KRH: 140 mM NaCl, 5.0 mM KCl, 1.0 mM MgCl₂, 2.0 mM CaCl₂, 6.0 mM glucose and 10 mM 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 × 10⁶ cells/ml and incubated for 20 min at room temperature to ensure complete de-esterification 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,

Figure 1 Agarose gel electrophoresis of RT-PCR products revealed the specific transcripts for angiotensin II type 1 receptor (AT1) in cultured normal human breast epithelial cells at passages 1, 2, 3 and 4. β -Actin levels normalised the amount of cDNA template used in each PCR reaction.

with a chopper interval of 0.5 s, and the emission monochromator was set at 510 nm. $[Ca^{2+}]_i$ was calculated in accordance with the equation of Grynkiewicz *et al.* (1985), using the software Spectra Manager provided by JASCO.

RT-PCR

Total RNA was extracted from cultured normal and cancerous epithelial breast cells obtained from three patients using the SV Total RNA Isolation System according to the manufacturer's instructions. For RT-PCR 1 μ g 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 μ g and 15 U M-MLV RT.

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 μ M specific primers and 2.5 U Taq polymerase. 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'-GGAAACAGCTTGGTGGTG-3' and 5'-GCACAATCGCCATAATTATCC-3' which correspond to sense and anti-sense, respectively, of bases 133–150 and 719–739 in the human AT1 sequence. 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. The RT samples were also used to generate β -actin PCR products and their amount was considered as internal controls.

Figure 2 (A) $[Ca^{2+}]_i$ transient peak evoked by increasing concentrations of angiotensin II (Ang II) in cultured epithelial breast cells. Letters (a–e) indicate Ang II concentrations (0.1, 1.0, 10, 100, 1000 nM). Each tracing is representative of five separate experiments performed on cells cultured from four different individuals. The arrow indicates Ang II administration. (B) $[Ca^{2+}]_i$ transient peak evoked by different concentrations of Ang II expressed as the increment above the basal level ($\Delta[Ca^{2+}]_i$). The dose-dependent curve is representative of five separate experiments performed on cells cultured from four different individuals. Different letters indicate statistical differences by Fisher's PLSD test (ANOVA factorial: $P < 0.0001$).

Subcellular fractionation

Cells in flasks were incubated with 100 and 1000 nM Ang II and with 1000 nM phorbol 12-myristate 13-acetate (PMA) in RPMI medium without FBS for 5, 10, 25 and 60 min at 37 °C; cells incubated with medium only were used as controls. The stimulation was stopped by transferring the flasks on ice. The cells were extracted with lysis buffer: 50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM DTT, 0.25 M sucrose, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin and sonicated on ice (3×10 s cycles). The mixture was centrifuged for 10 min at 800 g and the supernatant was saved and centrifuged at 100 000 g for 1 h; the supernatant was taken as the cytosol fraction. The pellet was resuspended in lysis buffer plus 1% Triton X-100 and centrifuged as before; the supernatant was collected as the membrane fraction. An aliquot of cellular lysate of the control was used to quantify the total

Table 2 $[Ca^{2+}]_i$ increase above basal level ($\Delta[Ca^{2+}]_i$) in normal epithelial breast cells in primary culture in different experimental conditions

		$\Delta[Ca^{2+}]_i$ (nM)	Student's <i>t</i> -test
A	Buffer with $CaCl_2$ + 100 nM Ang II	107 ± 5	
B	Buffer without $CaCl_2$ + 100 nM Ang II	117 ± 6	A vs B: <i>P</i> >0.05
C	Buffer with $CaCl_2$ + 1 µM nifedipine + 100 nM Ang II	116 ± 10	A vs C: <i>P</i> >0.05
D	Buffer with $CaCl_2$ + 1 µM TG	130 ± 29	A vs D: <i>P</i> >0.05
E	Buffer with $CaCl_2$ + 1 µM TG + 100 nM Ang II	15 ± 3.1	A vs E: <i>P</i> >0.005

TG, thapsigargin

Figure 3 Characterisation of receptor type involved in Ang II Ca^{2+} signalling. Cultured epithelial breast cells were treated with or without different concentrations of either AT1 (losartan) or AT2 (CGP42113A) inhibitors before adding 100 nM Ang II. Different letters indicate statistical differences by Fisher's PLSD test (ANOVA factorial: *P*<0.0001). The histogram is representative of five separate experiments performed on cells cultured from four different individuals.

expression of PKC isozymes. Protein concentration was measured by the Lowry protein assay.

