



## Angiotensin II Receptor Internalization and Signaling in Isolated Rat Hepatocytes

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**ABSTRACT.** Since angiotensin II (Ang II)-induced receptor internalization is required to maintain the production of certain intracellular signals in some target cells, we investigated the relationships between Ang II receptor endocytosis and the generation of second messengers in rat hepatocytes. The results of the present study demonstrate that in response to exposure of hepatocytes to Ang II, a decrease in surface Ang II receptors occurred, consistent with a rapid endocytosis of the receptor-bound hormone complex. Pretreatment of cells with okadaic acid (OA) did not have any effect on receptor-mediated internalization. In contrast, a marked reduction of the Ang II receptor endocytosis process occurred after treatment of hepatocytes with phenylarsine oxide (PAO), indicating that cysteine residues could be involved in receptor-mediated endocytosis. Stimulation of cells with Ang II blocked the generation of cyclic adenosine monophosphate (cAMP), which follows the stimulation of hepatocytes with forskolin. Moreover, Ang II increased both inositol 4,5-bisphosphate ( $IP_2$ ) and inositol 1,4,5-trisphosphate ( $IP_3$ ) generation, and enhanced intracellular calcium concentration ( $[Ca^{2+}]_i$ ). Exposure of cells to PAO did not alter the effect of Ang II on the accumulation of cAMP after forskolin stimulation, indicating that endocytosis of the agonist–receptor complex is not involved in adenylate cyclase inhibition. Conversely, PAO and OA markedly reduced  $IP_2$  and  $IP_3$  synthesis, and the plateau phase of Ang II-induced  $Ca^{2+}$  mobilization. The relationship between Ang II-induced endocytosis and the generation of phosphoinositols and increment in  $[Ca^{2+}]_i$  indicates that sequestration of the Ang II receptor is necessary to maintain the production of these intracellular signals in rat hepatocytes. *BIOCHEM PHARMACOL* 57:10: 1125–1131, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** angiotensin II receptors; angiotensin II receptor internalization; angiotensin II signaling; rat hepatocytes.

Ang II§ elicits a variety of physiological effects through its interaction with specific receptors located at the surface of the plasma membrane of target cells. Two distinct Ang II receptor subtypes, termed  $AT_1$  and  $AT_2$ , have been identified using non-peptide and peptide antagonists [1, 2]. The primary effector mechanism activated by  $AT_1$  receptor subtypes is the hydrolysis of phosphoinositides by PLC [3], which generates the second messengers  $IP_3$  and diacylglycerol (DAG). Each of these two metabolites activates a different pathway; DAG directly activates PKC, whereas  $IP_3$  mobilizes  $Ca^{2+}$  from the endoplasmic reticulum. Ang II is also able to inhibit adenylate cyclase activity via a *pertussis* toxin-sensitive G-protein [4].

In intact cells or tissues, exposure of  $AT_1$  receptors to agonist often leads to a rapid loss of receptor responsiveness.

Two mechanisms have been proposed for this process of desensitization. The first involves a rapid functional uncoupling of the receptors from G-proteins, mediated by phosphorylation of the receptor by specific kinases [5, 6]. The second mechanism is internalization, which transports receptors away from the cell surface, making them inaccessible to further agonist interaction [7, 8]. However, Ang II receptor endocytosis may also be required for the maintenance of several mechanisms of intracellular signaling. Thus, Ang II receptor sequestration is necessary for the maintenance of the sustained intracellular calcium signal and PKC activation in adrenal zona glomerulosa cells [9, 10], and PLC activation in the apical surface of proximal tubule cells [11], but not for  $IP_3$  release and PLC activation in adrenal zona glomerulosa and the basolateral surface of proximal tubule cells [10, 11].

On the basis of these observations, which show that differences exist between Ang II receptor internalization and the production of second messengers in various target cells, the purpose of this study was to investigate the relationship between Ang II receptor endocytosis and the generation of intracellular signals in Ang II-stimulated rat hepatocytes.

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§ Abbreviations: Ang II, angiotensin II, cAMP, cyclic adenosine monophosphate;  $[Ca^{2+}]_i$ , intracellular calcium concentration;  $IP_2$ , inositol 4,5-bisphosphate;  $IP_3$ , inositol 1,4,5-trisphosphate, OA, okadaic acid, PAO, phenylarsine oxide;  $IP_1$ , inositol monophosphate; PKC, protein kinase C; and PLC, phospholipase C.

