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Receptor-Mediated Endocytosis of Angiotensin II in Rat Myometrial Cells

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ABSTRACT. The events involved in the processing of the angiotensin II (Ang II)–receptor complex were studied in primary cultures of rat myometrial cells. Ang II bound to rat myometrial cells in a specific, time- and temperature-dependent fashion. Pretreatment with cycloheximide did not interfere with binding up to 3 hr, but inhibited increases in binding observed over longer periods. The [^{3}H]Ang II binding to intact cells was inhibited by dithiothreitol (DTT), and the rank order of potency of Ang II and nonpeptide antagonists to inhibit the [^{3}H]Ang II binding was Ang II > Losartan ≫ PD 123319 or CGP 42112B, indicating the presence of the AT_1 receptor type. Whereas most of the [^{3}H]Ang II binding at 4° was susceptible to acid or pronase treatment, binding at 35° was resistant to both treatments, suggesting an internalization of the Ang II–receptor complex. Phenylarsine oxide (PAO) and *N*-ethylmaleimide (NEM) caused a concentration-dependent inhibition when the binding assay was performed at 35°, but no effect was observed at 4°, indicating that these agents did not alter cell-surface binding but actually prevented the internalization process. Simultaneous treatment with 1 mM DTT or β-mercaptoethanol prevented the inhibitory effect of NEM, but only DTT could prevent the inhibition caused by PAO, indicating that two closely located sulfhydryl groups must be involved in the internalization process. Chloroquine (100 μM) inhibited the [^{3}H]Ang II dissociation from cells, and monensin (25 μM) induced a 30% inhibition of [^{3}H]Ang II binding (35°, 3 hr), suggesting endosomal processing of the Ang II–receptor complex with receptor recycling to the cell surface. These results indicate that Ang II binding to AT_1 receptors in rat myometrial cells is followed by internalization of the Ang II–receptor complex and recycling of the receptor to the cell surface. BIOCHEM PHARMACOL **54**;3:399–408, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. angiotensin; endocytosis; recycling; NEM; phenylarsine oxide; myometrial cells

The main active component of the renin-angiotensin system, the octapeptide Ang II[‖], exerts a wide variety of effects by interacting with specific cell surface receptors widely distributed in the organism. Two main types of angiotensin receptors have been described: the AT_1 type, which is responsible for the majority of the known effects of the peptide, and the AT_2 type, which has been related to a possible role of the peptide in development and cell differentiation [1].

In comparison to the available information about Ang II action in vascular tissues, less is known about its function in non-vascular smooth muscle. Ang II receptors have been described in the uteri of several species, and the receptor type expressed in this tissue depends on the species analyzed: in human and rabbit uteri the AT_2 receptor predominates, whereas in rat uterus a mixed population of both types is found [1]. The uterine receptors are regulated by reproductive hormones [2, 3], and myometrial AT_1 receptors are known to mediate the contractile response of the rat uterus to Ang II [4].

Furthermore, despite great advances in knowledge about the structure and signaling mechanisms after receptor activation, much remains to be clarified about the fate of the Ang II–receptor complex. After interaction of some agonists with their respective receptors on the cell surface, receptor-mediated endocytosis and one of the following mechanisms could take place: (a) dissociation of the complex with delivery of ligand to lysosomes and recycling of the receptor back to the cell surface [5]; (b) dissociation of the complex and delivery of both receptor and ligand to a lysosomal compartment where they are degraded [6]; (c) recycling of the receptor and the ligand back to the cell surface [7]; or (d) transcytosis of the complex in polarized cells [8].

Since internalization seems to be an important mechanism for regulating receptor function, and the fate of Ang II–receptor complex in uterine tissue is unknown, the present study was undertaken to verify the events involved

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‖ *Abbreviations:* Ang II, angiotensin II; βME, β-mercaptoetanol; DTT, dithiothreitol; EGF, epidermal growth factor; G protein, guanine nucleotide binding protein; ITS, insulin-transferrin-selenium; NEM, *N*-ethylmaleimide; and PAO, phenylarsine oxide.

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in the processing of the Ang II–receptor complex in a primary culture of rat myometrial cells.

MATERIALS AND METHODS

Materials

(5-L-Isoleucine), [tyrosyl-3,5-^{3}H(N)]AngII ([^{3}H]Ang II, 62.5 Ci/mmol) was purchased from DuPont/NEN (Boston, MA). The peptide antagonist nicotinic acid Tyr-(N^{α}-benzyloxycarbonyl-Arg)-Lys-His-Pro-Ile (CGP 42112B) was provided by Dr. M. de Gasparo (Ciba–Geigy, Basel, Switzerland). The nonpeptide antagonists Losartan (2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[[2′-(1*H*-tetrazol-5-yl) biphenyl-4-yl] methyl]imidazole, potassium salt; DuP 753) and PD 123319 (1-{[4-(dimethylamino)-3-methylphenyl] methyl}-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo [4,5-*c*] pyridine-6-carboxylic acid, ditrifluoroacetate, dihydrate) were provided by Dr. Robert Smith (DuPont, Wilmington, DE) and Dr. Joan A. Keiser (Parke Davis Pharmaceutical Research/Warner-Lambert Co., Ann Arbor, MI), respectively. The culture medium was Ham's F-12/Dulbecco Modified Eagle's Medium, 1:1 (Irvine Scientific, Santa Ana, CA), containing 14.3 mM sodium bicarbonate, 15 mM HEPES, and 20 mg/L gentamycin (Sigma Chemical Co., St. Louis, MO). Trypsin 1:250 and collagenase type IA were from Sigma. Matrigel was from Collaborative Biomedical Products (Bedford, MA). Monoclonal mouse anti-muscle actin (clone HHF 35) was from Dako (Glostrup, Denmark). All other drugs and reagents were from Sigma.

