Am J Physiol Endocrinol Metab
280: E462–E470, 2001.

Desensitization of angiotensin II: effect on $[Ca^{2+}]_i$, inositol triphosphate, and prolactin in pituitary cells

ARTURO GONZÁLEZ IGLESIAS, CECILIA SUÁREZ, CLAUDIA FEIERSTEIN, GRACIELA DÍAZ-TORGA, AND DAMASIA BECU-VILLALOBOS
Instituto de Biología y Medicina Experimental-Consejo Nacional de Investigaciones Cientificas y Técnicas, 1428 Buenos Aires, Argentina

Received 26 May 2000; accepted in final form 31 October 2000

González Iglesias, Arturo, Cecilia Suárez, Claudia Feierstein, Graciela Díaz-Torga, and Damasia Becu-Villalobos. Desensitization of angiotensin II: effect on $[Ca^{2+}]_i$, inositol triphosphate, and prolactin in pituitary cells. *Am J Physiol Endocrinol Metab* 280: E462–E470, 2001.—Activation of pituitary angiotensin (ANG II) type 1 receptors (AT_1) mobilizes intracellular Ca^{2+}, resulting in increased prolactin secretion. We first assessed desensitization of AT_1 receptors by testing ANG II-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response in rat anterior pituitary cells. A period as short as 1 min with 10^{-7} M ANG II was effective in producing desensitization (remaining response was 66.8 ± 2.1% of nondesensitized cells). Desensitization was a concentration-related event (EC_{50}: 1.1 nM). Although partial recovery was obtained 15 min after removal of ANG II, full response could not be achieved even after 4 h (77.6 ± 2.4%). Experiments with 5×10^{-7} M ionomycin indicated that intracellular Ca^{2+} stores of desensitized cells had already recovered when desensitization was still significant. The thyrotropin-releasing hormone (TRH)-induced intracellular Ca^{2+} peak was attenuated in the ANG II-pretreated group. ANG II pretreatment also desensitized ANG II- and TRH-induced inositol phosphate generation (72.8 ± 3.5 and 69.6 ± 6.1%, respectively, for inositol triphosphate) and prolactin secretion (53.4 ± 2.3 and 65.1 ± 7.2%), effects independent of PKC activation. We conclude that, in pituitary cells, inositol triphosphate formation, $[Ca^{2+}]_i$ mobilization, and prolactin release in response to ANG II undergo rapid, long-lasting, homologous and heterologous desensitization.

angiotensin type 1 receptor; thyrotropin-releasing hormone; calcium; homologous desensitization; protein kinase C

ANGIOTENSIN II (ANG II) type 1 (AT_1) receptors belong to the family of plasma membrane receptors that couple to guanine nucleotide regulatory proteins [guanine nucleotide protein-coupled receptors (GPCR)]. Two subtypes have been described: AT_{1A} and AT_{1B}. In the rat pituitary, activation of AT_{1B} receptors coupled to $G_{q/11}$ protein increases phospholipase C-β (PLC-β) activity, resulting in increased inositol 1,4,5-triphosphate [$Ins(1,4,5)P_3$] and diacylglycerol formation. A biphasic increase in intracellular calcium concentration ($[Ca^{2+}]_i$) triggered by $Ins(1,4,5)P_3$ ensues. In a previous study (16) we showed that the initial transient $[Ca^{2+}]_i$ spike, which is primarily due to $Ins(1,4,5)P_3$-mediated release of intracellular Ca^{2+}, is followed by a sustained $[Ca^{2+}]_i$ elevation that results from increased Ca^{2+} influx through voltage-sensitive calcium channels and capacitative Ca^{2+} entry. The initial $[Ca^{2+}]_i$ spike is terminated as $Ins(1,4,5)P_3$ concentration declines, intracellular Ca^{2+} stores become exhausted, and cytoplasmic Ca^{2+} is sequestered by intracellular organelles or pumped out from the cells. DAG, together with the increased $[Ca^{2+}]_i$, stimulates protein kinase C (PKC) activity. Activation of this pathway by ANG II in lactotrophs results in prolactin secretion (10).

It is well recognized that, in the presence of agonists, many transmembrane GPCRs not only display rapid activation of signal transduction but also rapid desensitization of response (31). Receptor desensitization is potentially a physiologically important process, as it provides a means of regulating continuous receptor stimulation. Rapid receptor desensitization does not necessarily involve receptor loss but rather receptor phosphorylation (36). This acute process (seconds to minutes) may be supplemented by internalization (minutes to hours) and by downregulation (hours to days) of membrane receptors.

