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Angiotensin AT_1 receptors in Clone 9 rat liver cells: Ca^{2+} signaling and c-*fos* expression

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Abstract

In C9 (Clone 9) liver cells, angiotensin II increased the intracellular Ca^{2+} content, inositol phosphate production and c-*fos* mRNA expression. Other angiotensins were also active with the order of potency being angiotensin II = angiotensin III ≫ angiotensin I > angiotensin IV. Losartan, but not PD 123177 (1-(4-amino-3-methyl)-5-diphenylacetyl-4,5,6,7-tetrahydro-1*H*-imidazo [4,5*c*]pyridine-6-carboxylic acid), blocked the effects of angiotensin II. Pertussis toxin did not alter these actions of angiotensin II. These data indicate that the effects were mediated through angiotensin AT_1 receptors involving pertussis toxin-insensitive G-proteins. Phorbol myristate acetate was also able to increase c-*fos* mRNA expression. The action of angiotensin II was consistently greater than that of the active phorbol ester. Staurosporine but not genistein inhibited this effect of angiotensin II. Angiotensin II- and phorbol myristate acetate-induced proto-oncogene mRNA expression was attenuated in cells incubated overnight with the active phorbol ester, which suggests a major role of protein kinase C.

Keywords: Angiotensin II; Angiotensin AT_1 receptor; C9 clone; Liver cell; c-*fos*

1. Introduction

Angiotensin II is an extremely potent octapeptide hormone that modulates the general metabolism and homeostasis of vertebrates by eliciting a variety of physiological actions on the cardiovascular system, the brain, kidney, adrenal glands, pituitary and liver among many other organs and systems (Inagami et al., 1994; Jackson and Garrison, 1996). It is also a growth factor capable of stimulating cell proliferation and oncogene expression in different cells (Millet et al., 1992; Viard et al., 1992; González-Espinosa and García-Sáinz, 1992, 1995, 1996; Zou et al., 1996).

The actions of this hormone are initiated by its interaction with two major types of receptors, i.e., angiotensin AT_1 and AT_2 receptors. These receptors have been characterized using selective nonpeptide antagonists such as Losartan (AT_1-selective) and PD 123177 (AT_2-selective) (Chiu et al., 1990; De Gasparo et al., 1995). An heterogeneous group of receptors for angiotensin II (atypical receptors) that have in common a much lower affinity for the nonpeptide antagonists has been proposed (De Gasparo et al., 1995).

Angiotensin AT_1 receptors mediate most of the known actions of angiotensin II. They are coupled to the phosphoinositide turnover/Ca^{2+} mobilization signal transduction pathway via pertussis toxin-insensitive G proteins (García-Sáinz and Macías-Silva, 1990; Bauer et al., 1991; Crawford et al., 1991). Depending on the cell system, they are also coupled to other phospholipases and to inhibition of adenylyl cyclase via pertussis toxin-sensitive G-proteins (Jard et al., 1981; Bauer et al., 1991).

An initial action usually observed in response to agents that modulate cell growth and proliferation, such as angiotensin II, is the expression of the so-called immediate early genes (Curran and Morgan, 1987; Ransone and Verma, 1990). Among these early genes are c-*fos* and c-*jun*, whose products, Fos and Jun, constitute AP-1 (Activation Protein-1). AP-1 is a transcription factor that is regarded as a nuclear third messenger that couples short-term stimulation to long-term action by regulating the expression of target genes (Curran and Morgan, 1987; Ransone and Verma, 1990).

In isolated rat hepatocytes, activation of angiotensin AT_1 receptors leads to stimulation of phosphoinositide

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turnover, Ca^{2+} signaling and increased activity of phosphorylase *a* (García-Sáinz and Macías-Silva, 1990; Bauer et al., 1991). There is also evidence that in these cells activation of angiotensin AT_1 receptors inhibits adenylyl cyclase activity (Bauer et al., 1991). In addition, it has been previously shown that stimulation of these receptors leads to an increased expression of the mRNA for c-*fos* (González-Espinosa and García-Sáinz, 1992, 1995, 1996). Protein kinase C and tyrosine protein kinases seem to mediate this action which involves both increases in gene transcription and modulation of mRNA degradation (González-Espinosa and García-Sáinz, 1995).

It is known that during primary culture rat hepatocytes lose their ability to increase the cytosol Ca^{2+} concentra-