Immunoblotting

An equal amount of protein was solubilized in sample buffer by boiling for 5 min and subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by electrotransfer onto a PVDF membrane (Amersham Pharmacia Biotech, Amersham, Bucks, UK). We used the monoclonal antibodies against PKC- α , - β 1, - δ and - ζ isozymes and the dilutions of the antibodies were 1:5000 for PKC- α , and 1:2000 for the others. The filter was incubated with the appropriate primary antibody and then with peroxidase–conjugated secondary antibodies. Proteins were detected using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech), and

Figure 4 Desensitisation of AT1 in cultured epithelial breast cells. (A) Calcium mobilisation in response to subsequent maximal Ang II stimulation (1000 nM) and 0.5% FBS. Each tracing is representative of five separate experiments of cells cultured from four different individuals. (B) Time course of phorbol 12-myristate 13-acetate (PMA)-induced inhibition of Ang II-induced $\Delta[Ca^{2+}]_i$. Cultured epithelial cells were incubated with 1000 nM PMA for 0, 5, 25 and 60 min before exposure to 100 nM Ang II. Different letters indicate statistical differences by Fisher's PLSD test (ANOVA factorial: *P*<0.0001). The histogram is representative of five separate experiments of cells cultured from four different individuals. (C) Effect of staurosporine on AT1 desensitisation. Calcium mobilisation in response to subsequent maximal Ang II stimulation (1000 nM) in cells pretreated with 1000 nM staurosporine for 15 min. Each tracing is representative of five separate experiments of cells cultured from four different individuals.

Figure 5 Effect of U73122, a phospholipase C (PLC) inhibitor, on the Ang II-induced $[Ca^{2+}]_i$ transient peak. Cultured epithelial breast cells were treated with 100 nM Ang II only and with different doses of U73122 before adding 100 nM Ang II. Different letters indicate statistical differences by Fisher's PLSD test (ANOVA factorial: $P < 0.0001$). The histogram is representative of cells cultured from four different individuals.

Figure 6 Densitometric analysis of protein kinase C (PKC)-α, -β1, -δ and -ζ isozymes in cellular lysates of breast cells. Different letters indicate statistical differences by Fisher's PLSD test ($P < 0.0001$). The histogram is representative of four different individuals.

semi-quantitative analysis of PKC isozyme bands was performed by scanning densitometry using the NIH Image 1.62 software.

Proliferation assay

Mammary cells were seeded at a density of 1.5×10^3 cells/well in 96-well plates in RPMI growth medium

with 10% FBS and incubated overnight at 37 °C in a humidified environment containing 5% CO₂ to allow the adherence. The medium was changed to FBS-free growth medium for 12–18 h to induce quiescence. Ang II solution was diluted in FBS-free growth medium. Increasing concentrations of Ang II (from 0.1 to 10 000 nM) or losartan (from 0.1 to 10 000 nM) were added to each well, except for one strip, used as control, and plates were incubated for 24 h.

Cell proliferation was assessed using the spectrophotometric MTT assay (Mosmann 1983). The MTT solution (5 mg/ml) was added to each well and incubated for 4 h at 37 °C. The medium was aspirated and the purple crystals of formazan formed were dissolved by the addition of isopropanol/HCl 0.04 M. Absorbances were measured on a spectrophotometer at 570 nm against blank (cells without MTT solution). The absorbances of the cells treated with Ang II and losartan were transformed to percentage with respect to the relative controls.

Statistics

Data are expressed as the means $\pm$ S.D. Differences between multiple groups were tested using analysis of the variance (ANOVA factorial) and checked for significance using the Fisher's protected least significant difference (PLSD) test.

Results

Ang II-induced $[Ca^{2+}]_i$ increase in breast cells in primary culture

By RT-PCR analysis we found that freshly seeded human breast epithelial cells expressed the AT1 receptor mRNA, and such mRNA was still retained at passage 4 without significant variations (Fig. 1).

