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Angiotensin II AT1 receptor stimulates Na^+–K^+ATPase activity through a pathway involving PKC-ζ in rat thyroid cells

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Angiotensin II (Ang II) receptor subtype 1, AT1, is expressed by the rat thyroid. A relationship between thyroid function and several components of the renin–angiotensin system has also been established, but the Ang II cellular effects in thyrocytes and its transduction signalling remain undefined. The aim of the present paper was to investigate the modulation of the activity of the Na^+–K^+ATPase by Ang II and its intracellular transduction pathway in PC-Cl3 cells, an established epithelial cell line derived from rat thyroid. Here we have demonstrated, by RT-PCR analysis, the expression of mRNA for the Ang II AT1 receptor in PC-Cl3 cells; mRNA for the Ang II AT2 receptor was not detected. Ang II was not able to affect the intracellular Ca^{2+} concentration in fura-2-loaded cells, but it stimulated the translocation from the cytosol to the plasma membrane of atypical protein kinase C-zeta (PKC-ζ) and -iota (PKC-ι) isoforms with subsequent phosphorylation of the extracellular signal-regulated kinases 1 and 2 (ERK1 and 2). Translocated atypical PKCs displayed temporally different activations, the activation of PKC-ζ being the fastest. PC-Cl3 cells stimulated with increasing Ang II concentrations showed dose- and time-dependent activation of the Na^+–K^+ATPase activity, which paralleled the PKC-ζ translocation time course. Na^+–K^+ATPase activity modulation was dependent on PKC activation since the PKC antagonist staurosporine abolished the stimulatory effect of Ang II. The inhibition of the ERK kinases 1 and 2 (MEK1 and 2) by PD098059 (2′-amino-3′-methoxyflavone) failed to block the effect of Ang II on the Na^+–K^+ATPase activity. In conclusion, our results suggest that Ang II modulates Na^+–K^+ATPase activity in PC-Cl3 cells through the AT1 receptor via activation of atypical PKC-ζ while the Ang II-activated PKC-ι appears to have other as yet unknown functions.

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The existence of a relationship between thyroid function and several components of the renin–angiotensin system (RAS), such as plasma renin activity, plasma renin substrate and aldosterone levels (Montiel *et al.* 1984, Ruiz *et al.* 1987, Catanzaro, 1995) has been established. Moreover, angiotensin II (Ang II) receptor densities in rat heart, liver, kidney and adrenal gland have been found to be altered in thyroid dysfunction (Marchant *et al.* 1993, Matillas *et al.* 1993). It has also been shown that the rat thyroid expresses the Ang II receptor subtype 1, AT1, but not the other subtype AT2 (Montiel & Jimenez, 1998).

In epithelial cells, Ang II may have a role in the maintenance of structure and functions including mitosis, tissue differentiation (Goldfarb, 1994; McEwan *et al.* 1996), and the regulation of water and electrolyte transport (Norris *et al.* 1991; Quan & Baum 1996). Ang II also appears to be potentially important as a growth factor or growth promoter, since it causes a rise in cytosolic free Ca^{2+} in a variety of cell types, a process linked to mitogenesis (Kuwahara *et al.* 2000; Hou *et al.* 2000; Shen *et al.* 2001). The activity of the Na^+–K^+ATPase appears to be greatly involved in the proliferation and differentiation of various cells, including keratinocytes (Shen *et al.* 2001), lymphocytes (Marakhova *et al.* 2000), astrocytes (Matsuda *et al.* 1996), 3T3 cells (Russo & Sweadner, 1993) and retinal pigment epithelium (Burke *et al.* 1991). Both *in vivo* and in cultured cells the Na^+–K^+ATPase activity is modulated by Ang II (Muscella *et al.* 1997, 2000; Buhagiar *et al.* 1999; Yingst *et al.* 2000; Zhang & Mayeux, 2001).

Research in the thyroid field regarding Na^+–K^+ATPase has stressed its functional role in iodide (I^-) uptake since the pump energises all the Na^+-coupled secondary transport systems and, therefore, the Na^+–I^- symport also, which is fundamental in thyroid hormone synthesis. The Na^+–K^+ATPase activity is also important in the physiological mechanisms of thyroglobulin concentration in the thyroid follicle and in the exit of water and ions out of the follicular lumen.

Whether Ang II exerts a regulating control on thyrocyte function has not yet been established. The purpose of this

study was to investigate firstly the existence of the Ang II receptors in the PC-Cl3 cell line, an established epithelial cell line derived from Fisher rat thyroid (Fusco *et al.* 1987) that expresses the typical markers of thyroid differentiation, secondly the effects of Ang II on the activity of Na^+–K^+ATPase and, thirdly, the intracellular transduction pathway.

METHODS

Materials

Fetal bovine serum (FBS), and glutamine were from Euroclone (Paignton, UK). Fura-2 AM, thapsigargin and Pluronic F-127 were from Molecular Probes (Eugene, OR, USA). Hydrocortisone, transferrin, L-glycyl-histidyl-lysine and somatostatin were from ICN Biomedicals (Costa Mesa, CA, USA). Coon's modified Ham's F12 medium, bovine serum albumin (BSA), PD098059 (2′-amino-3′-methoxyflavone), staurosporine and other reagents were from Sigma-Aldrich Co. (Milan, Italy).

Cell culture

PC-Cl3 rat thyroid cells were grown in Coon's modified Ham's F12 medium, supplemented with 5% fetal bovine serum, containing L-glutamine (2 mM), gentamicin (50 μg ml^{-1}) and a six-hormone mixture consisting of insulin (1 μg ml^{-1}), hydrocortisone (3.62 μg ml^{-1}), transferrin (5 μg ml^{-1}), L-glycyl-histidyl-lysine (20 ng ml^{-1}), somatostatin (10 ng ml^{-1}) and thyroid stimulating hormone (TSH; 1 mU ml^{-1}). The cells were maintained in a water-saturated atmosphere of 5% CO_2 and 95% air at 37°C. The cells were passaged every 7–8 days, and the culture medium was changed every 2–3 days. Fresh medium without FBS was always added 24 h prior to an experiment.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from PC-Cl3 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, DTT 10, dNTP 0.5; oligo(dT)15 primer 0.5 μg and 15 U Moloney murine leukaemia virus reverse transcriptase (Promega).

PCR was performed in (mM): Tris-HCl (pH 8.8) 50, KCl 50, $MgCl_2$ 1.5, DTT 2, dNTP 0.2, 0.1% Triton X-100, 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′-GGAAACAGCTTGGTGGTG-3′ and 5′-GCACAATCGCCAT AATTATCC-3′ which correspond to sense and anti-sense, respectively, of bases 133–150 and 719–739 in the human AT1 sequence. AT2 primers were 5′-ATAATGATTTGGGGATTCAG-3′ and 5′-TTTTAAGCCACCCAGATATT-3′ corresponding to bases sense 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.