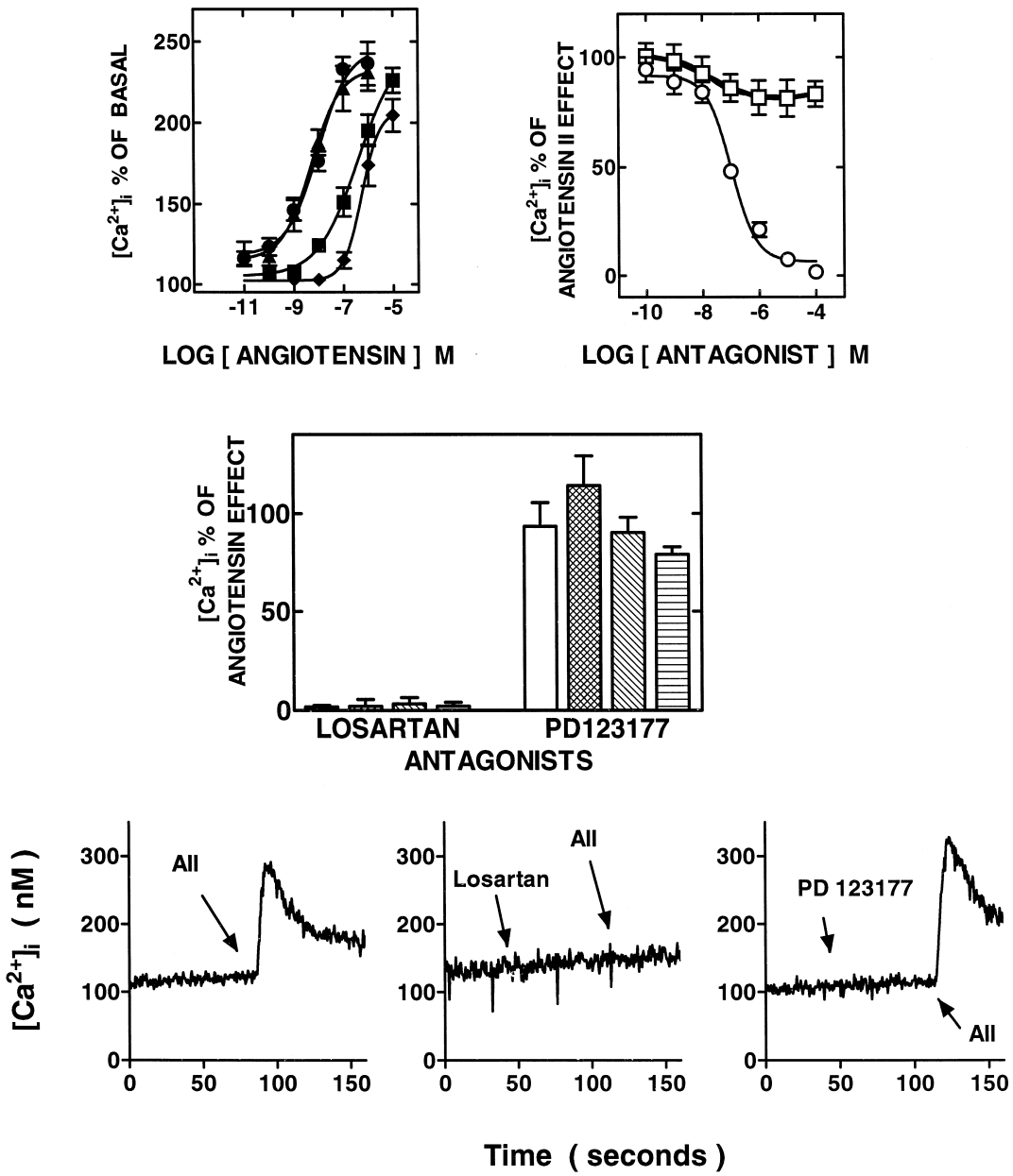

Fig. 1. Effect of angiotensin agonists and antagonists on intracellular Ca^{2+} ($[Ca^{2+}]_i$). Upper left panel: cells were incubated with different concentrations of angiotensin II (solid circles), angiotensin III (solid triangles), angiotensin I (solid squares) or angiotensin IV (solid diamonds). Data are presented as percent of basal $[Ca^{2+}]_i$, which was 137 ± 5 nM. Upper right panel: cells were incubated with the indicated concentration of Losartan (open circles) or PD 123177 (open squares) and challenged after 1–2 min with 100 nM angiotensin II. Data are presented as percent of the effect of angiotensin II. Middle panel: cells were incubated with 10 μM Losartan or 10 μM PD 123177 for 1 min and challenged with 1 μM angiotensin I (open bars), 100 nM angiotensin II (cross-hatched bars), 100 nM angiotensin III (hatched bars) or 10 μM angiotensin IV (lined bars). Data are presented as percent of the effect of each angiotensin in the absence of antagonist. In all cases the means are plotted and vertical lines represent the S.E.M. of 5–7 determinations using different cell preparations; when no error bars are presented they are within the symbol. Lower panel: representative tracings on the effects of 100 nM angiotensin II (A II), 10 μM Losartan and 10 μM PD 123177 on intracellular Ca^{2+}.

tion in response to angiotensin II, an effect mainly due to a drastic decrease in receptor density during culture (Bouscarel et al., 1990). Clone 9 is an epithelial cell line isolated from normal rat liver. Very recently, it was reported that angiotensin II induced the accumulation of inositol phosphates in C9 (Clone 9) liver cells and angiotensin AT_1 receptors were detected in radioligand binding studies (Kozlowski et al., 1993). This prompted us to characterize the effect of angiotensin on signal transduction and c-*fos* expression. Our results indicate that C9 cells could be a very useful model to study the actions of angiotensin II in a hepatic cell line. Nevertheless, differences were observed between the actions of angiotensin II in these cells and in freshly isolated liver cells.