Ang II induces an $[Ca^{2+}]_i$ spike response in a dose-dependent manner (ANOVA factorial: $P < 0.0001$), showing after 2–5 s the maximal increase at 100 nM Ang II (200 ± 20 nM; basal = 93 ± 11 nM) and a subsequent decay to resting levels after 63 ± 2 s (Fig. 2).

We used, for both fluorimetric and Western blotting experiments, cultured breast epithelial cells at passages 2 and 3 as we found that the Ang II-induced $[Ca^{2+}]_i$ increase, and ER and PgR concentrations, specific markers of the epithelial source of the breast cells, do not change significantly until the fourth culture passages (Table 1).

In order to determine the participation of plasma membrane Ca^{2+} channels in the effects of Ang II, cells were challenged with 100 nM Ang II both in Ca^{2+} -free KRH medium, with 2 mM MgCl₂ instead of 2 mM CaCl₂, and in the presence of nifedipine, an L-type membrane channel blocker. Neither Ca^{2+} removal nor

Figure 7 The translocation from cytosol to cellular membrane of PKC- α , - β 1, - δ and - ζ isozymes evoked by 100 and 1000 nM Ang II and by 1000 nM PMA incubated for 5, 10, 25 and 60 min. Results are expressed as percentage of PKC signals corresponding to bands from membranes of control cells (treated with medium only) (100%). The bars are representative of cells cultured from four different individuals. Different letters indicate statistical differences by Fisher's PLSD test $P < 0.0001$ for α , β , and δ and $P > 0.05$ for ζ .

10-min pretreatment with 1 μ M nifedipine attenuates Ang II-induced $\Delta[\text{Ca}^{2+}]_i$, indicating that extracellular Ca^{2+} entry is not involved (Table 2, letters B and C respectively). Pretreatment of cells for 5 min with 1 μ M thapsigargin, which depletes Ca^{2+} stores, abolishes the Ang II-induced $\Delta[\text{Ca}^{2+}]_i$, suggesting that Ca^{2+} is released from intracellular stores (Table 2, letter E).

AT1 receptor is involved in Ang II-induced $[\text{Ca}^{2+}]_i$ increase

In order to characterise the Ang II receptor involved in $[\text{Ca}^{2+}]_i$ increase, we incubated the cells for 5 min, before Ang II treatment, with increasing concentrations of losartan and CGP42113A (0.1, 10 and 1000 nM), specific inhibitors of AT1 and AT2 respectively. Only losartan reduces the Ang II-evoked $\Delta[\text{Ca}^{2+}]_i$ in a dose-dependent manner (ANOVA factorial: $P < 0.0001$) (Fig. 3), indicating that the Ang II-evoked Ca^{2+} signalling is induced by AT1.

The AT1 inhibitor, losartan, is still able to decrease the Ang II-induced $[\text{Ca}^{2+}]_i$ transient peak until the fourth passage (data not shown).

Desensitisation of AT1

Following initial exposure to 100 nM Ang II, the phase of Ca^{2+} response was allowed to proceed for 4 min and reach resting level, then a second maximal dose of Ang II (1000 nM) was added. As can be seen in Fig. 4A no further peaks of Ca^{2+} mobilisation are observed in response to Ang II. However, subsequent addition of 0.5% FBS provokes an $[\text{Ca}^{2+}]_i$ increase, indicating that intracellular Ca^{2+} stores are not discharged (Fig. 4A).

To investigate the function of phosphorylation in the modulation of the properties of AT1, we studied the role of PKC in the desensitisation of AT1 by using PMA. Human breast cells were pre-incubated for 5, 25, and

Figure 8 Ang II and PMA-induced maximal translocation of PKC- α , - β 1, - δ and - ζ isozymes to membrane fraction of breast cells. Serum-starved cells were stimulated with 100 and 1000 nM Ang II and 1000 nM PMA for 25 min. Control indicates cells treated with medium only. Cytosolic (C) and membrane (M) fractions were subjected to SDS-PAGE and Western blotting with anti-PKC antibodies.