Both AT_1 receptor subtypes (AT_{1A} and AT_{1B}) undergo rapid homologous desensitization in response to ANG II. This has been reported in the adrenal gland (28), vascular smooth muscle cells (2), rat cardiomyocytes (1), renal afferent arterioles (21), brain (14), and Chinese hamster ovary (CHO) cells (34). In particular, desensitization of ANG II receptors in the pituitary has not been documented. Different degrees and temporal patterns of desensitization have been reported for PLC-β-coupled receptors, and it is believed that desensitization may be a cell-specific phenomenon, regulated by differential expression of elements involved in the process. Moreover, it is well established that signaling and desensitization of GPCRs can be influenced by the cell type in which they are expressed, as in the case of the mouse thyrotropin-releasing hormone (TRH) recep-

Address for reprint requests and other correspondence: D. Becú-Villalobos, Instituto de Biología y Medicina Experimental, CONICET, V. Obligado 2490, (1428) Buenos Aires, Argentina (E-mail: dbecu@dna.uba.ar).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

tor (12). This raises the possibility that ANG II desensitization may be a function of the cell type rather than of AT_1 receptor structure.

We therefore assessed whether rapid desensitization of the AT_1 receptor occurs in anterior pituitary cells by use of repetitive stimulation of intracellular Ca^{2+} mobilization, inositol triphosphate generation, and prolactin secretion as end points. We also determined whether heterologous desensitization occurred by use of TRH as a second alternative stimulus and the participation of PKC in the desensitization process.

MATERIALS AND METHODS

Animals. Female 60-day-old Sprague-Dawley rats were housed in an air-conditioned room with lights on at 0700 and off at 1900. They had free access to laboratory chow and tap water. Female rats were checked by vaginal cytology for regular estrous cycles during ≥10 days previous to the experiment. Rats in diestrus were used.

Cell dispersion. Unless specified, all drugs were purchased from Sigma (St. Louis, MO).

Rats were killed by decapitation at 9:00 AM, and pituitaries were removed on ice, separated from the neurointermediate lobe, and placed in chambers containing freshly prepared Krebs-Ringer bicarbonate buffer (KRBGA) without Ca^{2+} or Mg^{2+}. Buffer contained 14 mM glucose, 1% bovine seroalbumin, MEM amino acids 2%, MEM vitamins 1% (GIBCO, Buenos Aires, Argentina), 2 mM glutamine, and Phenol Red 0.025%. It was previously gassed for 15 min with 95% O_2-5% CO_2 and adjusted to pH 7.35–7.40. Buffer was sterilized by filtration through a 0.45-μm pore diameter membrane (Nalgene). Pituitaries were washed three times with KRBGA and then cut into 1-mm pieces. Fragments obtained were washed and incubated in the same buffer containing 0.5% trypsin for 30 min at 37°C, with 95% O_2-5% CO_2. They were treated for two additional minutes with 50 μl DNase I (1 mg/ml, Worthington, Lakewood, NJ). Digestion was ended by addition of 1 mg/ml LBI. Fragments were dispersed in individual cells by gentle trituration through siliconized Pasteur pipettes. Resulting suspension was filtered through a nylon gauze (160 μm) and centrifuged for 10 min at 120 *g*. Before centrifugation, an aliquot of cellular suspension was taken to quantify hypophysial cell yield by use of a Neubauer chamber. Viability of cells, determined by trypan blue exclusion, was always >90%. In a series of experiments, cells were freshly used for intracellular Ca^{2+} measurements; alternatively, they were cultured for 4 days (as will be described).

Intracellular Ca^{2+} measurements. Measurements were made as previously described (11). Briefly, the tetraacetoxymethyl ester (AM) of fura 2 was used as a fluorescent indicator. The pellet of anterior pituitary cells of each experimental group was redispersed and incubated in a buffered saline solution (BSS: 140 mM NaCl, 3.9 mM KCl, 0.7 mM KH_2PO_4, 0.5 mM $Na_2HPO_4 \cdot 12H_2O$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20 mM HEPES, pH 7.4) in the presence of 1.5 μM fura 2-AM, 10 mM glucose, and 0.1% BSA. Cells were incubated for 30 min at 37°C in an atmosphere of 95% O_2-5% CO_2, a time during which fura 2 is trapped intracellularly by esterase cleavage. Cells were then washed twice in BSS without fura 2-AM and brought to a density of $1.7–2 \times 10^6$ cells/ml BSS. Fluorescence was measured in a spectrofluorometer (Jasco, Tokyo, Japan) provided with the accessory CA-261 to measure Ca^{2+} with continuous stirring, a thermostat adjusted to 37°C, and an injection chamber. Intracellular Ca^{2+} levels were registered every second by exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 505 nm was measured. In this way light intensities and their ratio (F_{340}/F_{380}) were followed. Drugs were injected (5 μl) into the chamber as a 100-fold concentrated solution without interruption of recording. The preparation was calibrated, and maximal fluorescence (F_{max}) was determined by addition of 0.1% Triton X-100 and minimal fluorescence (F_{min}) in the presence of 6 mM EGTA (pH adjusted to >8.3). $[Ca^{2+}]_i$ was calculated according to Grynkiewicz (17). Unless specified, basal values were considered those measured during 20 s before the addition of ANG II. Values were corrected for dye leakage, as described in Refs. 15 and 17, and for autofluorescence with unlabeled cells. Both dye leakage and autofluorescence were minimal.