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## MATERIALS AND METHODS

### Materials

myo-[2-<sup>3</sup>H]-Inositol (specific activity 20.5 Ci/mmol) and [<sup>125</sup>I]-Ang II (specific activity 2200 Ci/mmol) were purchased from Amersham International plc. and DuPont/NEN, respectively. Collagenase type A was from Boehringer Mannheim. Fura 2-acetoxymethyl ester was from Molecular Probes. The anion exchange resin AG 1-X8 (formate form) was from BioRad, and the kit for cAMP determination was obtained from Amersham International plc. Ang II, RPMI 1640 medium, proteinase inhibitors, pluronic F-127, and other drugs and reagents were from Sigma Chemical Co.

### Isolation and Incubation of Hepatocytes

Male adult Wistar rats (200–250 g weight) were housed under a schedule of 14-hr light:10-hr darkness and maintained on a normal laboratory diet with tap water available *ad lib*. Hepatocytes were isolated by perfusion of the liver with collagenase using a previously described method [12]. Cells were resuspended and maintained in RPMI 1640 medium, supplemented with 25 mM HEPES, 0.1% BSA, and 0.01% bacitracin, at 37° with continuous agitation (100 strokes/min). The gas phase was 95% O<sub>2</sub>/5% CO<sub>2</sub>. The viability of the isolated hepatocytes was evaluated by the trypan blue exclusion test (usually 85% of the cells excluded the stain).

### Internalization Assay in Hepatocytes

Cells were incubated with 100 nM Ang II for the indicated times to allow receptor internalization. Internalization and recycling of receptors was terminated by washing the cells with cold PBS. All subsequent manipulations were performed at 4°. Cell surface-bound Ang II was removed by exposure to 50 mM glycine, pH 3.0, containing 150 mM NaCl for 5 min, followed by two washes with PBS. Hepatocytes were then resuspended in 50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl, 6.5 mM MgCl<sub>2</sub>, 0.1% BSA and proteinase inhibitors (1 µg/mL of leupeptin and aprotinin, and 0.1 µg/mL of bacitracin and phenylmethylsulphonyl fluoride).

In another series of experiments, cells were preincubated in the absence or in the presence of 20 µM PAO or 100 nM OA for 10 min at 37°, and the proportion of surface Ang II receptor was determined 20 min after Ang II stimulation. Control groups were set up in which hepatocytes were treated only with 10 µL of ethanol solution, the vehicle for solubilization of drugs.

### [<sup>125</sup>I]-Ang II Binding to Surface Cells

To measure the Ang II receptor remaining on the cell surface, hepatocytes were incubated with 0.2 nM [<sup>125</sup>I]-Ang II for 3 hr at 4°. Non-specific binding was determined in

the presence of 10 µM unlabeled Ang II. Bound and free [<sup>125</sup>I]-Ang II were separated by centrifugation at 1000 g for 5 min. After a further wash in PBS, the radioactivity of the cells was measured in a γ-counter (Diagnostic Products Corporation).

### cAMP Assay

Extraction of cAMP from hepatocytes was performed according to Horton and Baxendale [13]. Briefly, 1 mL of ice-cold ethanol was added to 0.5 mL of cell suspension (10<sup>6</sup> cells). Cells were allowed to settle and were re-extracted twice. The combined supernatants were centrifuged (2000 g for 15 min at 4°) and evaporated under a stream of nitrogen at 45°. The dried residue was dissolved in 200 µL of assay buffer, and 50-µL aliquots were analyzed by radioimmunoassay with [8-<sup>3</sup>H]-adenosine 3',5'-cyclic phosphate as tracer and a binding protein purified from bovine muscle, which has a high specificity and affinity for cAMP. Separation of the protein-bound cAMP from the unbound nucleotide was achieved by adsorption of the free nucleotide onto coated charcoal, followed by centrifugation.