60 min with 1000 nM PMA before treatment with 100 nM Ang II. While PMA exposure does not change the resting level of $[Ca^{2+}]_i$ (data not shown), it does, however, significantly inhibit the transient elevation of $[Ca^{2+}]_i$ induced by Ang II (ANOVA factorial: $P < 0.001$). However, whereas pre-incubation for 25 min provoked a fourfold decrement in the Ang II-evoked $[Ca^{2+}]_i$ response, PMA was less effective after 60 min pre-incubation (Fig. 4B).

To provide further evidence for a role of PKC in the desensitisation of AT1, breast cells were pre-incubated for 15 min with 1000 nM staurosporine, an inhibitor of PKCs, before treatment with Ang II. Staurosporine does not change the resting level of $[Ca^{2+}]_i$ (data not

shown) neither does it change the elevation of $[Ca^{2+}]_i$ induced by the first stimulation with 100 nM Ang II. However, a second maximal dose of Ang II (1000 nM) provokes a further $[Ca^{2+}]_i$ increase (Fig. 4C), suggesting that desensitisation is mediated by activation of PKCs.

Phospholipid hydrolysis is involved in Ang II-induced $[Ca^{2+}]_i$ increase

We pretreated cells for 5 min with increasing concentrations of U73122 (1, 10, 100, 1000 and 5000 nM), a PLC inhibitor, before adding 100 nM Ang II. The PLC inhibitor decreases $\Delta[Ca^{2+}]_i$ in a dose-dependent manner (ANOVA factorial: $P < 0.001$), demonstrating the involvement of PLC in calcium signalling pathways (Fig. 5).

Expression of PKC isozymes and their differential activation

Human cultured epithelial breast cells at passages 2 and 3 express PKC- α , - β 1, - δ and - ζ isozymes in cellular lysates (Fig. 6). The expression of PKC- β 1 was the highest while the expression of PKC- δ was the lowest.

In control cells (cells treated with medium only), all the PKC isozymes were found mainly in the cytosol; only small quantities were found to be associated with the membrane fraction. The isozymes - α , - β 1 and - δ translocate to membrane from cytosol after Ang II or PMA treatment, while the PKC- ζ does not (Figs 7 and 8). In all cases the highest translocation is observed for 25-min treatment (Fig. 7).

Proliferative response of cultured epithelial breast cells to Ang II

Incubation for 24 h with Ang II stimulates cultured human breast epithelial cell proliferation in a dose-dependent manner (Fig. 9A) showing maximal response at 1000 nM Ang II (Fig. 9A). Pre-incubation with 1000 nM losartan inhibits the stimulating effects of 1000 nM Ang II (Fig. 9B). Incubation with losartan alone does not achieve any effect (data not shown).

Gö 6976, a specific inhibitor of the Ca^{2+} -dependent isozymes PKC- α and - β 1, was used in order to assess whether PKC activation was involved in the proliferative effect of Ang II. Incubation of cells (24 h) with increasing concentrations (1–100 000 nM) of Gö 6976 has no effect on cell growth (data not shown). Co-incubation of cells (24 h) with a fixed dose of Ang II (1000 nM) and increasing concentrations (1–100 000 nM) of Gö 6976 produces a significant and dose-dependent inhibition of the growth-stimulating effects of Ang II (ANOVA factorial: $P < 0.0001$) (Fig. 9C).

Discussion

While it is clear that human epithelial breast cells express AT1 (Inwang *et al.* 1997, Tahmasebi *et al.* 1998) and that

Ang II evokes in many cell types an $[Ca^{2+}]_i$ increment via AT1 (Zhang *et al.* 1994), no clear evidence of its signalling pathways in breast have been obtained to date. We show here for the first time that Ang II in cultured epithelial breast cells increases the $[Ca^{2+}]_i$ in a similar manner to other cell types (Diaz-Torga *et al.* 1998, Garcia-Sainz *et al.* 1998, Iversen & Arendshorst 1998, Shao *et al.* 1998, Gonzalez-Iglesias *et al.* 1999). In several cell types, the initial $[Ca^{2+}]_i$ elevation induced by Ang II is independent of extracellular Ca^{2+} influx and reflects the depletion of the

intracellular stores induced by 1,4,5-inositol trisphosphate (IP_3), whereas the plateau phase, if present, involves extracellular Ca^{2+} influx through membrane channels (Kuwahara *et al.* 2000). Since Ca^{2+} removal and nifedipine treatment did not change the Ang II-induced $[Ca^{2+}]_i$ increase in cultured breast epithelial cells, we suggest that extracellular Ca^{2+} entry is not involved; a similar cell response was also described in brain endothelial cells and in glomerular podocytes (Stanimirovic *et al.* 1996, Nitschke *et al.* 2000).