2. Materials and methods

Angiotensins (I, II, III and IV), captopril, staurosporine and phorbol myristate acetate (PMA) were obtained from Sigma. The angiotensin II nonpeptide antagonists, Losartan and PD 123177 (1-(4-amino-3-methyl)-5-diphenylacetyl-4,5,6,7-tetrahydro-1*H*-imidazo [4,5*c*]pyridine-6-carboxylic acid), were generous gifts from DuPont. Fura-2 AM (acetoxymethyl ester) was from Molecular Probes. Ham's F12 medium (Kaighn's modification), fetal bovine serum, trypsin, antibiotics and other reagents used for cell culture were from Gibco. Genistein was obtained from Research Biochemicals International. Myo-[2-^{3}H]inositol (22.9 Ci/mmol), [^{32}P]NAD^+ (800 Ci/mmol) and [^{32}P]dCTP (3000 Ci/mmol) were from New England Nuclear. Pertussis toxin was purified from vaccine concentrates (Sekura et al., 1983; García-Sáinz et al., 1992). The RNA-PCR (RNA-reverse transcription-polymerase chain reaction) GeneAmp kits were obtained from Perkin Elmer. The C9 cell line was obtained from the American Type Culture Collection. Restriction enzymes were from New England Biolabs. Nick translation kits and the c-*fos* Amprobe were from Amersham.

C9 cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 0.25 μg/ml amphotericin B at 37°C under a 95% air/5% CO_2 atmosphere. When the effect of pertussis toxin was tested the cells were incubated for 24 h with or without pertussis toxin (100 ng/ml). Pertussis toxin was present throughout the experiment. Pertussis toxin-catalyzed ADP-ribosylation was carried out as described previously (García-Sáinz et al., 1989).

To determine the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) the cells were loaded with 5 μM Fura-2/AM in Krebs–Ringer–HEPES containing 0.05% bovine serum albumin, pH 7.4 for 1 h at 37°C. Cells were detached by gentle trypsinization. Fluorescence measurements were carried as described (Vázquez-Prado et al., 1997); the 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 Grynkiewicz et al. (1985), using the software provided by AMINCO-Bowman.

C9 cells approaching confluence in 35-mm plates were labeled with [^{3}H]inositol (6 μCi/ml) for 18–24 h to study the production of inositol phosphates. The cells were washed twice with Krebs–Ringer–HEPES buffer containing 1.3 mM $CaCl_2$ and preincubated for 20 min in 2 ml of the same buffer containing 10 mM LiCl, at 37°C in a 5%

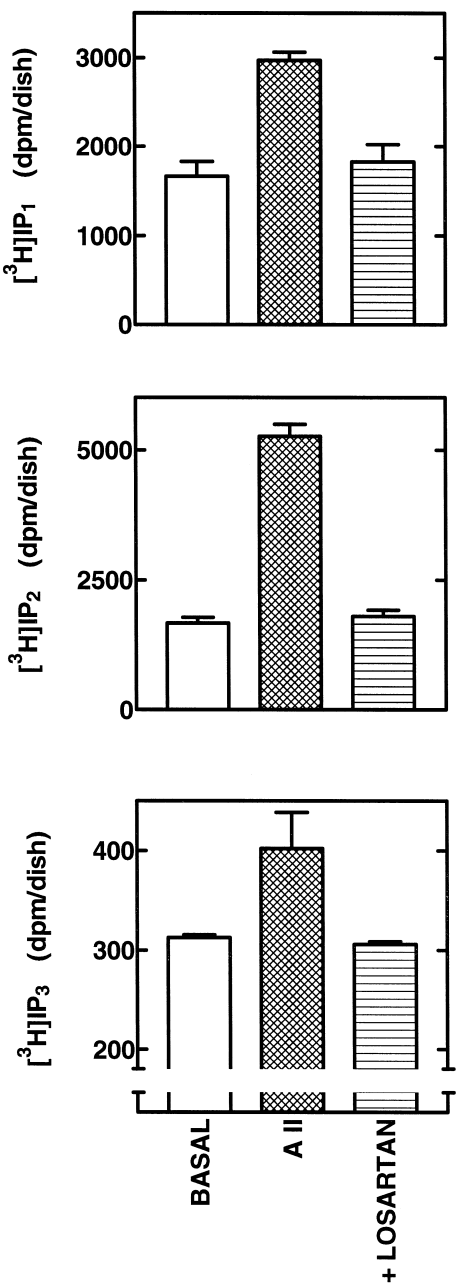

Fig. 2. Effect of angiotensin II and Losartan on inositol phosphate production. [^{3}H]Inositol-labeled cells were incubated in the absence of any agent (BASAL, open bars), 100 nM angiotensin II (A II, cross-hatched bars) or 10 μM Losartan + 100 nM angiotensin II (+Losartan, lined bars). Plotted are the means and vertical lines represent the S.E.M. of four determinations using different cell preparations.