Primary Culture of Rat Myometrial Cells

Female Wistar rats (2-months-old) were treated with estradiol hexahydrobenzoate (Benzoginoestril®, Sarsa, RJ, Brazil) (50 μg/0.1 mL, s.c.) for two consecutive days. Twenty-four hours after the last injection, the rats were decapitated, and the uterine horns were removed aseptically to plates containing culture medium. Myometrial cells were prepared as described by Maggi *et al.* [9]. Briefly, the longitudinal muscular layers were dissected from endometrium and submitted to treatment with a 2.5 mg/mL trypsin solution, for 15 min at 37°. The tissue was then washed with Hanks' balanced salt solution (without Ca^{2+} and Mg^{2+}), minced, and incubated with 300 U/mL collagenase, for 90 min at 37°. The final suspension of myometrial cells was washed five times with culture medium and plated in 12-well plates (Corning, NY), at a density of 5×10^5 to 10^6 cells per well. Cells were cultured in medium supplemented with 5% horse serum plus 2.5% newborn calf serum, and were grown in a humidified atmosphere of 95% air–5% CO_2 at 35° for 5 days. For electron microscopy studies, cells were grown on plates covered with 20% (v/v) Matrigel. Twenty-four hours before each experiment, the cells were transferred to serum-free medium containing ITS (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenium) and 100 ng/mL EGF. The confluence of cells at this stage was 90–95%, and the viability, as determined by trypan blue exclusion, was more than 90%. Protein concentration was determined according to Bradford [10].

Cell Characterization

The material obtained after each step involved in the preparation of the myometrial cells was fixed for 60 min at 4° with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. After a buffer rinse, the material was postfixed for 60 min at 4° with cacodylate-buffered 1% osmium tetroxide, contrasted for 30 min at 4° with 0.1% aqueous uranyl acetate, and dehydrated in graded ethanols. The ethanol was replaced with propylene oxide. The cell cultures were fixed as described above and detached from plates after addition of propylene oxide. The resulting material was finally embedded in Araldite. Semithin sections (0.8 μm) were stained with toluidine blue for light microscopic analysis, and ultrathin sections were stained with uranyl acetate and lead citrate for electron microscopy studies. Figure 1 shows the electron micrographs from the material obtained in each step of the myometrial cell preparation. The electron microscopy study showed that the myometrial cell was the predominant cell type and that it maintained the ultrastructural features of smooth muscle in the culture conditions [11].

Characterization of the cell cultures also was done by immunohistochemistry (peroxidase-antiperoxidase method) using a mouse anti-muscle actin monoclonal antibody as the primary antibody. A positive reaction was indicated by the appearance of a brown precipitate after reaction with hydrogen peroxide and 3,3′-diaminobenzidine hydrochloride (data not shown).

Binding Assay

Myometrial cells, maintained in serum-free medium without ITS and EGF, were used for binding assays. Cells (100–200 μg protein/well) were incubated at 35° (in medium supplemented with 5 mM $MgCl_2$, pH 7.4) or at 4° (in Hanks' balanced salt solution, with 5 mM $MgCl_2$, pH 7.4) with [^{3}H]Ang II (8 nM), in a final volume of 0.5 mL, for different periods of time (30 min to 12 hr). At specific time intervals, plates were placed in ice, medium was removed, and cells were rapidly rinsed three times with ice-cold PBS to remove unbound ligand. Then the cells were solubilized with 20% (v/v) Triton X-100, collected, and counted in a β-counter (Beckman LS6000 IC). Nonspecific binding was determined in the presence of 100 μM unlabeled Ang II, and contributed to 10–25% of the total binding. The dpm values for the total binding at equilibrium were in the range of 800–1500 at 4° and of 2000–3000 at 35°. All values are expressed as specific binding (total minus non-specific binding). The contribution of protein synthesis throughout the period of time studied was evaluated by treating the cells with cycloheximide (89 μM,