Here, we investigated the effects of AT1 and AT2 inhibitors on the Ang II-evoked $[Ca^{2+}]_i$ transient peaks. The finding that losartan, and not CGP42113A, was able to decrease the Ang II-induced $[Ca^{2+}]_i$ transient peak indicates that AT1 is responsible for the Ca^{2+} -signalling effects of Ang II in cultured breast epithelial cells. There is evidence that AT1 mRNA and protein expression may change in culture after passages (Ko *et al.* 1997); we found that the AT1 inhibitor, losartan, was still able to decrease the Ang II-induced $[Ca^{2+}]_i$ transient peak until the fourth passage indicating that a functional AT1 was still expressed; in addition, the AT1 mRNA expression did not change until the fourth passage. We show that repeated stimulation of cells with Ang II induced a desensitisation of AT1 after a few minutes of exposure to Ang II, in accordance with other studies (Barker *et al.* 1995, Balmforth *et al.* 1997, Feng *et al.* 1998). First, the finding that the treatment for 25 min with PMA decreased Ang II-induced $\Delta[Ca^{2+}]_i$ suggests that the desensitisation process may involve phosphorylation of AT1 by activated PKCs; in fact, in support of this hypothesis we demonstrate that staurosporine, an inhibitor of PKCs, is able to inhibit the desensitisation of AT1. Identical findings have been

Figure 9 (A) Effects of Ang II upon the proliferation of human cultured breast epithelial cells. Cells were grown for 24 h in serum-free media in the absence or presence of Ang II. All results are expressed as the percentage ratio over cells cultured in serum-free media only. The bars are representative of five separate experiments of cells cultured from four different individuals. Different letters indicate statistical differences by Fisher's PLSD test (ANOVA factorial: $P < 0.0001$). (B) Effects of the AT1 inhibitor losartan upon the Ang II (1000 nM)-stimulated proliferation of human cultured epithelial breast cells. Cells were grown for 24 h in serum-free media in the absence or presence of various concentrations of losartan added 30 min before the administration of 1000 nM Ang II. All results are expressed as the percentage ratio over cells cultured in medium only. All bars show the means and s.d. of cells cultured from four different individuals. Different letters indicate statistical differences by Fisher's PLSD test (ANOVA factorial: $P < 0.0001$). (C) Role of PKC in Ang II-induced proliferation of cultured human epithelial breast cells. Cells were grown for 24 h in serum-free medium containing 1000 nM Ang II in the absence or presence of various concentrations of Gö 6976. All results are expressed as the ratio over cells cultured in serum-free medium only. All bars show the means and s.d. of cells cultured from four different individuals. Different letters indicate statistical differences by Fisher's PLSD test (ANOVA factorial: $P < 0.0001$).

obtained in cultured guinea pig myocytes where inhibition of PKC blocked the Ang II homologous desensitisation (Shimuta *et al.* 1990) and in vascular smooth muscle cells where PKC stimulation enhanced it (Brock *et al.* 1985).