CO_2 atmosphere. Incubations with angiotensin II and/or Losartan were for 30 s and were terminated by the addition of 0.4 ml of 30% ice-cold perchloric acid. Supernatants were neutralized and [^{3}H]inositol phosphates (inositol monophosphate, IP_1; inositol bisphosphate, IP_2 and inositol trisphosphate, IP_3) were separated by Dowex AG1-X8 chromatography (Berridge et al., 1983).

Expression of c-*fos* was determined by using the reverse transcriptase-polymerase chain reaction (RT-PCR) as follows. Total RNA was obtained by the method of Chom-

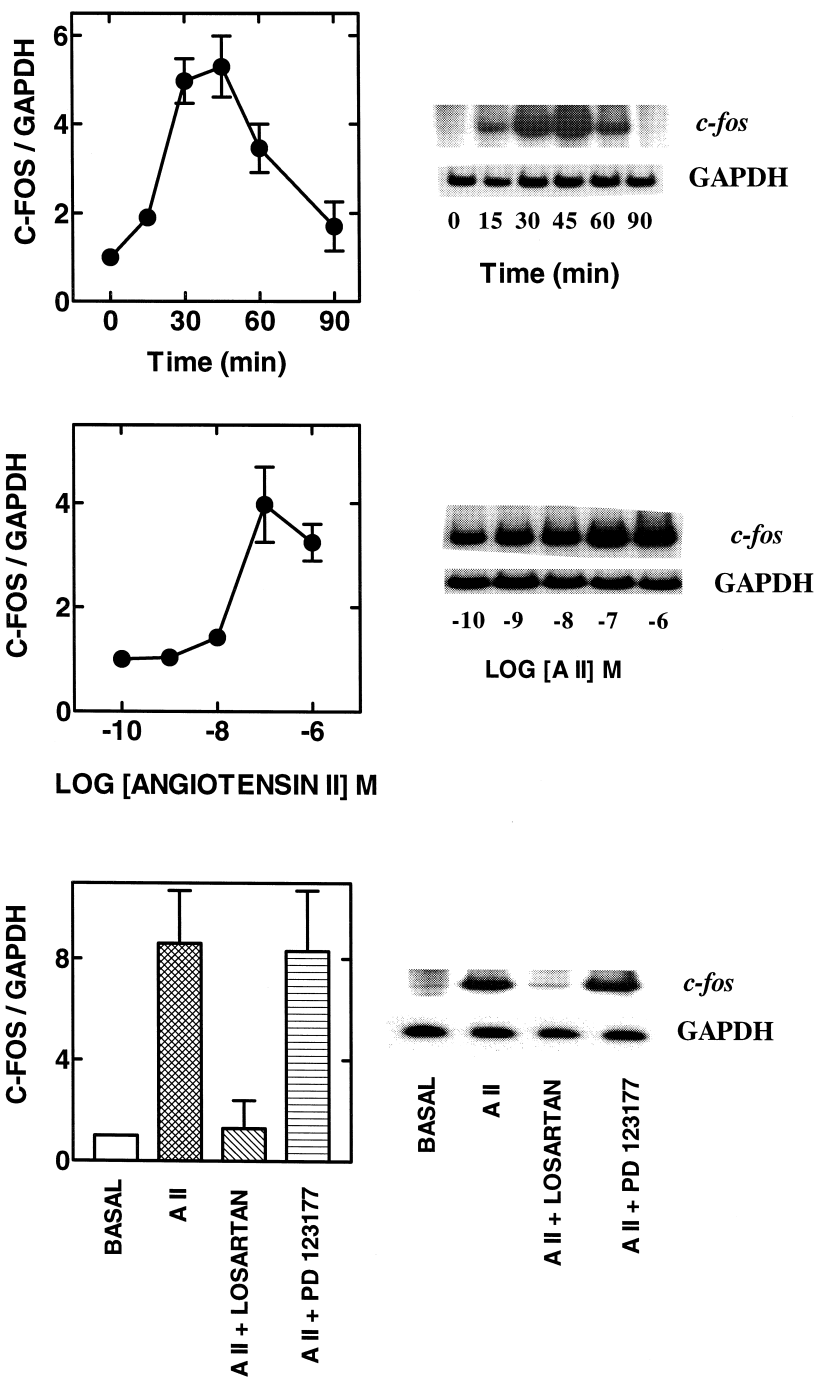

Fig. 3. Effect of angiotensin II and antagonists on c-*fos* expression. Upper panel: cells were incubated for the indicated times in the presence of 100 nM angiotensin II. Middle panel: cells were incubated for 45 min in the presence of different concentrations of angiotensin II. Lower panel: cells were incubated in the absence of any agent (BASAL, open bar), 100 nM angiotensin II (A II, cross-hatched bar), 100 nM angiotensin II + 10 μM Losartan (A II + LOSARTAN, hatched bar) or 100 nM angiotensin II + 10 μM PD 1231777 (A II + PD 123177, lined bar). Data are presented as the c-*fos*/GAPDH ratio which was considered as 1 (100%) in the absence of any stimulus. The means are plotted and vertical lines represent the S.E.M. of 4–7 determinations using different cell preparations. Representative autoradiographs are presented.