To assess the concentration of ANG II at which homologous desensitization occurs, experiments were conducted by applying an initial stimulus of ANG II in concentrations ranging from 1×10^{-10} to 1×10^{-7} M at *minute 1* and a second stimulus of 1×10^{-7} M ANG II 2 min later. $[Ca^{2+}]_i$ was monitored as described.

To determine the briefest period of ANG II exposure that would cause desensitization, 10^{-7} M ANG II or buffer was applied for varying periods (1, 5, 10, and 20 min) to cells in culture (see *Cell culture*). Stimulus was removed by extensive washing, and cells were resuspended with 0.01% trypsin and 0.02% BSS-EDTA and tested for 1×10^{-7} M ANG II-induced Ca^{2+} mobilization 2 h after first stimulus.

To determine the period needed to recover from desensitization of Ca^{2+} mobilization induced by ANG II, the following experiment was performed. Desensitization of the ANG II response was induced by exposing dispersed cells to 1×10^{-7} M ANG II for 10 min. Cells were then washed repeatedly to remove ANG II and maintained in ANG II-free medium for 15, 30, 60, 120, 180, or 240 min before the reintroduction of 10^{-7} M ANG II and testing of the Ca^{2+} response. Buffer-exposed cells were tested in parallel.

Effect of ionomycin and TRH on $[Ca^{2+}]_i$ in ANG II-desensitized cells. Desensitization of the ANG II response was elicited by exposing dispersed cells to 1×10^{-7} M ANG II for 10 min. Cells were then washed repeatedly to remove ANG II and maintained in ANG II-free medium for 2 h before application of 5×10^{-7} M ionomycin or 1×10^{-7} M TRH and testing of the $Ca^{2+}{}_i$ response. Control cells were pretreated with buffer and similarly treated thereafter.

Cell culture. Cell pellets were resuspended in DMEM, supplemented with 10% horse serum, 2.5% fetal calf serum, 1% MEM nonessential amino acids, 25,000 U/l of nystatin, and 25 ng/l gentamicin. Cells were plated in sterile tissue culture plates (Cluster 24, Corning; 500,000 cells per well) and incubated with 1 ml DMEM (GIBCO) in a metabolic incubator at 37°C with 5% CO_2-95% O_2.

Desensitization of ANG II-induced cellular inositol phosphate accumulation and prolactin secretion. After 4 days in culture, cells were washed twice with DMEM and F-12 Nutrient Mixture (GIBCO), supplemented with 1% BSA, 2 mM glutamine, and the same concentration of antibiotics, to remove all traces of serum. Fresh medium containing 4 μCi/ml *myo*-[2-^{3}H(N)]inositol (specific activity: 20 Ci/mmol; New England Nuclear, Boston, MA) was added, and cells were incubated at 37°C for 24 h. At the end of the labeling period, cells were washed twice with DMEM-F-12 supplemented with 1% BSA, 2 mM glutamine, 25,000 U/l nystatin, and 25 ng/l gentamicin. Cells were pretreated with buffer or first stimulated with 1×10^{-7} ANG II for 10 min, and stimulus was removed by washing. Thereafter, cells were further incubated for 105 min in DMEM-F12. Medium was

replaced, and cells were incubated with 20 mM LiCl for 15 min to allow accumulation of inositol phosphate species. A second stimulus of 1×10^{-9} or 1×10^{-7} M ANG II, 1×10^{-7} M TRH, or buffer (control) was then applied to the cells in duplicate. For analysis of prolactin secretion, samples were taken 30 min after drug administration. Samples were stored at −20°C until analyzed by RIA after appropriate dilution in 0.01 M PBS with 1% egg albumin. Cells were placed on ice, treated with 0.5 M cold perchloric acid, and scraped for inositol phosphate determination. Experiments were repeated four times. Times and concentrations were chosen according to our previous experience (4).