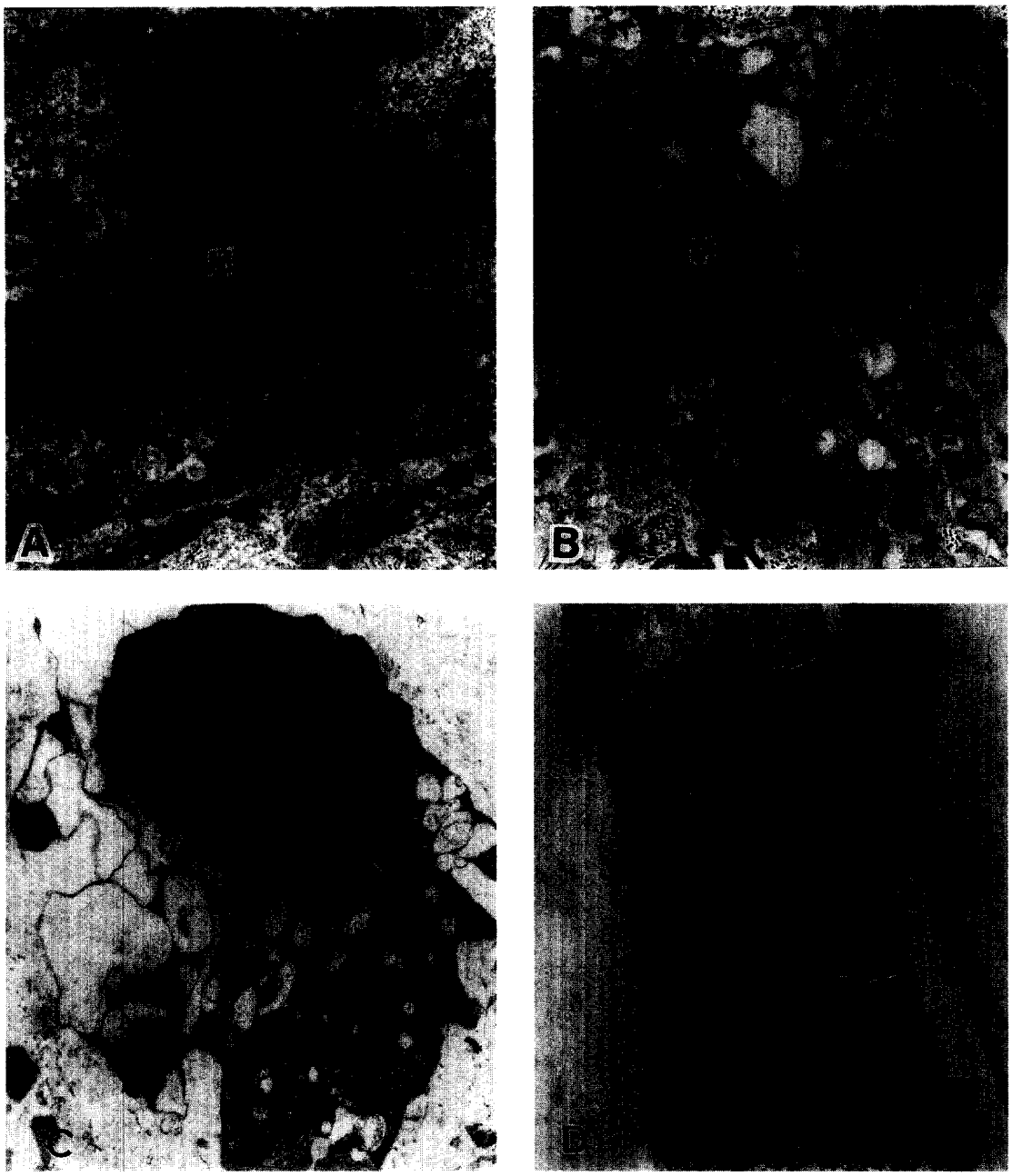

FIG. 1. Steps involved in the preparation of myometrial cells. (A) The longitudinal muscular layer before trypsin treatment. Muscular cells (M) inserted between collagen fibers (5000×). (B) Longitudinal muscular layer after trypsin treatment. Note the slackening of the structure probably caused by removal of the serosa layer by the enzymatic treatment (8000×). (C) Muscular cell, the predominant cell type in the suspension obtained after treatment with collagenase (8000×). (D) The myometrial cell after 5 days in culture. Observe the bundles of myofilaments (arrows) arranged in parallel lines at the periphery of the cell (20,000×).

at 35° for 1 hr), rinsing the cells three times with PBS, and performing the binding assay for different periods (30 min to 12 hr).

Competition studies were performed by incubating cells with [^{3}H]Ang II (about 8 nM) in the absence or in the presence of increasing concentrations of Ang II, Losartan, PD 123319, or CGP 42112B. The binding data were analyzed by the iterative non-linear regression analysis computer program GraphPad Prism (GraphPad Software Inc., 1994). The binding parameters K_i (the negative logarithm of the molar concentration of competitor at which 50% of the maximum binding is inhibited) and B_{max} (number of binding sites) were determined from the competition curves. The K_d (equilibrium dissociation constant) was deduced, since the radioligand and the competing ligand (Ang II) have the same affinity for the receptor; in this case, for a plot of bound versus total free ligand (radioligand plus inhibitor), the negative reciprocal of the slope is the K_d rather than the IC_{50}.

Effects of DTT and Sulfhydryl Modifying Agents on [^{3}H]Ang II Binding

The influence of DTT on [^{3}H]Ang II binding was evaluated by preincubating cells in the absence (control) or presence of DTT (1–10 mM) at 35°, and performing the binding assay in the absence or presence of DTT, for 3 hr at 35° or 6 hr at 4°. To evaluate the influence of -SH modifying agents on binding and/or internalization of [^{3}H]Ang II, the cells were incubated with PAO (5 and 10 μM) or NEM (10 and 100 μM) at 35° for 30 min, in the absence and presence of 1 mM DTT or βME. After treatment, the cells were rinsed three times with PBS, and the binding assay with [^{3}H]Ang II (8 nM) was performed for 3 hr at 35° or 6 hr at 4°.

Internalization Studies

To determine whether cell-associated ligand moved from the cell surface to the interior in a temperature-dependent fashion, cells were incubated with [^{3}H]Ang II (8 nM) for periods of 30 min to 6 hr at either 35° or 4°. The cells were rinsed, and removal of [^{3}H]Ang II bound to the cell surface was accomplished by exposure to acid [12] or to pronase [13]. In the acid treatment, cells were incubated with glycine buffer (50 mM glycine/150 mM NaCl, pH 3.0) for 10 min at 4°, placed in ice, and washed three times with cold PBS. In the pronase treatment, cells were incubated with 0.25% (w/v) pronase for 30 min at 4°, centrifuged at 710 *g* for 10 min, rinsed with PBS, and centrifuged three consecutive times. After each treatment, cells were solubilized with 20% Triton X-100, and the amount of radioactivity that remained in association with the cells was determined. The acid and pronase-treated cells were viable as determined by trypan blue exclusion and were able to bind [^{3}H]Ang II like control cells.