### Phosphoinositols Determination

Cells were labeled for 90 min with 20 µCi/mL myo-[2-<sup>3</sup>H]-inositol in RPMI 1640. Thereafter, the medium was removed and the cells were rinsed several times with PBS to remove free [<sup>3</sup>H]inositol. Cells were incubated at 37° for an additional 30 min in fresh medium, containing 10 mM LiCl, in the absence or in the presence of 20 µM PAO or 100 nM OA. After a 10-sec agonist stimulation, the reaction was terminated by addition of 0.5 mL of 10% trichloroacetic acid, which was then removed by five washes with diethyl ether. Phosphoinositols were separated using ion exchange chromatography [14]; inositol monophosphate (IP<sub>1</sub>), IP<sub>2</sub> and IP<sub>3</sub> were serially eluted with 5 mM disodium tetraborate, 180 mM sodium formate; 0.1 M formic acid, 0.4 M ammonium formate; and 0.1 M formic acid, 1 M ammonium formate, respectively. The radioactivity in the eluates was determined using a Beckman liquid scintillation spectrometer.

### Determination of [Ca<sup>2+</sup>]<sub>i</sub>

To measure the [Ca<sup>2+</sup>]<sub>i</sub>, the hepatocytes were loaded with fura 2-acetoxymethyl ester (final concentration 5 µM) at 37° with continuous shaking (100 cycles/min) for 15 min and in the presence of 20 µg/mL pluronic F-127 to encourage dispersion of the probe. After washing and centrifugation (50 g for 1 min) twice in a modified Krebs-Ringer buffer in which the bicarbonate was replaced by 20 mM HEPES, pH 7.4, the hepatocytes were incubated again at 37° for 5 min to facilitate hydrolysis of the esterified probe, and centrifuged one more time. The hepatocytes were resuspended in the same buffer containing

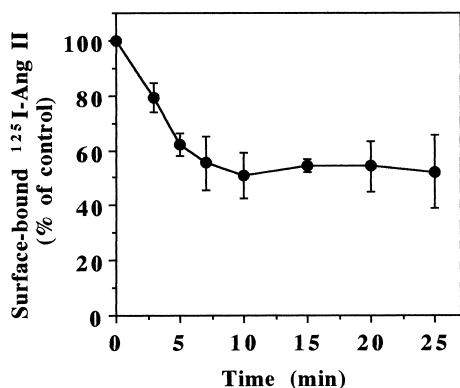


FIG. 1. Internalization of Ang II receptors in rat hepatocytes as a function of time at 37°. Cells were exposed to 100 nM Ang II, and surface Ang II receptors were determined at the time indicated as described in Materials and Methods. Results are expressed as the means  $\pm$  SD of four separate experiments.

0.5% BSA, and 2 mL of the cell suspension ( $10^6$  cells/mL) was placed in a fluorescence cuvette with continuous stirring. The fluorescence intensity was recorded at 510 nm in a F-2000 Hitachi spectrofluorimeter (Hitachi Ltd.) using a dual-excitation source at 340 and 380 nm. The maximal fluorescence was determined at the end of the assay by adding 10  $\mu$ L 10% SDS and the minimal fluorescence by adding 15  $\mu$ L 0.5 M EGTA solution, pH 9.0. The cytoplasmic  $Ca^{2+}$  concentration was calculated from the fluorescence ratio [15].

### Statistical Analysis

Results were expressed as means  $\pm$  SD and were analyzed by ANOVA followed by post-hoc analysis with the Fisher test. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

### Ang II Receptor Internalization

Initial experiments set out to investigate hormone–receptor complex internalization from the surface of hepatocytes as a function of time. Treatment of cells with 100 nM Ang II resulted in a rapid time-related loss of surface Ang II receptors, consistent with a rapid endocytosis of the receptor-bound hormone which was maximal within 10 min ( $50.4 \pm 5.7\%$  of control;  $P < 0.05$ ) and remained constant thereafter for 25 min. The time necessary to internalize 50% of the ligand–receptor complex ( $t_{1/2}$ ) was approximately 2.5 min (Fig. 1).

In the presence of 20  $\mu$ M PAO, a molecule that inhibits the internalization of several surface receptors [16, 17], the amount of surface Ang II receptor in hepatocytes stimulated with agonist was similar to that in unstimulated cells ( $85.6 \pm 10.5\%$  of control), while in the presence of 100 nM OA, a potent inhibitor of protein phosphatase 1 and protein phosphatase 2A, no inhibition of receptor endocytosis was observed following Ang II treatment ( $55.6 \pm 9.8\%$  of control;  $P < 0.05$ ).