The RT samples were also used to generate β-actin PCR products and their amount was considered as the internal control. β-Actin sense and anti-sense primers were as follows: 5′-CCTCTATGCC AACACAGT-3′ and 5′-AGCCACCAATCCACACAG-3′, respectively; the amplification profile was as reported above for AT1 and AT2.

Measurement of Na^+–K^+ATPase activity in PC-Cl3 cells

The enzyme activity was measured by using a coupled enzyme assay method (Nørby, 1988) with some modifications (Muscella *et al.* 2002). Briefly, the cells were grown on 96-well plates for 1 day in Ham's F12 medium plus 5% FBS and then the medium was replaced with 100 μl of serum-free medium for 24 h to induce quiescence. After incubation with the various agents, cells were immediately permeabilised by freezing for 10 min at −20°C. The reaction was started by addition of 200 μl of reaction mixture (mM: KCl 20, $MgCl_2$ 8.0, NaCl 100, EGTA 0.5, Tris 40, phosphoenol pyruvate 10, NADH 0.25, fructose-1,6-diphosphate 1.0, ATP 5, 1.1 U ml^{-1} lactate dehydrogenase, 0.9 U ml^{-1} pyruvate kinase, with or without 1 mM ouabain), directly in 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)$^{-1}$ h^{-1}.

Measurement of intracellular free calcium

Growth medium was changed to fresh FBS-free culture medium for 12–18 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) (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^{-1} 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, with a chopper interval of 0.5 s and the emission monochromator was set at 510 nm. Intracellular free calcium $[Ca^{2+}]_i$ was calculated according to the equation of Grynkiewicz (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 of Triton X-100 (0.01% final concentration) and 20 μl of EGTA (5 mM final concentration), respectively.

Western blot analysis

To obtain whole protein cell extracts for Western blot analysis of ERK1/2, PC-Cl3 cells were scraped in the following buffer (mM): 20 Tris-HCl, pH 8, containing 420 NaCl, 2 EDTA, 2 Na_3VO_4, 2% Nonidet P-40. Cells were then passed several times through a 20 gauge syringe and centrifuged at 16 000 *g* for 20 min at 4°C.

For analysis of PKC isoforms PC-Cl3 cells were scraped into buffer A (mM: 20 Tris-HCl, pH 7.5, containing 250 sucrose, 2 EGTA, 2 EDTA, 10 $MgCl_2$, 2 DTT and a mixture of protease inhibitors including 0.5 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} pepstatin), and after two freeze and thaw cycles, cytosolic and membrane fractions were separated by centrifugation at 100 000 *g* for 1 h at 4°C. The pellet was solubilised in buffer B (mM: 20 Tris-HCl, pH 7.5, 150 NaCl, 1 EGTA, 1 EDTA and protease inhibitors) containing 1% Nonidet P-40.

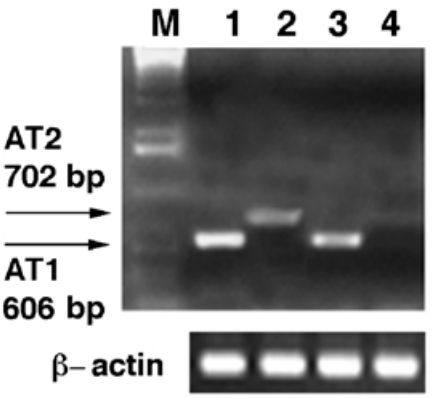

Figure 1. PC-Cl3 cells express angiotensin II receptor subtype AT1

Agarose gel electrophoresis of RT-PCR products revealed the specific transcripts for AT1 and AT2 of Ang II receptors in rat adrenal (lanes 1 and 2, respectively), and AT1 but not AT2 in PC-Cl3 cells (lanes 3 and 4, respectively). M, size markers: 100 bp ladder (Gibco BRL), appropriate sizes are indicated. β-Actin levels normalised the amount of cDNA template used in each PCR reaction. The figure is representative of four independent experiments.

Proteins were determined with the Bio-Rad protein assay kit 1 (Milan, Italy), using lyophilised bovine serum albumin as a standard.

Equal amounts of proteins (25 μg) from homogenates (ERK1/2) and from cytosolic or membrane fractions (for PKCs) were loaded and separated on 10 % SDS gels by electrophoresis and transferred to a nitrocellulose membrane. The sheet was blocked with 3 % non-fat dry milk in buffered saline. PKC isozyme proteins were detected using antibodies specific for different PKC isoforms (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Dually phosphorylated ERK1 and ERK2, corresponding to the active forms of the enzymes, were detected by a specific antibody (Promega). The proteins were detected with goat anti-rabbit IgG conjugated with peroxidase (Santa Cruz Biotechnology), using the ECL (Amersham Life Sciences Inc., Amerham, UK). As a control, the blots used for active ERK1/2 detection were then stripped and reprobed with another antibody (Promega) which recognises both active and basal forms of the ERK enzymes. The intensity of the bands was quantified by scanning densitometry using the NIH Image 1.62 software (NIH, USA). The area under the peak of the PKC isoform scanned (both cytosolic and membrane fractions) was determined, and the membrane-to-cytosol ratio was used to calculate fold translocation (or activation).

Statistical analysis

Experimental points represent the means ± standard deviation (S.D.) of 3–6 replicates. Statistical analysis was carried out using Student's *t* test for unpaired samples, and ANOVA. When indicated, *post hoc* tests (Bonferroni and Dunn) were also performed. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

AT1 and AT2 gene expression in PC-Cl3

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). By RT-PCR analysis we found that PC-Cl3 cells expressed the AT1 receptor mRNA; the mRNA for AT2 was absent (Fig. 1).

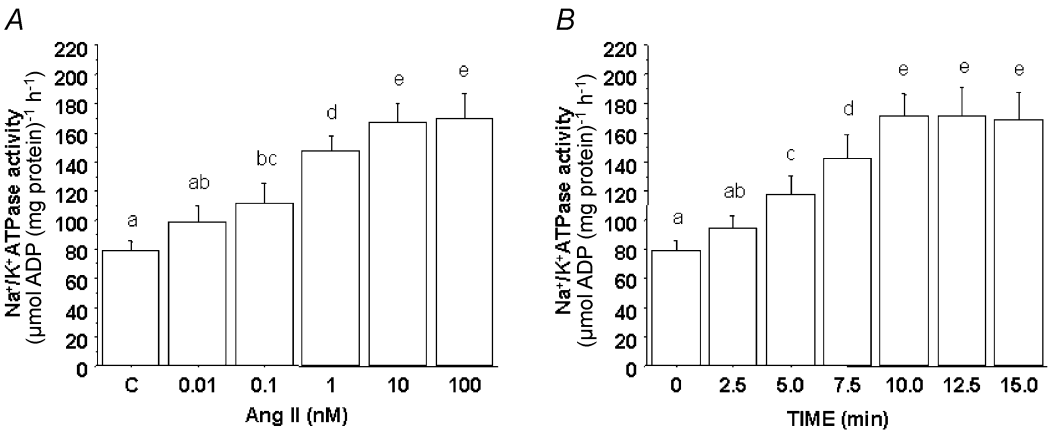

Figure 2. The effect of Ang II on Na^+–K^+ATPase activity is dose- and time-dependent

A, PC-Cl3 cells were incubated without or with increasing concentrations of Ang II for 10 min at 37 °C. *B*, cells were incubated without or with 10 nM Ang II for different time periods. The data are means ± S.D. of six different experiments run in triplicate. ANOVA for *A* and *B*: $P < 0.0001$. Values with shared letters are not significantly different according to Bonferroni and Dunn *post hoc* tests.