czynski and Sacchi (1987) with minor modifications. RNA integrity was routinely checked by electrophoresis on formaldehyde-containing agarose gels and visualization under UV light, using ethidium bromide staining. RT-PCRs were performed with total RNA, using a kit from Perkin Elmer, as described by Nishimura et al. (1992). The primers were: 5′-CACGACCATGATGTTCTCGG-3′ (coding sense) and 5′-AGTAGATTGGCAATCTCGGT-3′ (anticoding sense). These oligonucleotides allowed the amplification of a fragment of 567 bp corresponding to bases 128–695 of c-*fos* cDNA (GeneBank Accesion number X06769) (Curran et al., 1987). The amplification profile consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1.5 min (35 cycles), using 5 μg of total RNA. The identity of the fragment was characterized by its apparent size on ethidium bromide-stained polyacrylamide gels, Southern blot hybridization with Nick translation-labeled c-*fos* Amprobe and direct sequencing of the fragment cloned into the SrfI site of pCR-Script SK(+) plasmid. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined in each sample in which c-*fos* expression was assayed. GAPDH was determined also by RT-PCR using the primers 5′-TCC-CTCAAGATTGTCAGCAA-3′ (coding sense) and 5′-AGATCCACAACGGATACATT-3′ (anticoding sense). These oligonucleotides allowed the amplification of a fragment of 309 bp corresponding to bases 506–814 of GAPDH cDNA (GeneBank Accesion number X06769) (Fort et al., 1985). The amplification conditions were identical to those described above but 25 cycles were used. The identity of the fragment was characterized by its apparent size on ethidium bromide-stained polyacrylamide gels and Southern blot hybridization with Nick translation-labeled rat GAPDH complete cDNA (Fort et al., 1985). The RT-PCR