Inhibition of the Ang II–Receptor Complex Endosomal Processing

For dissociation studies, the cells were preincubated in the absence or presence of chloroquine (100 μM for 1 hr at 35°), and the binding assay was performed at 35° for 3 hr. Cells were then placed in ice and rinsed three times with PBS. The cell-associated radioactivity was determined immediately in some wells to obtain the dissociation at zero time. Pre-warmed medium (without or with 100 μM chloroquine) was added to the other wells, and the cells were reincubated at 35° for different periods of time. The cell-associated radioactivity was determined and compared with the value obtained at zero time. To evaluate the contribution of a receptor recycling mechanism to the overall binding, cells were preincubated (1 hr, 35°) in the absence (control) or presence of 25 μM monensin. Binding assay was performed in the absence of monensin, at 35° for 3 hr, or at 4° for 6 hr.

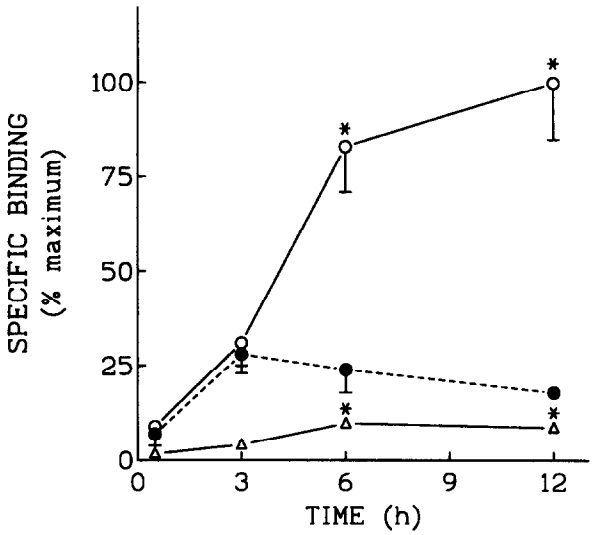

FIG. 2. Time–course of [^{3}H]Ang II specific binding to cultured myometrial cells. Cells were incubated with 8 nM [^{3}H]Ang II at 35° (○) or 4° (△) for the periods of time indicated. The influence of cycloheximide (●) was evaluated by pretreating the cells with cycloheximide (89 μM, 1 hr) and performing the binding assay at 35° for the periods indicated. Results are expressed as the means ± SEM of at least three experiments performed in duplicate and are the percentage of the binding in 12 hr. Key: (*) statistically different from the other points in the same curve (ANOVA, followed by the Newman–Keuls test, $P < 0.05$).

Analysis of Data

All binding assays were performed in duplicate or triplicate. Results are expressed as means ± SEM. Statistical comparisons were made using one-way ANOVA followed by the Newman–Keuls test for multiple comparisons, or by the two-tailed Student's *t*-test to compare a particular response in two groups. A P value < 0.05 was accepted as significant.

RESULTS

[^{3}H]Ang II Binding to Rat Myometrial Cells

The specific binding of [^{3}H]Ang II (8 nM) to cultured rat myometrial cells was time and temperature dependent (Fig. 2). Binding of [^{3}H]Ang II (8 nM) at 35° increased up to 6 hr. To evaluate whether protein synthesis could be involved during incubation, the effect of cycloheximide treatment on the time–course of [^{3}H]Ang II association to cells was studied. After a 1-hr cycloheximide pretreatment, maximal binding was reached in 3 hr, and further increases were inhibited. Binding at 4° reached a maximum in 6 hr that was about 10% of the binding at 35° (Fig. 2). Cycloheximide pretreatment did not interfere with [^{3}H]Ang II binding at 4°.

Figure 3 and Table 1 show the results obtained in competition experiments performed at 4° for 6 hr. Ang II inhibited [^{3}H]Ang II binding in a concentration-dependent fashion and revealed two receptor affinity states: a high affinity state (K_d = 0.97 ± 0.35 nM; B_{max} = 16.0 ± 4.2 fmol/mg protein) and a low affinity state (K_d = 963.80 ±

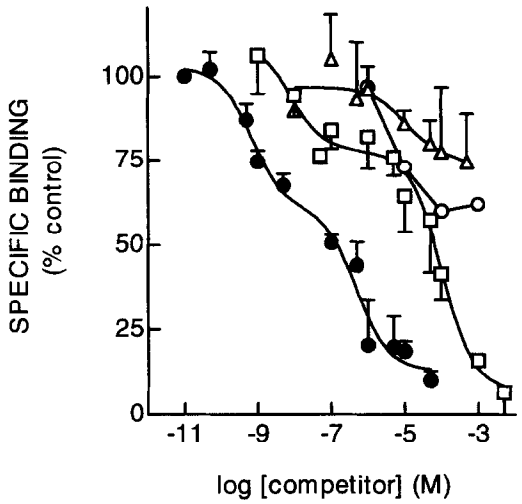

FIG. 3. Effect of increasing concentrations of Ang II (●), Losartan (□), PD 123319 (△) and CGP 42112B (○) on specific binding of [^{3}H]Ang II (about 8 nM, 4° for 6 hr) to intact cells. Results are expressed as the percentage of the control binding (in the absence of competitor). Each point represents the mean ± SEM from two to three experiments performed in triplicate.

359.90 nM; B_{max} = 13.1 ± 4.8 fmol/mg protein). Losartan also was able to inhibit [^{3}H]Ang II binding (K_i = 287.60 ± 134.90 nM, for the high affinity binding site), but PD 123319 and CGP 42112B could slightly inhibit [^{3}H]Ang II binding only at high concentrations (Fig. 3 and Table 1).