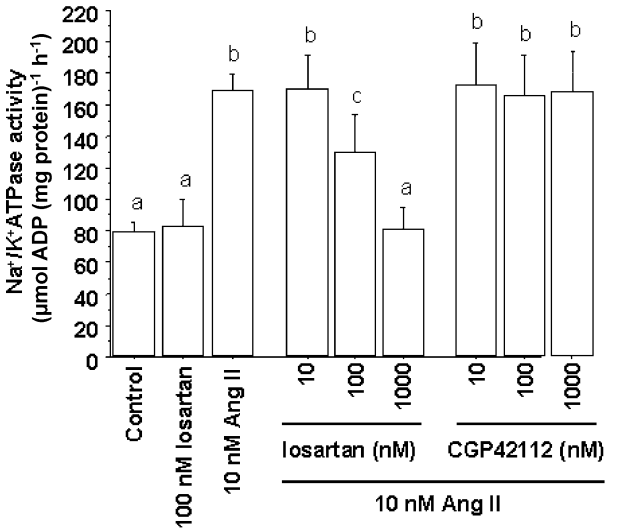

Figure 3. The AT1 receptor antagonist, losartan, prevents the Ang II-induced Na^+–K^+ATPase activity increase and does not affect its basal activity; CGP42112, an AT2 receptor antagonist, does not affect Ang II actions

PC-Cl3 cells were treated without or with increasing concentrations of losartan or CGP42112 and then with either control medium or medium containing 10 nM Ang II for 10 min at 37 °C. Results are means ± S.D. of three different experiments run in triplicate. Values with shared letters are not significantly different according to Bonferroni and Dunn *post hoc* tests.

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

The method used to measure Na^+/K^+ATPase activity in PC-Cl3 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 an increasing number of cells. The baseline activity of Na^+–K^+ATPase in PC-Cl3 cells was 79.7 ± 6.2 μmol ADP (mg protein)$^{-1}$ h^{-1} (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. 2*A*

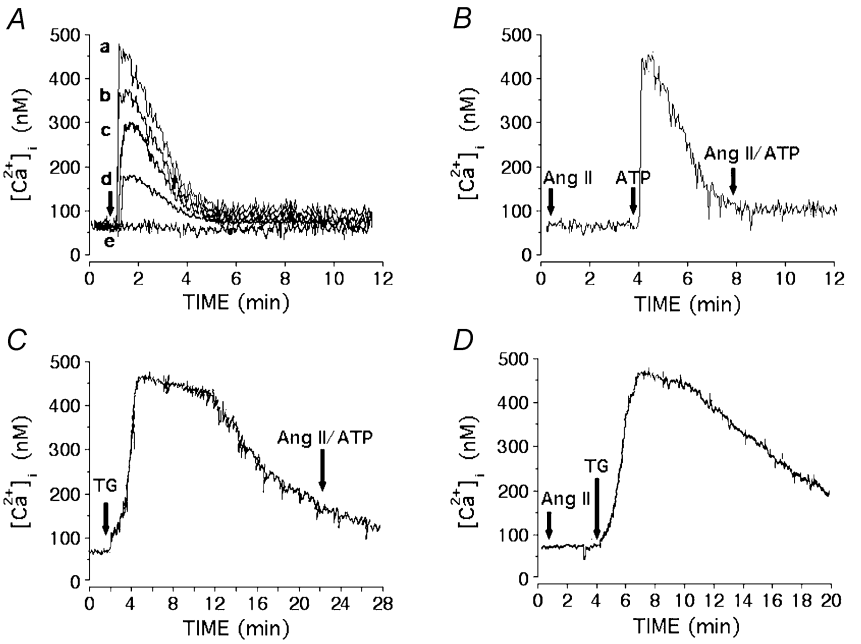

Figure 4. ATP, but not Ang II, elevates [Ca2+]i in PC-Cl3 cells

A, dose–response effect of ATP-induced increase in $[Ca^{2+}]_i$ in PC-Cl3 cells suspended in standard experimental medium containing 2 mM Ca^{2+}. Cells loaded with fura-2 were treated with various concentration of ATP. ATP was added at the indicated time at concentration of 100 (*a*), 10 (*b*), 1 (*c*) and 0.1 μM (*d*). Each tracing is representative of five separate experiments. Trace e represents the effect of Ang II (0.1–100 nM) on $[Ca^{2+}]_i$. The arrow indicates the addition of agonists. *B*, after Ang II administration (0.1–100 nM), 100 μM ATP was added at the indicated time to provoke an increment in $[Ca^{2+}]_i$; subsequent addition of Ang II and/or ATP did not have any effect. *C*, in standard experimental medium, 1 μM thapsigargin (TG) was added at the indicated time to mobilise the endoplasmic reticulum Ca^{2+} stores. ATP (100 μM) or Ang II (0.1–100 nM) was added afterwards. Results shown are representative of at least four experiments. *D*, after Ang II administration (0.1–100 nM), 1 μM thapsigargin (TG) was added at the indicated time to mobilise 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.

and *B*). The maximal response, in the subconfluent condition, was obtained after 10 min of incubation with 10 nM Ang II, and corresponded to a 2.15-fold increment in Na^+–K^+ATPase activity. Therefore, we chose the 10 nM concentration for 10 min of incubation in subsequent experiments. The ouabain-insensitive ATPase activity was not affected by any of the Ang II concentrations used (36.5 ± 4.3 μmol ADP (mg protein)$^{-1}$ h^{-1}). Saralasin (1 μM), a competitive Ang II antagonist, blocked the Ang II increase of Na^+–K^+ATPase activity (data not shown).

Cells were also incubated with receptor antagonists losartan (DuP753) and CGP42112 prior to treatment with Ang II. CGP42112, the AT2 antagonist, did not affect Ang II action, while the specific AT1 competitive antagonist, losartan, abolished the Ang II effect (Fig. 3). Losartan alone did not affect the basal Na^+–K^+ATPase activity.