### Ang II Effects on Second Messengers Generation

It was previously established that Ang II receptors can be coupled to distinct signal transduction pathways in response to Ang II. The time-courses of cAMP inhibition in response to the stimulation of hepatocytes with 100 nM Ang II are summarized in Fig. 2. Incubation with 10  $\mu$ M forskolin evoked a 5-fold increment in cAMP formation with respect to control (unstimulated cells). Ang II transiently and significantly blocked the forskolin-induced cAMP accumulation, indicating that this agonist regulates adenylate cyclase activity. The maximum inhibitory effect of Ang II occurred between 10 and 15 min after exposure (Fig. 2).

In contrast to the inhibitory effect of Ang II on cAMP accumulation, the agonist stimulated phosphoinositide metabolism in isolated rat hepatocytes (Fig. 3). The presence of 100 nM Ang II substantially stimulated phosphoinositol formation in cells as a consequence of PLC activation.  $IP_2$  and  $IP_3$  levels increased rapidly, whereas  $IP_1$  was not significantly elevated. Moreover, Ang II induced intracellular calcium mobilization with an initial transient increase in  $[Ca^{2+}]_i$  which was followed by a sustained plateau phase that persisted for at least 4 min of the observation period (Fig. 4).

### Effects of PAO and OA on the Generation of Second Messengers

To determine if the process of second messenger generation was related to the receptor-mediated endocytosis and/or receptor dephosphorylation, further studies were carried out in which both mechanisms were blocked by pretreatment of cells with PAO or OA. The Ang II-induced inhibition of forskolin-stimulated cAMP generation reported above was unaffected

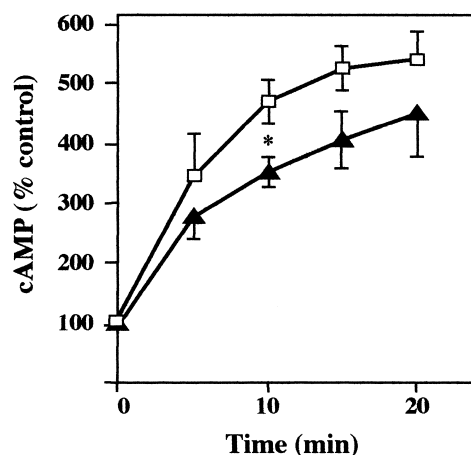


FIG. 2. Time-course of inhibition of forskolin-stimulated cAMP accumulation by Ang II. Cells were preincubated in the presence of 1 mM isomethylbutylxanthine for 15 min at 37°, and were then exposed to 10  $\mu$ M forskolin in the absence ( $\square$ ) or presence of 100 nM Ang II ( $\blacktriangle$ ). Results are expressed as the means  $\pm$  SD of three separate experiments. Statistical significance was defined as \* $P < 0.05$ .

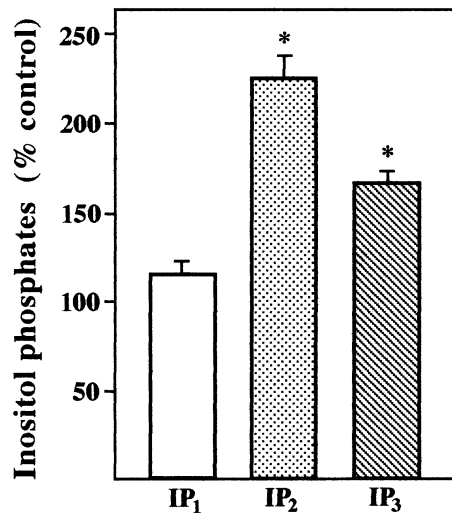


FIG. 3. Effect of Ang II on phosphoinositide production. Pre-labeled myo-[2-<sup>3</sup>H]-inositol hepatocytes were incubated in the presence of 10 mM LiCl for 30 min at 37° before Ang II stimulation (100 nM). Phosphoinositols were separated as described in Material and Methods. Results are expressed as the means  $\pm$  SD of three individual experiments. For probability calculations phosphoinositols levels were compared versus control. Statistical significance was defined as \* $P < 0.05$

by preincubation of hepatocytes with 20  $\mu$ M PAO for 10 min (Fig. 5). Conversely, pretreatment of cells with 100 nM OA significantly enhanced Ang II-induced inhibition of forskolin-stimulated cAMP production (Fig. 5).