Increasing concentrations of DTT also inhibited [^{3}H]Ang II binding at both 35°, 3 hr and 4°, 6 hr (Fig. 4).

Effect of PAO and NEM on [^{3}H]Ang II Binding and Internalization

Pretreatment of cells with increasing concentrations of the sulfhydryl-group reagents PAO and NEM had a concentration-dependent inhibitory effect on [^{3}H]Ang II association to cells at 35°, but did not interfere significantly with [^{3}H]Ang II association at 4° (Fig. 5A). Simultaneous incubation of PAO or NEM with 1 mM DTT prevented their inhibitory effect, whereas simultaneous incubation with βME only prevented the inhibitory effect of NEM

TABLE 1. Inhibition of [^{3}H]Ang II binding to rat myometrial cells by Ang II and nonpeptide antagonists

Competitor	N	K_i (nM)	
		High affinity	Low affinity
Ang II	3	0.97 ± 0.35	963.80 ± 359.90
Losartan	3	287.60 ± 134.90	>1000
PD 123319	3		>1000
CGP 42112B	2		>1000

Cells were incubated with [^{3}H]Ang II (about 8 nM) and increasing concentrations of each competitor, at 4° for 6 hr. Values are means ± SEM, and were derived from nonlinear regression computer analysis. N = the number of experiments performed in triplicate.

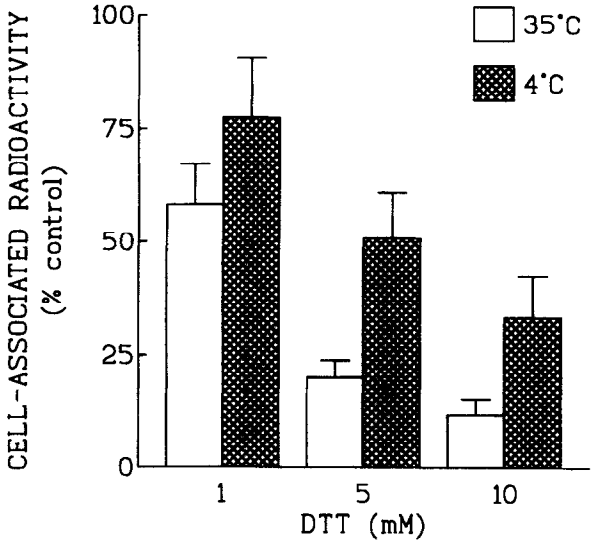

FIG. 4. Effect of increasing concentrations of DTT on [^{3}H]Ang II (8 nM) binding. Cells were preincubated with DTT (35°, 30 min), and the binding assay was performed in the presence of DTT at 35°, 3 hr (open bars) or at 4°, 6 hr (hatched bars). Results are expressed as a percentage of the control binding (in the absence of DTT) and are the means ± SEM of three to six experiments performed in duplicate.

(Fig. 5B). It should be mentioned that 1 mM DTT had only minimal effects on [^{3}H]Ang II association to cells when washed out before the binding assay.

Ang II–Receptor Complex Internalization

When the binding assay was performed for 6 hr at 35°, about 95% of the cell-associated radioactivity was resistant to acid treatment and 91% was resistant to pronase treatment. On the other hand, when the binding assay was performed for 6 hr at 4°, only 47% of cell-associated radioactivity was resistant to acid and 20% was resistant to pronase treatment (Fig. 6A). The transference of the ligand to a cell compartment resistant to these treatments seemed to occur rapidly, since after 30 min of binding at 35°, almost all cell-associated radioactivity was already resistant to both treatments (Fig. 6B). The resistance to acid and pronase treatment continued at 180 and 360 min, and a significant difference from control (without treatment) was detected at 180 min.

Ang II–Receptor Endosomal Processing

To investigate the endosomal pathway, dissociation studies were performed in the absence and presence of the acidotrophic amine chloroquine (100 μM) (Fig. 7A). [^{3}H]Ang II dissociation from cells was relatively slow ($T_{1/2}$ ~ 3.4 hr), and was inhibited by chloroquine. After 30, 90, and 180 min, dissociation was, respectively, 35, 45, and 53% in the absence, and 13, 19, and 33% in the presence, of chloroquine.