Since Ang II exerts its stimulatory effects through the PLC/Ca²⁺ signalling pathway, the latter being involved in PKCs activation, we investigated the effects of Ang II on membrane translocation of PKCs. Among the eleven PKC isoforms expressed in mammalian cells we paid attention to four isozymes: the 'conventional' Ca²⁺-dependent, PKC- α and - β 1 isozymes, the 'novel' Ca²⁺-independent, diacylglycerol (DAG)-dependent PKC- δ isozyme, and the 'atypical' Ca²⁺-DAG-independent PKC- ζ isozyme. Following exposure of cells to Ang II, PKC- α , - β 1 and - δ translocated significantly from the cytosol to the membrane, and a 25-min long stimulation was required to achieve the maximal translocation. It therefore appears that the effect of Ang II on the translocation of PKCs from cytosol to membrane develops slowly compared with the rapid time course of Ca²⁺ mobilisation. The reason for this remains unknown but a similar delay in PKC translocation has also been observed in rat pinealocytes challenged with noradrenaline (Ho *et al.* 1999) and in hippocampal neurones stimulated with glutamate (Buchner *et al.* 1999). The phorbol ester, PMA, known to strongly activate conventional and atypical PKCs, was used to activate PKC isoforms and to compare it with the membrane translocation induced by Ang II. Similarly to Ang II, PMA also maximally stimulates translocation of PKC- α , - β 1 and - δ after 25 min; a similar time-course was also shown in rat liver WB cells (Maloney *et al.* 1998) and in mammalian eggs (Raz *et al.* 1998).

Data from the literature has shown only the PKC total activity expressed by breast cancer biopsies and by surrounding normal tissue (Bignon *et al.* 1990), and the expression of PKC- α , - β 1, - δ , - ζ , - ϵ and - η in MCF-7 breast carcinoma cell line (Ways *et al.* 1995, Shanmugam *et al.* 1999). To our knowledge, the present study provides the first indication of the presence of PKC isozymes, and that Ang II activates some of them in human cultured breast epithelial cells. Ang II causes hypertrophy and hyperplasia via AT1 (Tian *et al.* 1995) and increases the expression of immediate early genes such as *c-fos*, *c-jun* and *c-myc* (Griendling *et al.* 1997). These effects have been well documented in adrenal glomerulosa cells (Hauger *et al.* 1978), in renal mesangial cells and vascular smooth muscle cells (Huckle & Earp 1994). Consistently, in the present study, Ang II stimulated proliferation of breast epithelial cells through AT1. Moreover, PKC activation has been shown to regulate cell cycle progression in MCF-7 cells (Lafon *et al.* 1995) and in other cell lines (Piacentini *et al.* 2000, Zhao *et al.* 2000). The sensitivity of the Ang II-induced proliferative responses in breast cells to Gö 6976 is consistent with these effects being induced through the activation of PKC- α / β 1.

It is concluded that in human breast epithelial cells, Ang II elicits Ca²⁺ responses. Through the activation of AT1, Ang II elevates [Ca²⁺]_i and translocates PKCs, which in turn activate cell proliferation. Although the mechanism by which Ang II affects cell proliferation remains not fully understood, our findings obtained in cultured cells strongly suggest the peripheral actions of Ang II in mitogenic processes in human breast.

Acknowledgements

We are grateful to the Italian MIUR for project grant support (Programma Operativo del Piano 'Biomedicina'-Cluster C04 - progetto no. 19).