Exposure of cells to 20  $\mu$ M PAO or 100 nM OA significantly reduced the generation of both IP<sub>2</sub> and IP<sub>3</sub> that follows Ang II stimulation (Table 1). Furthermore, preincubation of the cells with 100 nM OA slightly decreased the initial peak response to Ang II, but markedly reduced the sustained phase of the  $[Ca^{2+}]_i$  response by up to 70%. (Fig. 6). Similarly, pretreatment of hepatocytes with 20  $\mu$ M PAO did not significantly reduce the rapid and

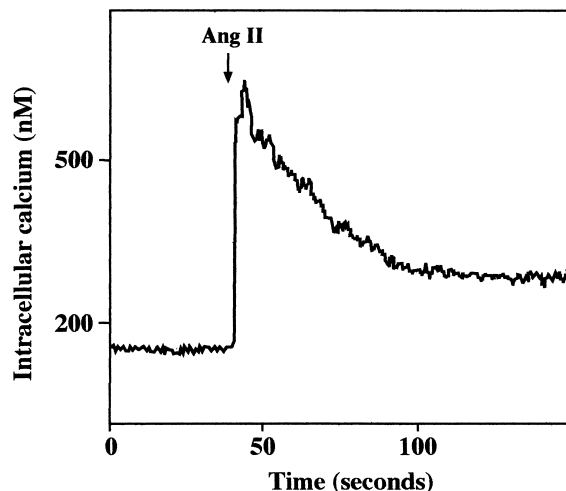


FIG. 4. Representative tracing of the  $[Ca^{2+}]_i$  response to 100 nM Ang II in rat hepatocytes.

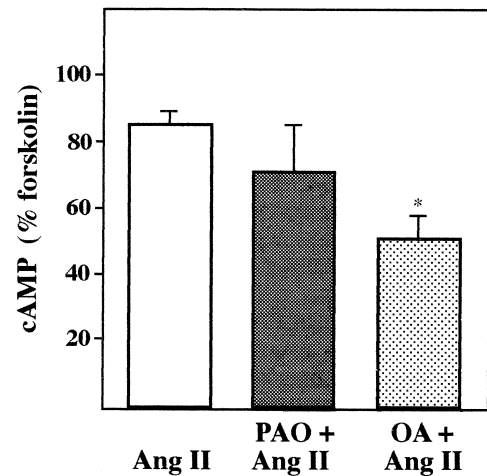


FIG. 5. Effect of Ang II and Ang II plus PAO or OA on forskolin-stimulated cAMP accumulation. Hepatocytes were preincubated in the presence of 1 mM isomethylbutylxanthine at 37° for 15 min, and were then incubated in the absence or presence of 20  $\mu$ M PAO or 100 nM OA for 10 min at 37°. cAMP concentration was determined after a 10-min stimulation with 100 nM Ang II and 10  $\mu$ M forskolin. Results are expressed as the means  $\pm$  SD of three individual experiments. For probability calculations, cAMP concentrations were compared versus forskolin treated cells. Statistical significance was defined as \* $P < 0.05$

transient  $[Ca^{2+}]_i$  response and dose-dependently decreased the sustained phase in response to Ang II (Fig. 6).

## DISCUSSION

It is well known that the binding of peptide hormones to their cell surface receptors induces several membrane and intracellular events that express the hormone action. Moreover, after ligand binding many G-protein-coupled receptors undergo a process of internalization [18, 19]. The internalization of the AT<sub>1</sub> receptor has been previously described in various target cells, including vascular smooth muscle cells, adrenal glomerulosa cells, or proximal tubule cells [9, 11, 20]. In agreement with these observations, the results of the present study demonstrate that in response to exposure of hepatocytes to Ang II, a decrease in surface Ang II receptors occurs, consistent with rapid endocytosis of the receptor-bound hormone. This mechanism could be

TABLE 1. Effect of PAO and OA on phosphoinositide responses to Ang II in isolated rat hepatocytes

Phosphoinositols	PAO	OA
IP <sub>1</sub>	96.4 $\pm$ 8.9	99.4 $\pm$ 2.9
IP <sub>2</sub>	84.7 $\pm$ 12.3*	83.1 $\pm$ 7.8*
IP <sub>3</sub>	74.2 $\pm$ 10.9*	77.5 $\pm$ 12.3*

After incorporation of myo-[2-<sup>3</sup>H]-inositol and subsequent incubation with 10 mM LiCl for 30 min at 37°C, cells were exposed to 20  $\mu$ M PAO or 100 nM OA for 10 min at 37°C before 100 nM Ang II stimulation. Results are means  $\pm$  SD of three separate experiments, and are presented as percentages of Ang II-treated cells. Statistical significance was defined as \* $P < 0.05$ .