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

The basal level of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was 70 ± 4 nM (mean ± S.E.M.; $n = 30$). The effects of Ang II (0.1, 1, 10 and 100 nM) on the $[Ca^{2+}]_i$ were not significant in all the experimental conditions used (in the presence and absence of extracellular Ca^{2+}; in normal or serum-starved cells; in cells incubated with an agonist, ATP, able to increase the $[Ca^{2+}]_i$) (Fig. 4).

As a positive control for the fluorimetric studies extracellular ATP was used, which is known to evoke $[Ca^{2+}]_i$ transients in PC-Cl3 cells following stimulation of phospholipase C through P2Y receptors (Marsigliante *et al.* 2002). Indeed, ATP (1–200 μM), produced a dose-dependent increase in $[Ca^{2+}]_i$ above basal levels (Fig. 4*A*). A threshold increase in $[Ca^{2+}]_i$ was observed at 1 μM with a maximum at 100 μM ATP (up to 490 ± 19 nM). No further increase was observed at 200 μM ATP. Various concentrations of Ang II added to PC-Cl3 prior to ATP exposure did not alter the ATP-evoked $[Ca^{2+}]_i$ transients (Fig. 4*B*). The microsomal Ca^{2+} ATPase inhibitor thapsigargin (TG) was used to empty internal stores: in the presence of external Ca^{2+}, 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. 4*C*). Ang II administered before TG did not alter its effects on the elevation of $[Ca^{2+}]_i$ (Fig. 4*D*).

Expression and translocation of PKCs in PC-Cl3 cells

The expression of different PKC isozymes in PC-Cl3 cells was determined by Western blotting with isozyme-specific antibodies. PKC-α, -β1, -δ, -ϵ, -ι and -ζ appear to be expressed at significant levels, since corresponding antibodies detected immunoreactive proteins expressed in PC-Cl3 cell lysates (data not shown); isozymes β2 and γ were not detected. The specificity of the immunoreactivity of PKCs was verified by absorption of antibodies with isozyme-specific peptide antigen at 10 ng ml^{-1}.

The relative distribution of the PKC isozymes among the cytosolic and membrane fractions was determined; all PKC isozymes were mostly in the cytosolic fraction, with various amounts in the membrane fraction (Fig. 5).

In order to investigate the action of Ang II on PKCs in PC-Cl3 cells, translocation of PKC isoforms from the cytosol to cellular membranes was analysed. Western blot analysis was performed using PC-Cl3 cells exposed to 0.1, 1, 10 and 100 nM Ang II for different incubation times (0.5, 2.5, 5, 10 and 20 min). A translocation of PKC-ζ and PKC-ι to PC-Cl3 membranes was observed. Ang II (10 nM) induced the maximal association of both PKC-ζ and -ι with the membrane, with concurrent losses from the cytosolic fraction; isozyme translocations did not increase further with the use of higher doses of hormone. Ang II stimulation caused differential translocation of atypical PKC isoforms. PKC-ζ was translocated by a 2.5 min Ang II stimulation and returned to the cytosol after 20 min, whereas PKC-ι was activated only by a 20 min Ang II stimulation (Fig. 6).

Interestingly, the preincubation of cells with the PKC inhibitor staurosporine (10 μM for 30 min) did not inhibit the Ang II-evoked translocation of atypical PKC-ζ and -ι (10 and 20 min Ang II stimulations, respectively) (data not shown). This is in accordance with results obtained by others (Tang & Sharp, 1998).

The distribution of PKC-α, -β1, -δ and -ϵ appeared to be unchanged upon Ang II treatment over the same time periods used for atypical PKC translocations (data not shown).

Effects of Ang II on the activation state of ERK1/2 in PC-Cl3 cells

Since atypical PKCs are known to activate, in some cells, the mitogen-activated protein kinase (MAPK) pathway, we studied the effects of Ang II treatment on ERK1/2 in

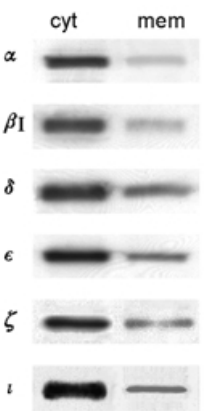

Figure 5. Representative immunoblots of protein kinase C isoforms in PC-Cl3 cells
Cyt and mem represent the cytosolic and the membrane-associated PKCs, respectively. PKC isozymes βII and γ were undetectable. The figure is representative of four independent experiments.

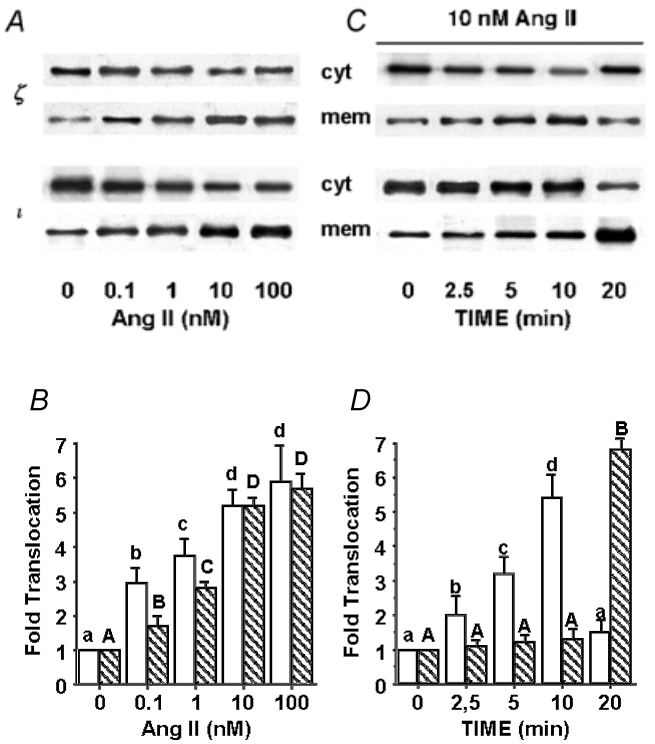

Figure 6. PKC-ζ and PKC-ι translocation from cytosol (cyt) to membrane (mem) induced by Ang II in PC-Cl3 cells

A, cells were treated without or with increasing concentrations of Ang II and immunoblotting was performed with PKC-ζ and PKC-ι isozyme-specific antibodies; incubation times were 10 min for PKC-ζ and 20 min for PKC-ι. *B*, fold translocation of atypical PKC isoforms (-ζ, open columns and -ι, hatched columns) provoked by increasing Ang II concentrations. *C*, cells were incubated without or with 10 nM of Ang II for the indicated periods and immunoblotting was performed with PKC-ζ and PKC-ι isozyme specific antibodies. *D*, fold translocation of atypical PKC isoforms (-ζ, open columns and -ι, hatched columns) provoked by 10 nM Ang II at various incubation times. *A* and *C*, the figures are representative of four independent experiments. *B* and *D*, PKC isoforms from control cells were considered to be 1.0-fold activated. Values are means ± S.D.; $n = 4$ Western blot analyses, each from different cell culture experiments. Area under the peak of the PKC isoform scanned (both cytosolic and membrane fractions) was determined, and the membrane-to-cytosol ratio was used to calculate fold translocation (or activation). Values with shared letters are not significantly different according to Bonferroni and Dunn *post hoc* tests. Capital letters refer to PKC-ι, small letters to PKC-ζ.