To investigate whether the receptor cycling process

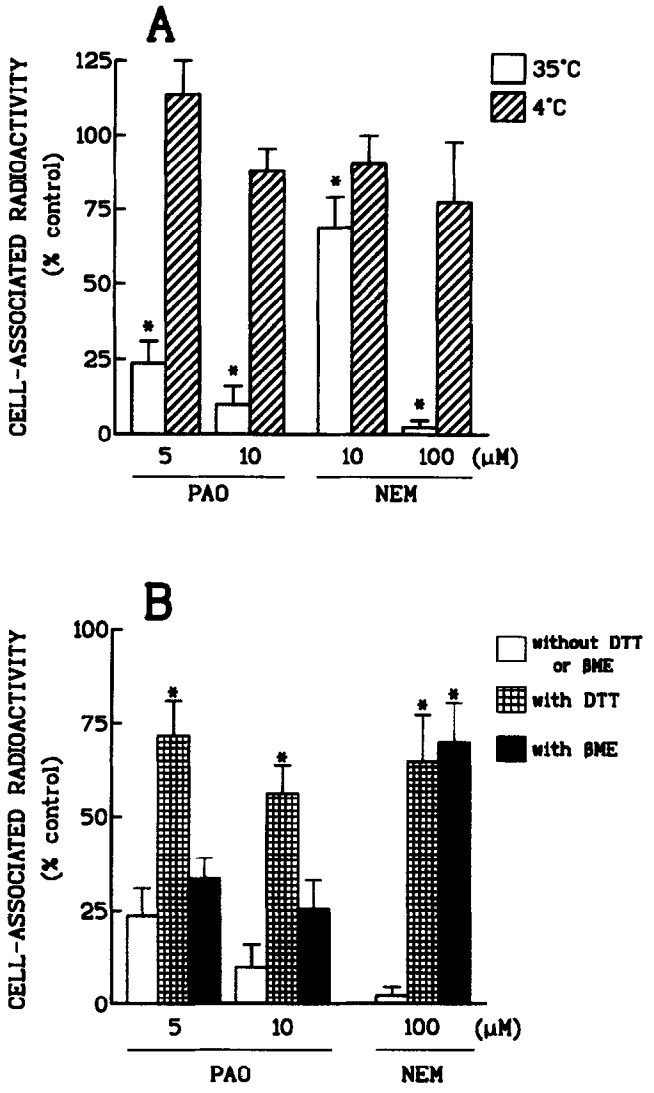

FIG. 5. Effects of PAO and NEM on cell-associated radioactivity. (A) Cells were treated with either PAO or NEM for 30 min and then were rinsed, and the binding assay with [^{3}H]Ang II (8 nM) was performed (35°, 3 hr or 4°, 6 hr). Key: (*) statistical difference between the values obtained at 35° and 4° (Student's t-test, $P < 0.05$). (B) Effect of PAO or NEM in the absence and presence of 1 mM DTT or βME on cell-associated radioactivity (35°, 3 hr). Key: (*) statistical difference between values obtained in the absence and presence of DTT or βME (Student's t-test, $P < 0.05$). Values shown are means ± SEM of three to six experiments performed in duplicate and are the percentage of the control binding (treatment with buffer).

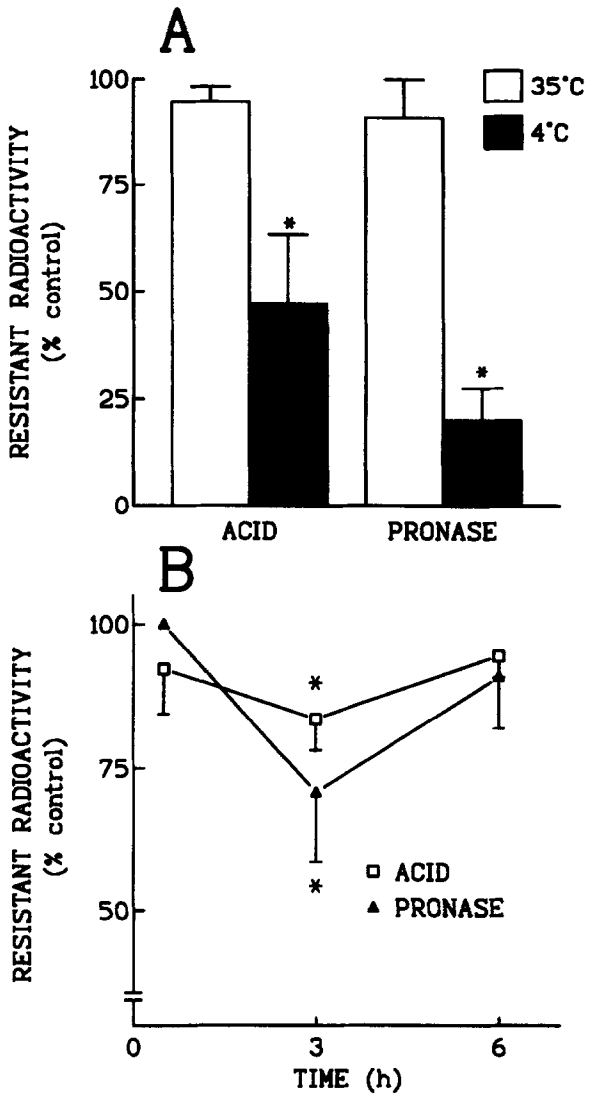

FIG. 6. Resistance of cell-associated radioactivity to acid or pronase treatment. (A) Cells were incubated with [^{3}H]Ang II (8 nM, at 35° or 4°, for 6 hr), rinsed, and subjected to treatment with acid or pronase. Key: (*) statistical difference between values obtained at 35° and 4° (Student's t-test, $P < 0.05$). (B) Cells were incubated with [^{3}H]Ang II (8 nM, 35°, for 30 min to 6 hr), rinsed, and subjected to acid or pronase treatment. The percentage of the cell-associated radioactivity resistant to these treatments is shown. Key: (*) statistically different from control (Student's t-test, $P < 0.05$). Results are means ± SEM of three to six experiments performed in duplicate and are the percentage of control binding (treatment with buffer).

contributed to the increase in ligand association at 35°, binding studies were performed at 4° or at 35° with cells pretreated with monensin (25 μM) (Fig. 7B). This treatment produced a decrease of about 30% in the percentage of [^{3}H]Ang II associated with cells at 35°. In contrast, binding at 4° was not affected by monensin.

DISCUSSION

In the present study, we attempted to evaluate the events involved after interaction of Ang II and its receptor in a primary culture of rat myometrial cells that maintained ultrastructural features of smooth muscle (Fig. 1). Association of [^{3}H]Ang II with cells was specific, time dependent, and greater at 35° than at 4° (Fig. 2). At 35° the binding increased up to 6 hr, but after treatment with cycloheximide, equilibrium was reached in 3 hr. Therefore, receptor neosynthesis or synthesis of another protein(s) important for binding seemed to contribute to increased binding during prolonged periods (Fig. 2). Equilibrium of [^{3}H]Ang II binding was reached in 6 hr at 4° and was not affected by cycloheximide.