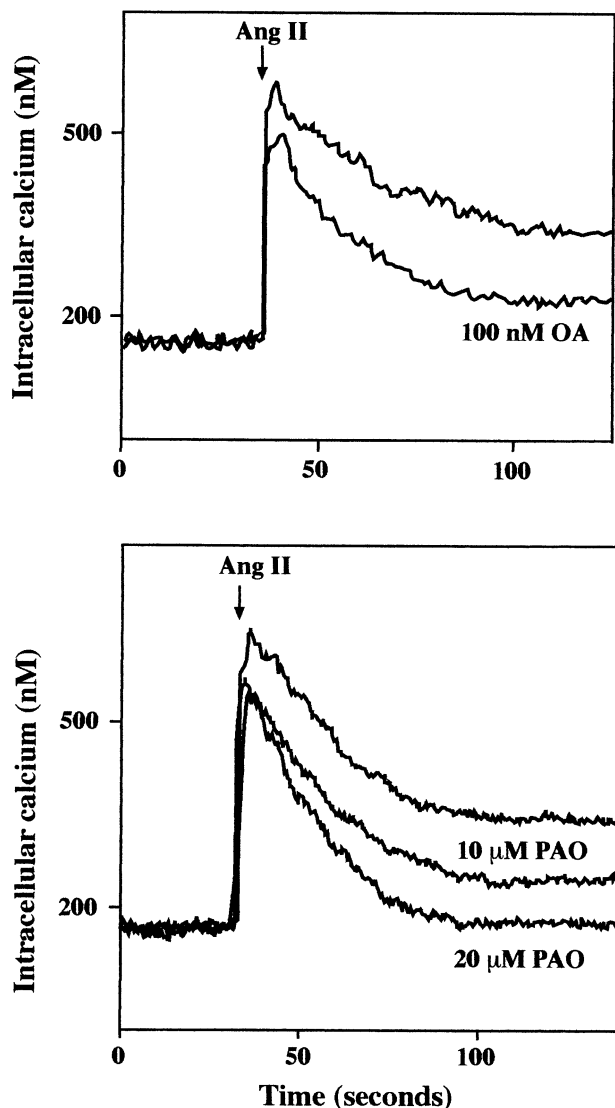


FIG. 6. Effect of PAO and OA on the Ang II-stimulated  $[Ca^{2+}]_i$  response in isolated rat hepatocytes. Fura-2-loaded cells were pretreated with OA (100 nM) or PAO (10–20  $\mu$ M) for 10 min at 37° prior to the addition of 100 nM Ang II.

related to hormone degradation and/or the recycling of functional receptors [21, 22].

At least four morphologically distinct receptor-mediated endocytosis pathways, namely clathrin-coated vesicles, caveolae, non-coated vesicles and macropinocytosis, have been characterized. These pathways are differentially regulated and their relative contribution to receptor internalization varies among cells [23]. The exact cellular mechanism by which the Ang II receptors are normally internalized remains unclear. In contrast to other G-protein-coupled receptors, such as the  $\beta_2$ -adrenergic receptor whose internalization appears to occur through clathrin-coated pits [19], the Ang II type 1A receptor does not require dynamin for sequestration, and the  $AT_{1A}$  receptor is internalized via the dynamin-dependent pathways only upon overexpression of  $\beta$ -arrestin [24]. These findings

indicate that other receptor-mediated endocytosis mechanisms could be involved in Ang II receptor internalization.

We show herein that treatment of cells with OA, a selective pharmacological stimulator of the caveolae endocytosis pathway [25], did not have any effect on Ang II receptor internalization, revealing no contribution of this mechanism to Ang II receptor sequestration in isolated rat hepatocytes.

The molecular domains required for Ang II receptor internalization have been studied extensively. It is known that the deletion of 50 amino acids from the carboxyl terminus abolishes receptor internalization [26]. Moreover, a serine/threonine-rich region including Leu<sup>337</sup> in the cytoplasmic tail of the  $AT_1$  receptor form a motif that is important for agonist-induced internalization of the receptor [27]. However, other molecular domains appear to be involved in the endocytosis of the Ang II receptor. According to previous observations in smooth muscle cells and adrenal glomerulosa cells [9, 20], treatment of hepatocytes with PAO, a trivalent arsenic reagent which forms stable ring structures with molecules containing two adjacent sulphhydryl groups [17], markedly reduced Ang II receptor endocytosis. This effect has been shown to be reversed by dithiothreitol [9, 28], an agent able to reduce disulfide bridges, suggesting that the cysteine residues present in each of the four extracellular loops of the receptor could also be involved in the Ang II receptor internalization process.