PC-Cl3 cells. To examine MAPK activation, PC-Cl3 cells were either left untreated or stimulated with 0.1, 1, 10 or 100 nM Ang II for various incubation times (1–20 min). Extracts of cells analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting showed a dose- and time-dependent activation of ERK1/2 (Fig. 7*A* and *B*). To test whether PKC-ζ was involved in Ang II-induced phosphorylation of ERK1/2, PC-Cl3 cells were pre-treated with staurosporine (0.1, 1 and 10 μM), which interferes with the catalytic domain of protein kinases (Ishii *et al.* 1996). Staurosporine at the highest dose completely inhibited the Ang II-evoked phosphorylation

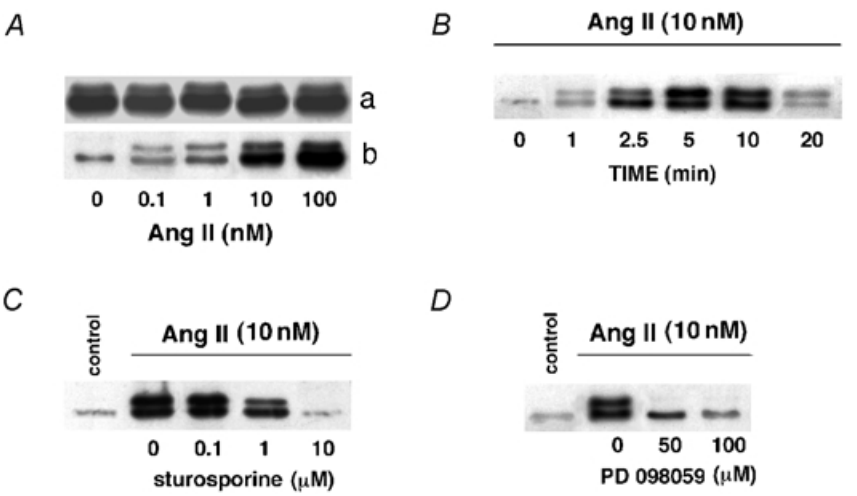

Figure 7. The Ang II-induced ERK1/2 phosphorylation is inhibited by staurosporine and MEK inhibitor PD098059

A, quiescent PC-Cl3 cells were stimulated without or with increasing concentrations of Ang II for 10 min. Panel b indicates the level of active (dually phosphorylated) ERK1/2, while panel a illustrates total ERK levels as detected using an antibody which recognises active and basal forms of the ERK enzymes. *B*, cells were treated without or with Ang II for the indicated periods; only phosphorylated ERK1/2 is shown. *C* and *D*, cells were preincubated with increasing concentrations of staurosporine or PD098059 for 30 min and stimulated with 10 nM Ang II for 10 min; only phosphorylated ERK1/2 is shown. The same results were obtained in other independent experiments ($n = 3$) and representative autoradiographs are shown.

of ERK1/2 (Fig. 7*C*). PD098059, a specific inhibitor of the activation of the MAPK/ERK kinases 1 and 2 (MEK1/2) (Alessi *et al.* 1995), was used in order to determine whether the MAPK pathway was involved in Ang II-induced effects on ERK1/2 phosphorylation. PC-Cl3 cells pre-treated with 50 or 100 μM PD098059 for 15 min did not show the Ang II-induced phosphorylation of ERK1/2 (Fig. 7*D*).

Effects of Ang II on Na^+–K^+ATPase activity: role of protein kinase C

To test whether PKC-ζ was involved in Ang II-induced effects on Na^+–K^+ATPase activity, PC-Cl3 cells were pre-treated with staurosporine (0.1, 1, 5 and 10 μM), which has a half-maximal inhibitory constant (IC_{50}) greater than 1.5 μM for PKC-ζ, whereas for other PKC isozymes the IC_{50} is 110 nM or lower (Ishii *et al.* 1996). The PKC antagonist did not significantly alter the basal activity of the Na^+–K^+ATPase (data not shown), but completely inhibited, at the highest dose, the Ang II effect on Na^+–K^+ATPase activity (Fig. 8).

Cytosolic and membrane fractions were prepared from untreated cells or cells treated with 10 nM Ang II for 10 min; in these fractions a PKC-ζ translocation was observed as reported above. In the same fractions the activity of the Na^+–K^+ATPase was also assayed. After Ang II treatment a significant increase in the Na^+–K^+ATPase activity was found in the membrane fraction (from 403 ± 27 to 808 ± 39 μmol ADP (mg protein)$^{-1}$ h^{-1}, $n = 3$, Student's t test: $P < 0.0001$). Conversely, the Na^+–K^+ATPase activity in the cytosolic fraction was 20 ± 8 μmol ADP (mg protein)$^{-1}$ h^{-1} in both Ang II-treated and untreated cells ($n = 3$).

PD098059 was used in order to determine whether the MAPK pathway was involved in Ang II-induced effects on Na^+–K^+ATPase activity. PC-Cl3 cells pre-treated with PD098059 (10, 50 and 100 μM) for 15 min did not change the basal activity of the Na^+–K^+ATPase (data not shown), and did not affect the effect of Ang II on the activity of the Na^+–K^+ATPase (Fig. 8).

DISCUSSION

While it is clear that rat thyroid expresses AT1 (Montiel & Jimenez, 1998) and that Ang II evokes in many cell types a $[Ca^{2+}]_i$ increment via AT1 (Berry *et al.* 2001), no clear evidence of AT1 signalling pathways in the thyrocyte has been obtained to date. Our study is the first showing that the rat thyroid PC-Cl3 cells express mRNA for the AT1 receptor subtype.

Since the AT1 receptor usually activates phosphatidylinositol turnover (Kuwahara *et al.* 2000; Hou *et al.* 2000; Shen *et al.* 2001) or membrane-located Ca^{2+} channels (Gebke *et al.* 1998) we performed experiments to determine whether Ang II also caused a $[Ca^{2+}]_i$ rise in PC-Cl3 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 $[Ca^{2+}]_i$ above basal levels, in a dose-dependent manner. This result is consistent with other studies showing that in PC-Cl3 cells, ATP increases $[Ca^{2+}]_i$ through binding to a purinergic P2Y receptor (Marsigliante *et al.* 2002). The demonstration that Ang II is ineffective in eliciting a rise in $[Ca^{2+}]_i$ may suggest that it activates other transduction pathways, but also raises the question of whether the AT1 receptor is functional or not in PC-Cl3 cells. We therefore aimed to assess AT1 functionality by looking 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; Buhagiar *et al.* 1999). We therefore assessed the ability of Ang II to modulate the Na^+–K^+ATPase activity of PC-Cl3 cells. We show that AT1 expressed by PC-Cl3 cells indeed regulates the Na^+–K^+ATPase activity, as demonstrated in other cell types (Muscella *et al.* 1997, 2000, 2002; Mondorf *et al.* 2000; Yingst *et al.* 2000; Zhang & Mayeux, 2001), since the specific AT1 receptor subtype competitive antagonist losartan completely blocked the Ang II effect.