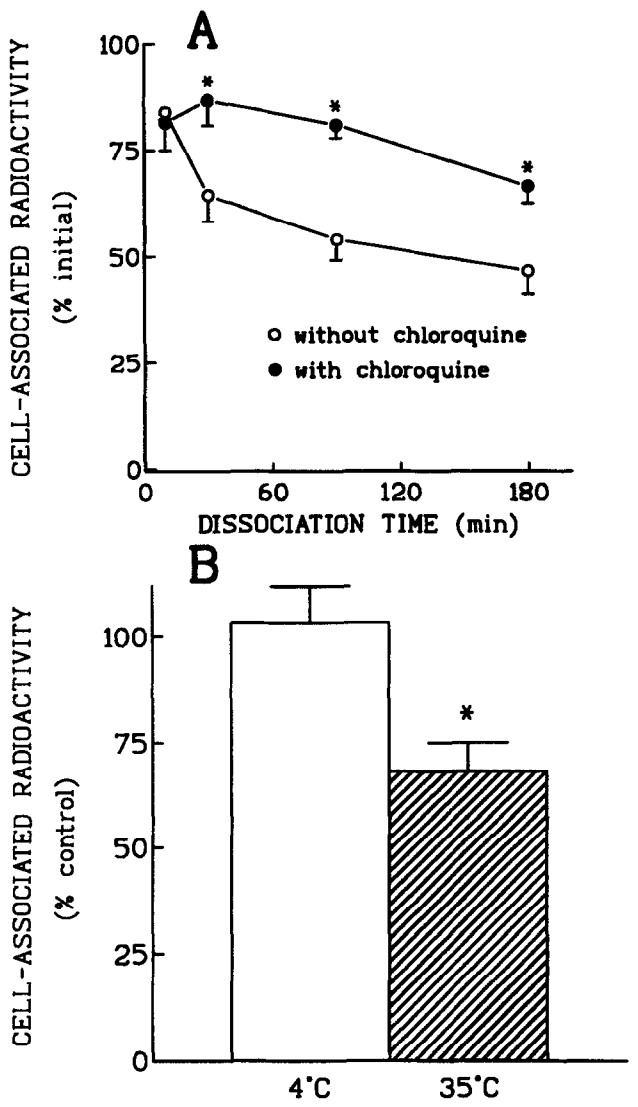

FIG. 7. Endosomal processing of the Ang II–receptor complex in myometrial cells. (A) Effect of chloroquine on the dissociation kinetics. The binding assay with [^{3}H]Ang II (8 nM, 35°, 3 hr) was performed in the absence (○) or presence (●) of chloroquine (100 μM), cells were rinsed, fresh medium (without or with chloroquine) was added, and the associated radioactivity was determined after the periods indicated and compared with the initial cell-associated radioactivity (dissociation at zero time). Key: (*) statistical significance between values obtained without or with chloroquine (Student's *t*-test, $P < 0.05$). (B) Effect of monensin on [^{3}H]Ang II association to cells. Cells were preincubated without (control) or with monensin (25 μM, 35°, 1 hr) before the binding assay (35°, 3 hr or 4°, 6 hr). Key: (*) significant difference between temperatures (Student's *t*-test, $P < 0.05$). Results are means ± SEM of three to seven experiments performed in duplicate.

Competition studies performed with intact cells at 4° to evaluate only the cell surface receptors showed that the [^{3}H]Ang II binding was specifically displaced by Ang II. The analysis of the homologous competition data revealed the presence of two affinity binding sites: a high affinity (K_d = 0.97 ± 0.35 nM) and a low affinity (K_d = 963.80 ± 359.90 nM) binding site. The displacement experiments were performed in the presence of a high radioligand concentration, leading to significant labeling of both low and high affinity sites. It must be noted that this situation is sometimes encountered with tritiated ligands, which, because of their relatively low specific activity, are used at concentrations that are high as compared with the K_d value of high affinity sites [14]. However, this concentration did not interfere with the calculation of the binding constant; the K_d obtained from rat myometrial cells was in close agreement with data in the literature for displacement of [^{125}I]Ang II binding in rat uterus membrane preparations [15]. The heterologous competitive experiments with selective nonpeptide antagonists revealed that the main receptor type in the culture is AT_1, since Losartan (K_i = 287.60 ± 134.90 nM, for the high-affinity binding site), but not PD 123319 or CGP 42112B, could compete for [^{3}H]Ang II binding (Fig. 3 and Table 1). Therefore, the biphasic shape of the displacement curves observed with Ang II and Losartan did not seem to be due to a possible heterogeneity of Ang II receptors but to a unique receptor type in two different affinity states.

Furthermore, it is well known that the two main Ang II receptor types can be differentiated by their sensitivity to DTT, since AT_1 is sensitive and AT_2 is resistant to inactivation by DTT [16]. In rat myometrial cells in culture, binding of [^{3}H]Ang II at both 35° and 4° was inhibited by DTT in a concentration-dependent manner (Fig. 4), and could be abolished completely by 30 mM DTT (data not shown). This is in accord with the data obtained with the nonpeptide antagonists, showing that AT_1 is the predominant receptor type in myometrial cells in culture. In rat uterine tissue, both types of Ang II receptors have been demonstrated [15], but it is possible that, in culture conditions, the AT_1 type is predominantly expressed. A similar phenomenon has already been observed with cultured vascular smooth muscle cells, which express only the AT_1 receptor type, whereas both types are present in the tissue itself [15], and with cultured fetal fibroblasts, where a gradual increase of AT_1 and a decrease of AT_2 receptor type occur during primary culture or after subculture [17].