Although the dissociation between the internalization and signaling of mutant Ang II receptors suggests that the internalization and the generation of second messengers have different structural requirements [29], receptor endocytosis could be an important mechanism for the continuity of activation of intracellular signals, contributing to the more sustained effects observed after hormonal stimulation [9–11, 20]. The biological significance of Ang II internalization in rat hepatocytes, as well as in other cell types, is not sufficiently known.

Three different Ang II-induced signal transduction pathways mediated by the  $AT_1$  receptor have been identified: inhibition of adenylate cyclase activity [14], activation of PLC resulting in  $IP_3$  formation [3] and activation of  $Ca^{2+}$  mobilization [30]. In agreement with these findings we show here that the Ang II receptors expressed in rat hepatocytes are coupled to all three of these pathways. In fact, stimulation of cells with Ang II transiently blocked forskolin-induced cAMP accumulation, increased  $IP_3$  generation and enhanced  $[Ca^{2+}]_i$  through mobilization of intracellular  $Ca^{2+}$  from stores and  $Ca^{2+}$  influx across the plasma membrane.

To determine whether receptor-mediated endocytosis was related to the process of second messenger generation, we inhibited the process of internalization by PAO treatment. In contrast to previous observations, which showed that PAO selectively inhibits the sustained phase of diacylglycerol accumulation [20], the exposure of rat hepatocytes to this agent induced no change in the inhibitory

effect of Ang II on the accumulation of cAMP. These results suggest that endocytosis of the agonist-receptor complex is not necessary to regulate the production of this second messenger.

Here, we show that OA, the inhibitor of phosphoprotein phosphatases, enhanced the inhibition of cAMP production by Ang II in forskolin-stimulated rat hepatocytes. Since it has been shown that OA increases intracellular phosphodiesterase (PDE) activity in rat adipocytes [31], it is also possible that OA increases PDE activity and consequently decreases cAMP in rat hepatocytes.

Recently, it was determined using a specific monoclonal antibody to the first extracellular domain of the mammalian Ang II type 1 receptor that Ang II receptor internalization was required for PKC activation but not for IP<sub>3</sub> release in rat adrenal zona glomerulosa cells [10]. However, we found that retention of the Ang II receptors in the plasma membrane surface of hepatocytes markedly reduced intracellular IP<sub>2</sub> and IP<sub>3</sub> levels, consistent with an inhibition of PLC activity. These results are in agreement with the hypothesis that phosphoinositide hydrolysis is initiated within the plasma membrane but may continue in endocytic vesicles, thereby contributing to maintain the production of this intracellular signal [9]. Similarly, treatment of cells with OA, which increases the phosphorylation state of numerous phosphoproteins [32], decreased IP<sub>2</sub> and IP<sub>3</sub> levels, indicating that inhibition of Ang II receptor and/or coupled G-protein dephosphorylations are a critical event in intracellular signal transduction.

The Ang II-induced Ca<sup>2+</sup> mobilization has been extensively examined in several target cells. Ang II evokes a rapid and transient increase in [Ca<sup>2+</sup>]<sub>i</sub> resulting from both mobilization of intracellular Ca<sup>2+</sup> stores, mediated by IP<sub>3</sub> and Ca<sup>2+</sup> influx across Ca<sup>2+</sup> channels, and a subsequent lower and sustained phase that is dependent on Ca<sup>2+</sup> influx through the channels [9, 30]. Our results confirm the existence of both Ca<sup>2+</sup> phases of mobilization in rat hepatocytes, and also show that only the second phase is selectively inhibited by PAO and OA. These findings affirm the importance of Ang II receptor-complex endocytosis and agonist-induced receptor phosphorylation in intracellular signal generation and receptor desensitization, respectively.

In summary, the close correlation between Ang II-induced internalization and both phosphoinositols production and [Ca<sup>2+</sup>]<sub>i</sub> mobilization suggests that endocytosis of the agonist-receptor complex is necessary to maintain the production of these intracellular signals in rat hepatocytes.

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