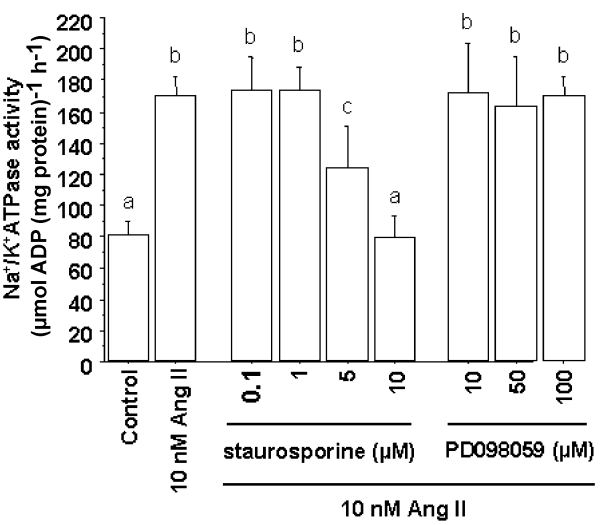

Figure 8. Ang II-induced increase in Na^+–K^+ATPase activity is blocked by PKC antagonists but not by MEK inhibitor

PC-Cl3 cells were treated with increasing concentrations of staurosporine or PD098059 for 30 min and then with either control medium or medium containing Ang II for 10 min. Results are means ± S.D. of three different experiments run in triplicate. Values with shared letters are not significantly different according to Bonferroni and Dunn *post hoc* tests.

In the follicular lumen the precursor of the thyroid hormones, thyroglobulin, is concentrated (up to 100–400 mg ml^{-1}; Smeds, 1972) and stored via a concentration process ensured by the extrusion of electrolytes and water out of the thyroid follicle; indeed, an apical–basal directed transport of fluid has been shown in pig thyrocytes (Pearson *et al.* 1988). Since the transepithelial flux of Na^+ determines transepithelial secondary flux of water, certain transport systems, including Na^+–K^+ATPase and Na^+–H^+ antiporter, are involved in these processes. Moreover, the Na^+–K^+ATPase is strictly implicated in I^- uptake by the Na^+/I^- symport, which is fundamental for the synthesis of thyroid hormones. Na^+–K^+ATPase is an important molecular target of hormonal regulation. In this regard, a regulatory mechanism for Na^+/K^+ATPase activity in intact cells is phosphorylation by PKC of the Na^+–K^+ATPase α-subunit. Two PKC phosphorylation sites (Ser16 and Ser23) have been identified in all cloned Na^+–K^+ATPase α-subunits (Fisone *et al.* 1995; Féraille *et al.* 1999). In response to PKC phosphorylation of its α-subunit, Na^+–K^+ATPase activity was stimulated (Carranza *et al.* 1996; Vasilets, 1997), inhibited (Chibalin *et al.* 1998*a*,*b*) or unchanged (Beron *et al.* 1997; Feschenko & Sweadner, 1997). These discrepancies may be accounted for in part by the presence of both indirect effects of PKC phosphorylation such as internalisation of active Na^+–K^+ATPase units and direct effects of phosphorylation such as an increase in apparent affinity for sodium. However, the two PKC phosphorylation sites might be targets for different PKC isozymes and/or produce different physiological effects. We found that in response to Ang II administration and subsequent PKC-ζ activation, the Na^+–K^+ATPase activity was significantly stimulated. Similarly, it has also been shown that in cortical tubule cells Ang II stimulated the Na^+/H^+ antiporter activity via selective translocation of PKC-ζ (Karim *et al.* 1999).

These data are the first to show a functional relationship between Ang II and both atypical PKCs. Translocating PKCs in PC-Cl3 cells displayed a temporally different activation, suggesting that each subspecies may have a temporally different targeting mechanism, contributing to their specific functions. While PKC-ζ is targeted to the Na^+–K^+ATPase, the Ang II-activated PKC-ι appears to have other and still unknown functions. We have no indication in the present study of the mechanisms of stimulation of PKC-ζ and -ι by Ang II. Both are members of the atypical PKC (aPKC) subfamily (Liu & Heckman, 1998), and cannot be activated by Ca^{2+}, phorbol esters or diacylglycerol, but they appear to be regulated by other important lipid cofactors such as phosphatidylinositol 3,4,5-P3, and ceramide (Lozano *et al.* 1994; van Blitterswijk, 1998) which are generated following cell activation by inflammatory cytokines and growth factors (Kolesnick & Kronke, 1998). While Ang II is able to induce ceramide synthesis, but through the type 2 (AT2) receptor (Lehtonen *et al.* 1999) which is a subtype absent in our cells, Ang II is also able to activate the monomeric G protein Ras via AT1 (Eguchi *et al.* 1999). In this regard, PKC-ζ is subjected to modulation by protein regulators and physically interacts with Ras (Diaz-Meco *et al.* 1994; Wooten *et al.* 1996); the interaction of PKC-ζ with Ras, in an agonist-dependent manner, is required for activation of ERK1/2 by Ang II in vascular smooth muscle cells (Liao *et al.* 1997). Recent evidence indicates that the activation of the MAPK pathway by classical and novel PKCs involves Ras activated factor (Raf) activation (Schonwasser *et al.* 1998), whereas the PKC-ζ actions are Raf-independent but mediated by MEK (Schonwasser *et al.* 1998). In other words, PKC-ζ constitutes an essential pathway parallel to Raf for the activation of the MAPK cascade. We show here that this happens also in PC-Cl3 cells under AT1 activation, but that the MAPK cascade is not involved in the modulation of the Na^+–K^+ATPase activity.

In conclusion, we have demonstrated the expression of a functional AT1 receptor in PC-Cl3 cells and a lack of effect of Ang II on $[Ca^{2+}]_i$. While this result is in contrast to those obtained in many cell types which exhibit Ca^{2+} mobilisation leading to mitogenesis, it is consistent with other studies showing for example that Ang II activation of ERK is independent of Ca^{2+} mobilisation (Li *et al.* 1998; Yang *et al.* 1999), 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) or 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 Ca^{2+} signalling (Muscella *et al.* 2002).