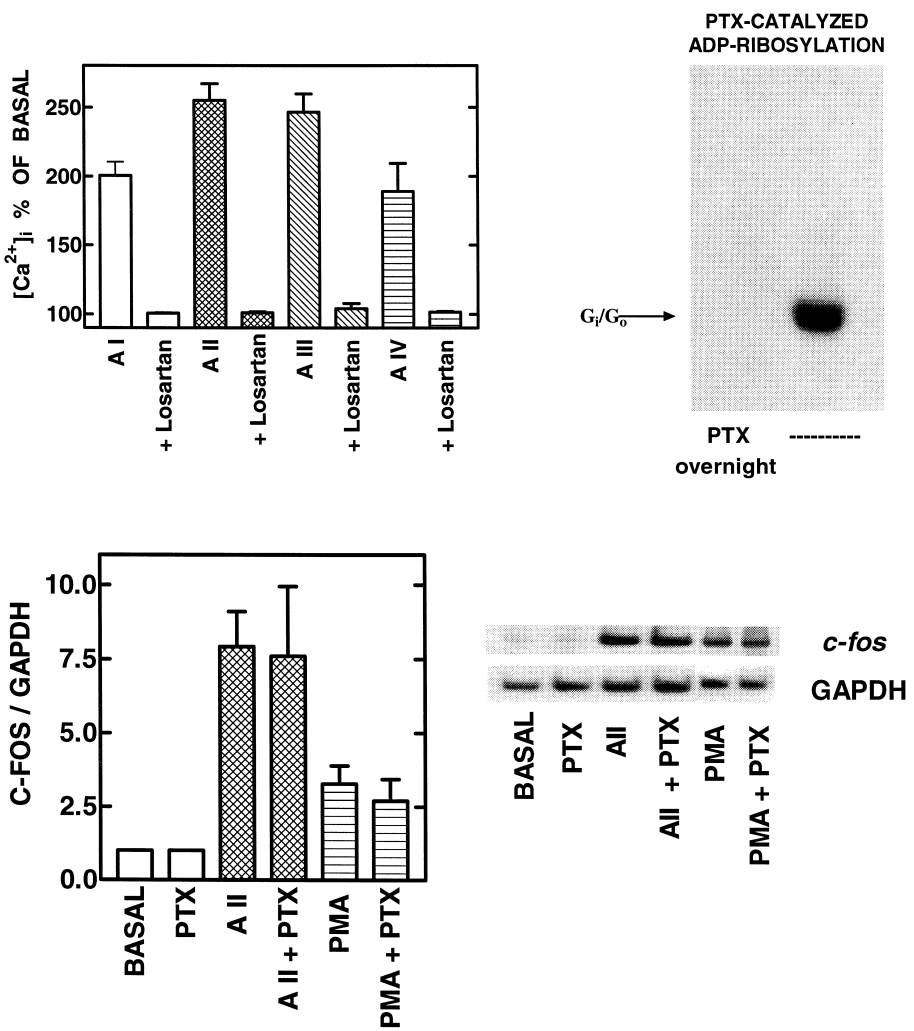

Fig. 4. Effect of pertussis toxin on the actions of angiotensin on intracellular Ca^{2+} and c-*fos* mRNA expression. Upper left panel: cells treated with pertussis toxin were incubated in the presence of 1 μM angiotensin I (A I, open bars), 100 nM angiotensin II (A II, cross-hatched bars), angiotensin III (A III, hatched bars) or 3 μM angiotensin IV (A IV, lined bars). Where indicated 10 μM Losartan was added 1 min before the agonists. Data are presented as percent of basal $[Ca^{2+}]_i$, which was 120 ± 7 nM. Upper right panel: representative autoradiograph of pertussis toxin-catalyzed ADP-ribosylation of membranes from cells preincubated overnight in the absence (---) or presence of pertussis toxin. Lower panel: cells were preincubated in the absence or presence of pertussis toxin (PTX) and were challenged with 100 nM angiotensin II (cross-hatched bars) or PMA (lined bars). The means are plotted and vertical lines represent the S.E.M. of 4–7 determinations using different cell preparations. A representative autoradiograph is presented.

reactions were supplemented with 1 μCi/tube of [^{32}P]dCTP and the products were electrophoresed on 7.5% polyacrylamide gels; the gels were dried and the amount of label incorporated into the product was quantified by PhosphorImager analysis. Gels were also exposed to X-OMAT X-ray-film (Kodak) at −80°C with an intensifying screen. At least five independent experiments were performed for each treatment.

In all cases the results represent the means ± S.E.M. of at least four experiments using different cell cultures. K_i values were calculated according to Cheng and Prusoff (1973). Data were analyzed and plotted by using commercial software (Prism 2.01, GraphPad Software). When autoradiographs were analyzed, a representative experiment is shown.

3. Results

Angiotensin II induced a dose-dependent immediate increase (~2.5-fold) in $[Ca^{2+}]_i$ with an EC_{50} of ~5 nM (Fig. 1, upper left panel). The effect of other angiotensins was tested: angiotensin III also increased $[Ca^{2+}]_i$ with similar efficacy and potency (Fig. 2, upper left panel) but angiotensin I and angiotensin IV were much less potent (EC_{50} values of ~300 nM and ~500 nM, respectively) than angiotensin II. The effect of 100 nM angiotensin II was dose dependently inhibited by the angiotensin AT_1 receptor antagonist Losartan (IC50 ~100 nM, K_i ~4.7 nM). In contrast, the AT_2 antagonist, PD 123177 was without effect (Fig. 1, upper right panel). The effects of the different angiotensins were inhibited by Losartan but not