References

- Balmforth AJ, Lee AJ, Shepherd FH, Warburton P, Donnelly D & Ball SG 1997 G-protein-coupled receptors for peptide hormones: angiotensin II receptors. *Biochemical Society Transactions* **25** 1041-1046.
- Barker S, Kapas S, Fluck RJ & Clark AJL 1995 Effects of the selective protein kinase C inhibitor RO 31-7549 on human angiotensin II receptor desensitisation and intracellular calcium release. *FEBS Letters* **369** 263-266.
- Bataller R, Gines P, Nicolas JM, Gorbis MN, Garcia-Ramallo E, Gasull X, Bosch J, Arroyo V & Rodes J 2000 Angiotensin II induces contraction and proliferation of human hepatic stellate cells. *Gastroenterology* **118** 1149-1156.
- Bignon E, Ogita K, Kishimoto A & Nishizuk Y 1990 PKC subspecies in ER-positive and -negative human breast cancer cell lines. *Biochemical and Biophysical Research Communications* **171** 1071-1078.
- Brock TA, Rittenhouse SE, Powers CW, Ekstein LS, Gimbrone MA Jr & Alexander RW 1985 Phorbol ester and 1-oleoyl-2-acetyl-glycerol inhibit angiotensin activation of phospholipase C in cultured vascular smooth muscle cells. *Journal of Biological Chemistry* **260** 14158-14162.
- Buchner K, Adamec E, Beermann ML & Nixon RA 1999 Isoform-specific translocation of protein kinase C following glutamate administration in primary hippocampal neurons. *Molecular Brain Research* **64** 222-235.
- Diaz-Torga G, Gonzalez Iglesias A, Achaval-Zaia R, Libertun C & Becu-Villalobos D 1998 Angiotensin II-induced Ca²⁺ mobilisation and prolactin release in normal and hyperplastic pituitary cells. *American Journal of Physiology* **274** E534-E540.
- Feng X, Zhang J, Barak LS, Meyer T, Caron MG & Hannun YA 1998 Visualisation of dynamic trafficking of a protein kinase C betaII/green fluorescent protein conjugate reveals differences in G protein-coupled receptor activation and desensitisation. *Journal of Biological Chemistry* **273** 10755-10762.
- Garcia-Sainz JA, García-Caballero A & Gonzalez-Espinosa C 1998 Angiotensin AT1 receptors in Clone 9 rat liver cells: Ca²⁺ signalling and c-fos expression. *European Journal of Pharmacology* **362** 235-243.
- Gonzalez-Iglesias A, Diaz-Torga G, Lux-Lantos V, Libertun C & Becu-Villalobos D 1999 Calcium influx and intracellular stores in angiotensin II stimulation of normal and hyperplastic pituitary cells. *American Journal of Physiology* **277** E455-E463.
- Griendling KK, Ushio-Fukai M, Lassegue B & Alexander RW 1997 Angiotensin II signalling in vascular smooth muscle. New concepts. *Hypertension* **29** 366-373.
- Gryniewicz G, Poenie M & Tsien RY 1985 A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260** 3440-3450.