Finally, since AT1 stimulation activates PKC-ζ and modulates the activity of the Na^+–K^+ATPase, one could speculate that the Na^+–K^+ATPase is a general target of Ang II in epithelial cells, thyrocytes included.

REFERENCES

Alessi DR, Cuenda A, Cohen P, Dudley DT & Saltiel AR (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J Biol Chem* **270**, 27489–27494.

Aperia A, Holtback U, Syrén ML, Svensson LB, Fryckstedt J & Greengard P (1994). Activation/deactivation of renal Na^+/K^+ATPase: a final common pathway for regulation of natriuresis. *FASEB J* **8**, 436–439.

Beron J, Forster I, Béguin P, Geering K & Verrey F (1997). Phorbol 12-myristate 13-acetate down-regulates Na^+/K^+ATPase independent of its protein kinase C site: decrease in basolateral cell surface area. *Mol Biol Cell* **8**, 387–398.

Berry C, Touyz R, Dominiczak AF, Webb RC & Johns DG (2001). Angiotensin receptors: signaling, vascular pathophysiology, and interactions with ceramide. *Am J Physiol Heart Circ Physiol* **281**, H2337–2365.

Buhagiar KA, Hansen PS, Gray DF, Mihailidou AS & Rasmussen HH (1999). Angiotensin regulates the selectivity of the Na^+/K^+ pump for intracellular Na^+. *Am J Physiol Cell Physiol* **277**, C461–468.

Burke JM, Jaffe GJ & Brzeski CM (1991). The effect of culture density and proliferation rate on the expression of ouabain-sensitive Na^+/K^+ATPase pumps in cultured human retinal pigment epithelium. *Exp Cell Res* **194**, 190–194.

Carranza ML, Féraille E & Favre H (1996). Protein kinase C-dependent phosphorylation of the Na^+/K^+ATPase α-subunit in rat kidney cortical tubules. *Am J Physiol Cell Physiol* **271**, C136–143.

Catanzaro DF (1995). The molecular biology of renin gene expression. In *Hypertension: Pathophysiology, Diagnosis and Management*, ed. Laragh JH & Brenner BM, pp. 1589–1605. Raven Press, New York.

Chibalin AV, Pedemonte CH, Katz AI, Féraille E, Berggren PO & Bertorello AM (1998*a*). Phosphorylation of the catalytic α-subunit constitutes a triggering signal for Na^+/K^+ATPase endocytosis. *J Biol Chem* **273**, 8814–8819.

Chibalin AV, Zierath JR, Katz AI, Berggren PO & Bertorello AM (1998*b*). Phosphatidylinositol 3-kinase-mediated endocytosis of renal Na^+/K^+ATPase a subunit in response to dopamine. *Mol Biol Cell* **9**, 1209–1220.

Diaz-Meco MT, Lozano J, Municio MM, Berra E, Frutos S, Sanz L & Moscat J (1994). Evidence for the *in vitro* and *in vivo* interaction of Ras with protein kinase C zeta. *J Biol Chem* **269:**31 706–31 710.

Eguchi S, Iwasaki H, Ueno H, Frank GD, Motley ED, Eguchi K, Marumo F, Hirata Y & Inagami T (1999). Intracellular signaling of angiotensin II-induced p70 S6 kinase phosphorylation at Ser(411) in vascular smooth muscle cells. Possible requirement of epidermal growth factor receptor, Ras, extracellular signal-regulated kinase, and Akt. *J Biol Chem* **274**, 36 843–36 851.

Féraille E, Carranza ML, Gonin S, Béguin P, Pedemonte C, Rous-Selot M, Caverzasio J, Geering K, Martin PY & Favre H (1999). Insulin-induced stimulation of Na^+/K^+ATPase activity in kidney proximal tubule cells depends on phosphorylation of the α-subunit at Tyr-10. *Mol Biol Cell* **10**, 2847–2859.

Feschenko MS & Sweadner KJ (1997). Phosphorylation of Na^+/K^+ ATPase by protein kinase C at Ser18 occurs in intact cells but does not result in direct inhibition of ATP hydrolysis. *J Biol Chem* **272**, 17 726–17 733.

Fisone G, Snyder GL, Fryckstedt J, Caplan MJ, Aperia A & Greengard P (1995). Na^+/K^+ATPase in the choroid plexus. Regulation by serotonin/protein kinase C pathway. *J Biol Chem* **270**, 2427-2430.

Fusco A, Berlingieri MT, Di Fiore PP, Portella G, Grieco M & Vecchio G (1987). One- and two-step transformations of rat thyroid epithelial cells by retroviral oncogenes. *Mol Cell Biol* **7,** 3365–3370.

Gebke E, Muller AR, Jurzak M & Gerstberger R (1998). Angiotensin II-induced calcium signalling in neurons and astrocytes of rat circumventricular organs. *Neuroscience* **85**, 509–520.

Goldfarb DA (1994). The renin-angiotensin system. New concepts in regulation of blood pressure and renal function. *Urol Clin North Am* **21**, 187–194.

Grynkiewicz G, Poenie M & Tsien RY (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**, 3440–3450.

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 Physiol Scand* **168**, 301–309.

Ishii H, Jirousek MR, Koya D, Tagagi C, Xia P, Clermont A, Bursell SE, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP & King GL (1996). Amelioration of vascular dysfunctions in diabetics rats by an oral PKC-β inhibitor. *Science* **272**, 728–731.

Karim ZG, Chambrey R, Chalumeau C, Defontaine N, Warnock ND, Paillard M & Poggioli J (1999). Regulation by PKC isoforms of Na^+/H^+ exchanger in luminal membrane vesicles isolated from cortical tubules. *Am J Physiol Renal Physiol* **277**, F773–778.

Kazanietz MG, Caloca MJ, Aizman O & Nowicki S (2001). Phosphorylation of the catalytic subunit of rat renal Na^+/K^+ATPase by classical PKC isoforms. *Arch Biochem Biophys* **388**, 74–80.

Kolesnick RN & Kronke M (1998). Regulation of ceramide production and apoptosis. *Ann Rev Physiol* **60**, 643–665.

Kuwahara M, Miyaji T, Tsubone H & Kuwahara M (2000). Angiotensin type 1 receptor-mediated increase in cytosolic Ca^{2+} and proliferation in mesothelial cells. *Eur J Pharmacol* **388**, 21–27.

Lehtonen JY, Horiuchi M, Daviet L, Akishita M & Dzau VJ (1999). Activation of the de novo biosynthesis of sphingolipids mediates angiotensin II type 2 receptor-induced apoptosis. *J Biol Chem* **274**, 16 901–16 906.

Li X, Lee JW, Graves LM & Earp HS (1998). Angiotensin II stimulates ERK via two pathways in epithelial cells: protein kinase C suppresses a G-protein coupled receptor-EGF receptor transactivation pathway. *EMBO J* **17**, 2574–2583.