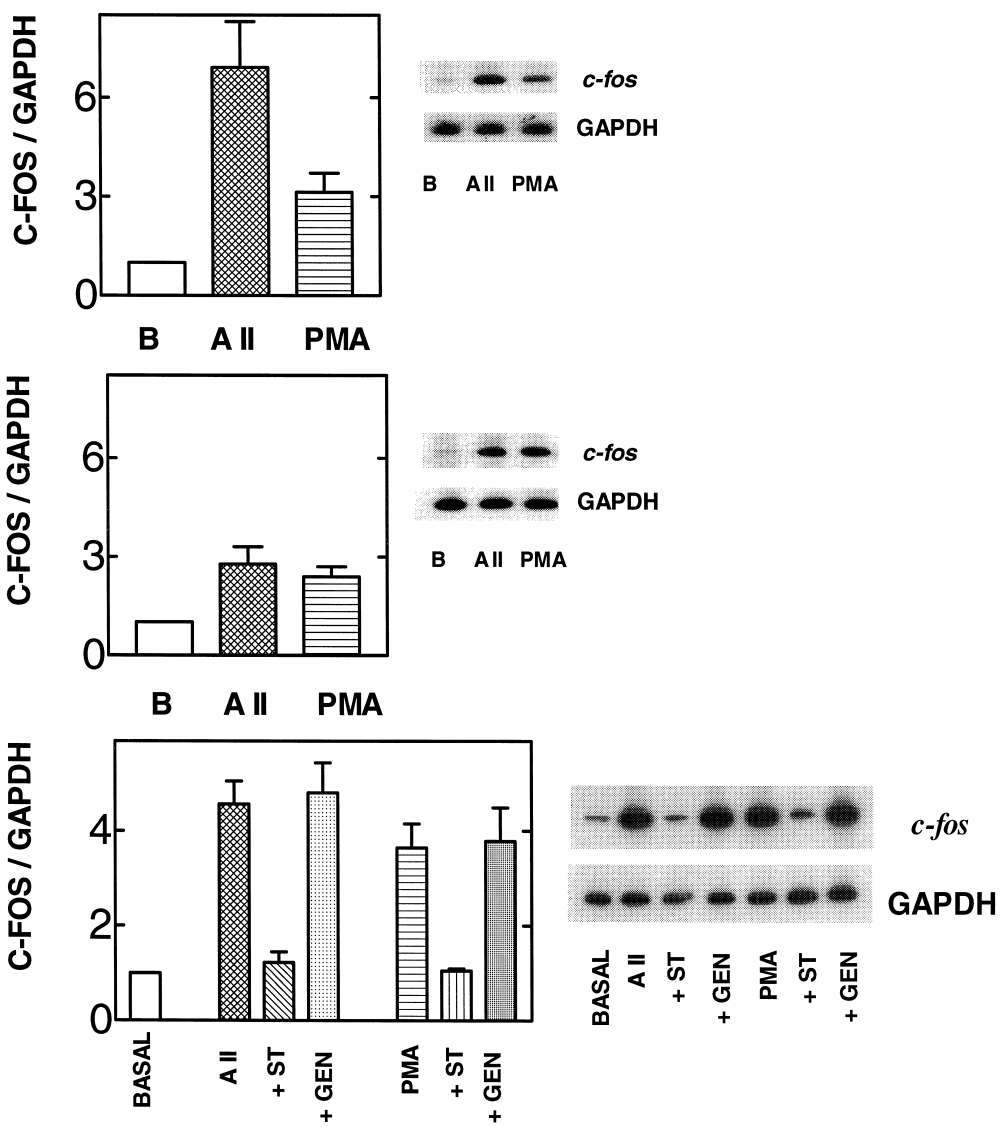

Fig. 5. Effect of PMA and angiotensin III on c-*fos* mRNA expression. Cells were preicubated in the absence (upper and lower panels) or presence of 1 μM PMA for 24 h. The cells were washed and challenged with 100 nM angiotensin II (cross-hatched bars) or 1 μM PMA (lined bars). Where indicated, cells were incubated with the agonists in the presence of 1 μM staurosporine (+ST) or 10 μM genistein (+GEN). The means are plotted and vertical lines represent the S.E.M. of 4–7 determinations using different cell preparations. Representative autoradiographs are presented.

by PD 123177 (Fig. 1, lower panel). The antagonists by themselves were without effect on basal $[Ca^{2+}]_i$ (data now shown). Captopril 100 μM was without effect on basal $[Ca^{2+}]_i$ and did not modify the actions of angiotensin I or angiotensin II on this parameter (data not shown).

The ability of 100 nM angiotensin II to increase the production of inositol phosphates was next studied. It was observed that angiotensin II increased the production of $[^3H]IP_1$, $[^3H]IP_2$ and $[^3H]IP_3$ from inositol-labeled cells as early as 30 s after agonist addition (Fig. 2). At longer times, the effect of angiotensin II was mainly observed on $[^3H]IP_1$ and $[^3H]IP_2$ but no effect was observed on $[^3H]IP_3$ (data not shown). The effect of angiotensin II was completely blocked by Losartan (Fig. 2); the antagonist by itself was without effect on this parameter (data not shown).

We next examined the ability of angiotensin II to increase c-*fos* expression by using Northern blot analysis. However, this approach was not successful since we were unable to detect basal c-*fos* expression and only a weak signal was observed under stimulated conditions. In order to circumvent this technical problem, we performed RT-PCR reactions supplemented with $[^{32}P]$dCTP, as indicated in Materials and Methods, to detect the expression of the mRNA of this proto-oncogene. Conditions were defined in order to obtain semi-quantitative data in a consistent way, with signal linearity with respect to the amount of sample (at least 10-fold). As indicated, the identity of the product was confirmed by its size, using Southern analysis and direct sequencing. In addition, in all experiments, the expression of the mRNA of GAPDH was also studied. This is an enzyme of intermediary metabolism, whose expression usually remains stable under most conditions and represents a general control for basal gene expression and mRNA recovery and stability.

As it can be observed in Fig. 3, angiotensin II induced a marked (5- to 7-fold) stimulation of c-*fos* mRNA expression without altering that of GAPDH. The effect of angiotensin II was rapid, reaching a maximum in 30–45 min and decreasing after this time (Fig. 3, upper panel). It was dose dependent with an EC_{50} of $\sim$30 nM, (Fig. 3, middle panel) and it was blocked by Losartan but not by PD 123177 (Fig. 3, lower panel). The antagonists by themselves were without effect on this parameter (data not shown).

We next examined the effect of pertussis toxin on the actions of angiotensin II. It can be observed in Fig. 4, upper right panel, that treatment of the cells with pertussis toxin completely blocked pertussis toxin-catalyzed $[^{32}P]$ADP-ribosylation in membranes in vitro. This data indicate that $>95\%$ of the available substrates (putatively G_i/G_o isoforms) were covalently modified by the toxin. Under these conditions, we studied the ability of angiotensin II to increase $[Ca^{2+}]_i$ and c-*fos* expression. Pertussis toxin treatment by itself was without effect on these parameters and did not alter the effects of angiotensin II (Fig. 4).

The effect of protein kinase C activation on c-*fos* mRNA expression was studied. It was observed that PMA induced 2- to 3-fold increase in the expression of this proto-oncogene (Figs. 4 and 5). The effect of PMA was unaffected by pertussis toxin treatment (Fig. 4) but was blocked by the serine/threonine protein kinase inhibitor, staurosporine, but not by the tyrosine kinase inhibitor, genistein. (Fig. 5, lower panel). In many cells, prolonged treatment with PMA leads to protein kinase C depletion (downregulation). Incubation of C9 cells with 1 μM PMA overnight reduced the subsequent effect of this agent on c-*fos* mRNA expression, but did not abolish it (Fig. 5, middle panel). Interestingly, when the effect of angiotensin II was tested, it was observed that overnight treatment with PMA decreased the effect of the vasopressor peptide on c-*fos* expression (Fig. 5, middle panel). Similarly, it was observed that staurosporine, but not genistein, markedly decreased the effect of angiotensin on this parameter (Fig. 5, lower panel).