Cysteine residues present in other receptors, such as the β-adrenoceptor, the EGF receptor, and the vasopressin receptor, seem to be related to the binding of the agonist and/or to the internalization of the receptor–agonist complex [18–20]. Furthermore, internalization of the angiotensin receptor in other cells is sensitive to treatment with PAO [21–23], a trivalent arsenic reagent, which forms stable ring structures with molecules containing two adjacent sulfhydryl groups [18]. To evaluate the importance of cysteine residues on [^{3}H]Ang II binding and/or internalization, cells were pretreated with the sulfhydryl-modifying agents NEM and PAO before the binding assay at 35° or 4°. Both PAO and NEM reduced, in a concentration-dependent fashion, the associated radioactivity at 35°. However, unlike DTT, they did not interfere with the associated radioactivity at 4°, indicating that these agents did not alter cell-surface binding but actually prevented the internaliza-

tion process (Fig. 5A). Although the addition of βME, a monofunctional -SH reagent, prevented the inhibitory effect of NEM but not of PAO, DTT prevented the inhibitory effect of both compounds (Fig. 5B). This suggests that these effects were elicited through specific action on -SH groups and that at least two adjacent -SH groups must be involved in the internalization process.

It remains to be determined whether these -SH groups are located in the angiotensin receptor or in some other associated structure, such as a G protein. Korner *et al.* [24] proposed the existence of an -SH group in a G protein associated with the β-adrenoceptor, which would be exposed after the hormone–receptor–G protein interaction; alkylation of this group by NEM would impair the return of the system to the resting state, locking the hormone to the receptor–G protein complex. However, a different mechanism was observed in the present study, since the inhibition caused by NEM or PAO did not require previous treatment with the hormone to expose the -SH-sensitive sites. Indeed, the involvement of G proteins in receptor trafficking is controversial. Mutants of the β-adrenoceptor, angiotensin receptor, and muscarinic receptor that are unable to couple to G proteins still retain their full capacity for internalization [25–30]. On the other hand, G protein coupling seems to be needed for internalization of the TRH receptor [31]. NEM and PAO could also be acting on other elements important for vesicular trafficking, such as the NEM-sensitive fusion protein and other NEM-sensitive factors, more recently described as specific mediators of other vesicular transport processes [32–35].

The possibility of an internalization phenomenon, suggested by the experiments with NEM and PAO, was confirmed by acid washing with glycine, which promotes the dissociation of the hormone from the cell surface receptor, and by proteolytic treatment with pronase, which promotes the hydrolysis of the peptide hormone bound to the cell surface, as well as of the extracellular receptor domains [36]. Both treatments were efficient in removing radioactivity associated to the cell surface and demonstrated that the radioactive ligand moved to a cell compartment resistant to both treatments at 35° but not at 4° (Fig. 6A). These results indicate, therefore, that internalization occurs in rat myometrial cells as observed in other cell types [21, 23, 37, 38]. This transference seemed to occur rapidly, since at 30 min of incubation almost all associated radioactivity was resistant to both treatments (Fig. 6B). The fluctuations observed in the percentage of binding resistance to acid or to pronase treatment during the period of time studied could be attributable to a change in the surface receptor number due to a recycling mechanism.

To determine the fate of the Ang II–receptor complex after internalization, studies were done with agents that interfere with vesicular traffic by alteration of the endosomal pH [39]: the acidotrophic amine chloroquine, which elevates endosomal pH and impairs dissociation of the complex, and the carboxylic ionophore monensin, which intercalates into membranes and impairs transmembrane ion gradients. The kinetics of dissociation of [^{3}H]Ang II from cells was slow, and it was further delayed by chloroquine, suggesting that an endosomal pathway could process the complex (Fig. 7A). Endosomal processing of angiotensin receptors has already been suggested in rat vascular smooth muscle cells studied by biochemical methods [22], and a specific gold probe has been localized by electron microscopy analysis in structures related to endosomes [40]. The endosomal processing of the Ang II–receptor complex in the rat myometrial cells seems to be followed by recycling of the receptor to the cell surface, in a form still capable of binding, since interruption of vesicular traffic with monensin induced a 30% inhibition of [^{3}H]Ang II binding at 35° for 3 hr (Fig. 7B). Receptor neo-synthesis could not be responsible for increasing binding in this protocol, since, as already discussed, cycloheximide had no effect on [^{3}H]Ang II binding in periods of incubation up to 3 hr (Fig. 2).

In summary, Ang II bound to AT_1 receptors in rat myometrial cells in culture in a specific, time- and temperature-dependent fashion. Interaction of Ang II with its receptor was followed by a receptor-mediated endocytosis process, which seems to involve at least two very closely located -SH groups. The Ang II–receptor complex seems to undergo endosomal processing with receptor recycling to the cell surface. This system is now better understood, allowing further studies concerning reproductive hormone regulation of Ang II function in the myometrium.

The biological significance of Ang II internalization in the rat myometrial cell, as well as in other cell types, is unknown. This process could represent a mechanism for the termination of hormone action and regulation of the number of cell-surface receptors. Conversely, it could be important in hormone action; in rat vascular smooth muscle cells and in adrenocortical cells it has been suggested that endocytosis would be a mechanism important to the continuity of activation of intracellular signals, contributing to the more sustained effects observed after hormonal stimulation [21, 23, 41, 42]. The process could also represent a mechanism through which the hormone gains access to the intracellular compartment and interacts with already described intracellular Ang II binding sites [43–45]. Further studies are necessary to evaluate the involvement of these mechanisms in the biological actions of the peptide in the uterus.

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