Liao DF, Monia B, Dean N & Berk BC (1997). Protein kinase C-zeta mediates angiotensin II activation of ERK1/2 in vascular smooth muscle cells. *J Biol Chem* **272**, 6146–6150.

Liu WS & Heckman CA (1998). The sevenfold way of PKC regulation. *Cell Signal* **10**, 529–542.

Lozano J, Berra E, Municio MM, Diaz-Meco MT, Dominguez I, Sanz L & Moscat J (1994). Protein kinase C zeta isoform is critical for kappa B-dependent promoter activation by sphingomyelinase. *J Biol Chem* **269**, 19200–19202.

McEwan PC, Lindop GB & Kenyon CJ (1996). Control of cell-proliferation in the rat adrenal-gland in vivo by the renin–angiotensin system. *Am J Physiol Endocrinol Metab* **34**, E192–198.

Marakhova II, Vinogradova TA & Toropova FV (2000). Na/K-pump and the cell response to mitogenic signal: regulatory mechanisms and relation to the blast transformation of human blood lymphocytes. *Membr Cell Biol* **14**, 253–261.

Marchant C, Brown L & Sernia C (1993). Renin–angiotensin system in thyroid dysfunction in rats. *J Cardiovasc Pharmacol* **22**, 449–455.

Marsigliante S, Elia MG, Greco S, Di Jeso B, Muscella A, Storelli C (2002). Increase of $[Ca^{2+}]_i$ via activation of ATP receptors in PC-Cl3 rat thyroid cell line. *Cell Signal* **14**, 61–67.

Matillas MJ, Montiel M & Jimenez E (1993). Angiotensin II (AII) and adrenal gland AII receptors in rats with congenital hypothyroidism. *J Endocrinol* **137**, 231–238.

Matsuda T, Murata Y, Tanaka K, Hosoi R, Hayashi M, Tamada K, Takuma K & Baba A (1996). Involvent of Na^+/K^+ATPase in the mitogenic effect of insulin-like growth factor-I on cultured rat astrocytes. *J Neurochem* **66**, 511–516.

Mondorf UF, Geiger H, Herrero M, Zeuzem S & Piiper A (2000). Involvement of the platelet-derived growth factor receptor in angiotensin II-induced activation of extracellular regulated kinases 1 and 2 in human mesangial cells. *FEBS Lett* **472**, 129–132.

Montiel M & Jimenez E (1998). Characterization of angiotensin II receptors (binding and mRNA) in the rat thyroid gland. *J Mol Endocrinol* **20**, 299–304.

Montiel M, Jimenez E, Narvaez JA & Morell M (1984). Aldosterone and plasma renin activity in hyperthyroid rats: effects of propranolol and propylthiouracil. *J Endocrinol Invest* **7**, 559–562.

Muscella A, Aloisi F, Marsigliante S & Levi G (2000). Angiotensin II modulates the activity of the Na^+/K^+ATPase in cultured rat astrocytes via AT1 receptor and PKC-δ activation. *J Neurochem* **74**, 1325–1331.

Muscella A, Greco S, Elia MG, Storelli C & Marsigliante S (2002). Angiotensin II stimulation of Na^+/K^+ATPase activity and cell growth by calcium-independent pathway in MCF-7 breast cancer cells. *J Endocrinol* **173**, 315–323.

Muscella A, Marsigliante S, Carluccio MA, Vinson GP & Storelli C (1997). Angiotensin II AT1 receptors and Na^+/K^+ATPase in human umbilical vein endothelial cells. *J Endocrinol* **155**, 587–593.

Nørby JG (1988). Coupled assay of Na^+/K^+ATPase activity. In *Methods in Enzymology*, vol. 156, part P, ed. Colowick SP & Kaplan NO, p.116. Academic Press, New York.

Norris B, Gonzalez C, Concha J, Palacios S & Contreras G (1991). Stimulatory effect of angiotensin-II on electrolyte transport in canine tracheal epithelium. *Gen Pharmacol* **22**, 527–531.

Pearson J, Bourke JR, Manley SW, Huxham GJ, Matainaho T, Gerard C, Verrier B & Mauchamp J (1988). Electrophysiological correlates of fluid transport in cultured porcine thyroid cells. *J Endocrinol* **119**, 309–314.

Quan A, & Baum M (1996). Endogenous production of angiotensin-II modulates rat proximal tubule transport. *J Clin Invest* **97**, 2878–2882.

Ruiz M, Montiel M, Jimenez E & Morell M (1987). Effect of thyroid hormones on angiotensinogen production in the rat *in vivo* and *in vitro*. *J Endocrinol* **115**, 311–315.

Russo JJ & Sweadner KJ (1993). Na^+/K^+ATPase subunit isoform pattern modification by mitogenic insulin concentrazion in 3T3-L1 preadipocytes. *Am J Physiol Cell Physiol* **264**, C311–316.

Schonwasser DC, Marais RM, Marshall CJ & Parker PJ (1998). Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol Cell Biol* **18**, 790–798.

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-1-induced proliferation of skin keratinocytes. *Diabetes* **50**, 255–264.

Smeds S (1972). A microgel electrophoretic analysis of the colloid proteins in single rat thyroid follicles. II. The protein concentration of the colloid single rat thyroid follicles. *Endocrinology* **91**, 1300–1306

Tang SH & Sharp GWG (1998). Atypical protein kinase C isozyme mediates carbachol-stimulated insulin secretion in RINm5F cells. *Diabetes* **47**, 905–912.

van Blitterswijk WJ (1998). Ceramide conditionally activates atypical protein kinase C, Raf-1 and KSR through binding to their cysteine-rich domains. *Biochem J* **331**, 679–680.

Vasilets LA (1997). Diversity of regulatory phosphorylation of the Na^+/K^+ATPase from mammalian kidneys and *Xenopus* oocytes by protein kinases: characterization of the phosphorylation site for protein kinase C. *Cell Physiol Biochem* **7**, 1–18.

Wooten MW, Seibenhener ML, Matthews LH, Zhou G & Coleman ES (1996). Modulation of zeta-protein kinase C by cyclic AMP in PC12 cells occurs through phosphorylation by protein kinase A. *J Neurochem* **67**, 1023–1031.

Yang LJ, Guo YL, Trygankova O, Li QY, Maloney JA, Steinhauer M & Williamson JR (1999). Epidermal growth factor and angiotensin II regulation of extracellular signal-regulated protein kinase in rat liver epithelial WB cells. *Biochem Pharmacol* **57**, 425–432.

Yingst DR, Davis J & Schiebinger R (2000). Inhibitors of tyrosine phosphatases block angiotensin II inhibition of Na^+ pump. *Eur J Pharmacol* **6**, 49–52.

Zhang C & Mayeux PR (2001). NO/cGMP signaling modulates regulation of Na^+/K^+ATPase activity by angiotensin II in rat proximal tubules. *Am J Physiol Renal Physiol* **280**, F474–479.

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