- Hauger RL, Aguilera G & Catt KJ 1978 Angiotensin II regulates its receptor sites in the adrenal glomerulosa zona. *Nature* **271** 176–178.
- Ho AK, Hashimoto K, Matowe W & Chik CL 1999 Adrenergic regulation of translocation of protein kinase C isozymes in rat pinealocytes. *Molecular and Cellular Endocrinology* **150** 169–178.
- Hou M, Pantev E, Moller S, Erlinge D & Edvinsson L 2000 Angiotensin II type 1 receptors stimulate protein synthesis in human cardiac fibroblasts via a Ca^{2+} -sensitive PKC-dependent tyrosine kinase pathway. *Acta Physiologica Scandinavica* **168** 301–309.
- Huckle WR & Earp HS 1994 Regulation of cell proliferation and growth by angiotensin II. *Progress in Growth Factor Research* **5** 177–194.
- Inwang ER, Puddefoot JR, Brown CL, Goode AW, Marsigliante S, Ho MM, Payne JG & Vinson GP 1997 Angiotensin II type 1 receptor expression in human breast tissues. *British Journal of Cancer* **75** 1279–1283.
- Iversen BM & Arendshorst WJ 1998 ANG II and vasopressin stimulate calcium entry in dispersed smooth muscle cells of preglomerular arterioles. *American Journal of Physiology* **274** F498–F508.
- Ko Y, Glodny B, Stier S, Totzke G, Nickenig G, Düsing R, Sachinidis A & Vetter H 1997 Angiotensin type-1 (AT1) receptor gene expression in primarily cultured human arterial umbilical endothelial cells. *Biochemical Pharmacology* **53** 417–421.
- Kuwahara M, Miyajia T, Tsubone H & Kuwahara M 2000 Angiotensin II type 1 receptor-mediated increase in cytosolic Ca^{2+} and proliferation in mesothelial cells. *European Journal of Pharmacology* **388** 21–27.
- Lafon C, Mazars P, Guerrin M, Barboule N, Charcosset JY & Valette A 1995 Early gene responses associated with transforming growth factor- β 1 growth inhibition and autoinduction in MCF-7 breast adenocarcinoma cells. *Biochimica et Biophysica Acta* **1266** 288–295.
- Liu WS & Heckman CA 1998 The sevenfold way of PKC regulation. *Cellular Signalling* **10** 529–542.
- Lundergan WP, Ferry D, Kobayashi H & Snowdowne KW 1999 Angiotensin-II increases cytoplasmic calcium, cell number and total DNA for human periodontal ligamental cells *in vitro*. *Journal of Periodontal Research* **34** 223–228.
- Maloney JA, Tsygankova O, Sztot A, Yang L, Li Q & Williamson JR 1998 Differential translocation of protein kinase C isozymes by phorbol esters, EGF, and ANG II in rat liver WB cells. *American Journal of Physiology* **274** C979–C982.
- Mosmann T 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65** 55–63.
- Nitschke R, Henger A, Ricken S, Gloy J, Muller V, Greger R & Pavenstadt H 2000 Angiotensin II increases the intracellular calcium activity in podocytes of the intact glomerulus. *Kidney International* **57** 41–49.
- Piacentini L, Gray M, Monbo NY, Chentoufi J, Bergman M & Karliner JS 2000 Endothelin-1 stimulates cardiac fibroblast proliferation through activation of protein kinase C. *Journal of Molecular Cell Cardiology* **32** 565–576.
- Raz T, Eliyahu E, Yesodi V & Shalgi R 1998 Profile of protein kinase C isozymes and their possible role in mammalian egg activation. *FEBS Letters* **431** 415–418.
- Shanmugam M, Krett NL, Maizels ET, Cutler RE, Peters CA, Smith LM & O'Brien ML 1999. Regulation of protein kinase C δ by estrogen in the MCF-7 human breast cancer cell line. *Molecular and Cellular Endocrinology* **148** 109–118.
- Shao Q, Seward L, Zahradka P & Dhalla NS 1998 Ca^{2+} mobilization in adult rat cardiomyocytes by angiotensin type 1 and 2 receptors. *Biochemical Pharmacology* **55** 1413–1418.
- Shen S, Alt A, Wertheimer R, Gartsbein M, Kuroki T, Ohba M, Braiman L, Sampson SR & Tennenbaum T 2001 PKC δ activation: a divergence point in the signaling of insulin and IGF-I-induced proliferation of skin keratinocytes. *Diabetes* **50** 255–264.
- Shimuta SI, Kanashiro CA, Oshiro ME, Paiva TB & Paiva AC 1990 Angiotensin II desensitisation and Ca^{2+} and Na^{+} fluxes in cultured intestinal smooth muscle cells. *Journal of Pharmacology and Experimental Therapeutics* **253** 1215–1221.
- Stanimirovic D, Morley P, Ball R, Hamel E, Mealing G & Durkin JP 1996 Angiotensin II-induced fluid phase endocytosis in human cerebromicrovascular endothelial cells is regulated by the inositol-phosphate signalling pathway. *Journal of Cellular Physiology* **169** 455–467.
- Tahmasebi M, Puddefoot JR, Inwang ER, Goode AW, Carpenter R & Vinson GP 1998 Transcription of the prorenin gene in normal and diseased breast. *European Journal of Cancer* **34** 1777–1782.
- Tian Y, Balla T, Baukal AJ & Catt KJ 1995 Growth responses to angiotensin II in bovine adrenal glomerulosa cells. *American Journal of Physiology* **268** E135–E144.
- Vinson GP, Puddefoot JR, Ho MM, Barker S, Mehta J, Saridogan E & Djahanbakhch O 1995 Type I angiotensin II receptors in rat and human sperm. *Journal of Endocrinology* **144** 369–378.
- Ways DK, Kukoly CA & de Vente J 1995 MCF-7 breast cancer cells transfected with protein kinase C- α exhibit altered expression of other protein kinase C isoforms and display a more aggressive neoplastic phenotype. *Journal of Clinical Investigation* **95** 1906–1915.
- Zhang J, Van Meel JC, Pfaffendorf M, Zhang J & Van Zwieten PA 1994 Endothelium-dependent, nitric oxide-mediated inhibition of angiotensin II-induced contractions in rabbit aorta. *European Journal of Pharmacology* **262** 247–253.
- Zhao Y, Neltner BS & Davis HW 2000 Role of MARCKS in regulating endothelial cell proliferation. *American Journal of Physiology* **279** C1611–C1620.

Received in final form 23 March 2002

Accepted 15 April 2002