4. Discussion

Our present results indicate that C9 cells can be used to study the actions of angiotensin II in a hepatic cell line in culture. This is of interest since cultured hepatocytes do not respond to this vasopressor peptide (Bouscarel et al., 1990; and unpublished data). This is an important limitation since procedures that require long term incubations, such as pertussis toxin treatment or overnight treatments with protein kinase C activators, cannot be performed. C9 cells represent an interesting alternative because they are derived from normal liver and retain an epithelial morphology (Weinstein et al., 1975). It is known that these cells are responsive to other hormones such as insulin, oxytocin and thyroid hormones (Haber et al., 1988; Barhoumi et al., 1996; Shetty et al., 1996).

Angiotensin II induced rapid increases in $[Ca^{2+}]_i$ and $[^3H]$inositol phosphates. The order of potency of the different angiotensins was as expected for an angiotensin II receptor, although it was surprising that angiotensin I and angiotensin IV induced a relatively large increase in $[Ca^{2+}]_i$. We considered the possibility that the action of angiotensin I could be due to its conversion to angiotensin II. However, the insensitivity to captopril and the fact that the effect of angiotensin I was immediate indicated that this was not the case. In addition, Losartan completely blocked the action of all the different angiotensins. The apparent affinity for this nonpeptide antagonist was very high, as reflected by the K_i value, which indicates that the actions were mediated through angiotensin AT_1 receptors. This is consistent with what has been observed in rat hepatocytes (García-Sáinz and Macías-Silva, 1990: Bauer et al., 1991).

The effect of angiotensin II on $[^3H]$inositol phosphate production was studied at early times in order to observe

the accumulation of the second messenger, IP_3. As indicated, at later times IP_2 and IP_1 were accumulated, even in the presence of LiCl, as observed in other systems.

Our main interest was c-*fos* mRNA expression. In C9 cells the time course of action and the dose–response curves for angiotensin II were similar to those observed in freshly isolated hepatocytes (González-Espinosa and García-Sáinz, 1992). The almost 10-fold difference in EC_{50} values observed for the actions on $[Ca^{2+}]_i$ and c-*fos* mRNA expression is not surprising because it has been observed in freshly isolated hepatocytes and could be due to the different time course of the effects, hormone degradation or intrinsic differences in the signaling events involved, as discussed elsewhere (García-Sáinz et al., 1995). The ability of Losartan, but not PD 123177, to block angiotensin II action clearly indicate that in C9 cells this effect is mediated through angiotensin AT_1 receptors.

The ability of angiotensin II to increase c-*fos* mRNA expression was pertussis toxin-insensitive, which strongly suggests that G_i/G_o were not involved. We have been unable to address this aspect in liver cells because pertussis toxin requires a relatively long time to penetrate the cells and exert its action. The administration of pertussis toxin to whole rats markedly altered liver c-*fos* mRNA expression (unpublished data) and, as mentioned, the action of angiotensin II cannot be properly studied in cultured hepatocytes. It is interesting to mention that the effect of angiotensin II on c-*fos* mRNA expression involves both an increase in gene transcription and modulation of the half-life of mRNA (González-Espinosa and García-Sáinz, 1996). Angiotensin II modulates the synthesis and expression of mRNA for angiotensinogen (Klett et al., 1990, 1993). Interestingly, the effect of angiotensin on angiotensinogen is pertussis toxin-sensitive. It is related to the ability of angiotensin II to inhibit adenylyl cyclase and is mainly due to stabilization of angiotensinogen mRNA (Klett et al., 1990, 1993).

In our previous studies with isolated liver cells, the effect of angiotensin II was partially inhibited by staurosporine and also by genistein, which suggests that protein kinase C and protein tyrosine kinases have a role (González-Espinosa and García-Sáinz, 1995). There is evidence that protein tyrosine kinases participate in mediating some of the long-term actions of angiotensin II (Huckle et al., 1990; Marrero et al., 1994, 1995; Earp et al., 1995) but not in others, which seem to be mediated mainly by protein kinase C. In the present experiments a major role of protein kinase C was also evidenced by the ability of staurosporine and overnight pretreatment with PMA to clearly decrease the effect of the peptide hormone. We were unable to detect any action of genistein. Staurosporine was able to completely block the effect of angiotensin II but it seems unlikely that the protein kinase C family of enzymes exclusively mediate the effect of angiotensin II on proto-oncogene mRNA since the effect of the hormone was consistently greater than that of a maximally effective concentration of PMA (1 μM). It is known that staurosporine is only relatively selective for protein kinase C and that this agent can inhibit other protein kinases. Therefore, it seems possible that kinases other than protein kinase C may be involved in the action of angiotensin II and could be inhibited by staurosporine. However, the identity of these putative protein kinases remains to be determined.

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