Inositol phosphate accumulation. Inositol phosphates were measured as previously described (3), with minor modifications. Well contents were transferred to tubes and centrifuged for 10 min at 3,000 rpm (4°C). Pellets were kept for DNA measurement. The supernatants were neutralized (0.72 M KOH-0.6 M $HKCO_3$) and chromatographed on Dowex columns (AG-1-X8, 200–400 mesh, formate form, Bio-Rad, Buenos Aires, Argentina) to elute inositol monophosphate (InsP), diphosphate ($InsP_2$), and triphosphate ($InsP_3$). Phosphate esters were eluted by the stepwise addition of solutions containing increasing levels of formate. Specifically, they were sequentially eluted with 10 mM inositol (for free [^{3}H]-inositol), 0.1 M formic acid and 0.2 M ammonium formate (for InsP), 0.1 M formic acid and 0.4 M ammonium formate (for $InsP_2$), and 0.1 M formic acid and 1.0 M ammonium formate (for $InsP_3$). Two-milliliter aliquots of each wash fraction were mixed with 6 ml Optiphase "Hisafe" 3 (Wallac Oy, Turku, Finland) and counted in a liquid scintillation counter.

PKC downregulation. PKC was downregulated in vitro by a 24-h pretreatment of cultured cells with 1 μM phorbol 12-myristate 13-acetate (PMA). Homologous and heterologous desensitization of prolactin response by ANG II pretreatment was tested as we have described. Downregulation of PKC was confirmed by the inability of a 30-min stimulation with 1 μM PMA (PMA30) to release prolactin in PMA-pretreated cells (PMA24), (PMA30: 274.5 ± 52.0; PMA30+PMA24: 9.4 ± 12.6 prolactin increase in ng/ml, $P = 0.023$).

RIA. Prolactin was measured by RIA using kits provided by the National Institute of Diabetes and Digestive and Kidney Diseases. Results are expressed in terms of prolactin PRL RP_3. Intra- and interassay coefficients of variation were 7.2 and 12.8%, respectively.

Statistical analyses. Results are expressed as means ± SE. For $[Ca^{2+}]_i$ measurements, peak values were analyzed by one-way ANOVA for repeated measures. Individual means were then compared by Duncan's test (desensitized vs. control). Prolactin secretion and inositol phosphate generation in vitro were analyzed by two-way ANOVA for repeated measures for the effects of drug treatment and group (control or desensitized). If an F of interaction was found significant, individual means were compared by Scheffé's test; if it was not significant, groups of means were analyzed by the same test. Basal prolactin and inositol phosphates were analyzed by Student's t-test. $P < 0.05$ was considered significant.

RESULTS

Desensitization of ANG II-mediated increase in $[Ca^{2+}]_i$. The amplitude of the $[Ca^{2+}]_i$ spike depended on ANG II concentration, reaching an apparent maximum at 10 nM. At higher concentrations, the $[Ca^{2+}]_i$ response occurred earlier and more synchronically, with a steeper upstroke (Fig. 1). The EC_{50} for peak amplitude was 2.3 nM.

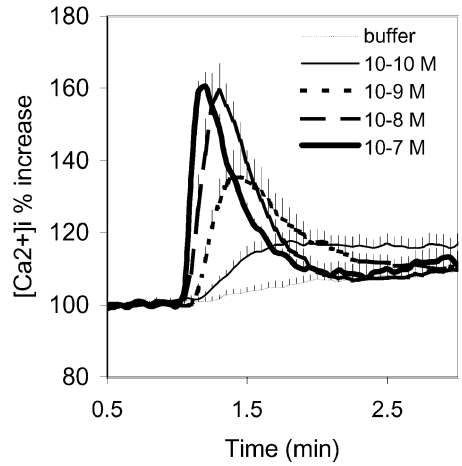

Fig. 1. Effect of various angiotensin (ANG) II concentrations (1×10^{-10} to 1×10^{-7} M) on %increase of intracellular calcium concentration ($[Ca^{2+}]_i$) over basal levels in dispersed anterior pituitary cells. Basal levels were considered as the average of $[Ca^{2+}]_i$ 20 s before the ANG II stimulus, which was applied at *minute 1*; basal $[Ca^{2+}]_i$ averaged 174 ± 35 nM. Lines represent the average for each time point, with thin lines on top or below, 1 SE; $n = 6$ for each concentration. (In all figures, n indicates nos. of experiments on different days, each performed in replicate from a batch of cells derived from ≥3 rats.)

To determine the ANG II concentration needed to elicit maximal desensitization, we tested the effect of pretreatment with increasing concentrations of ANG II on the ability of 1×10^{-7} M ANG II to stimulate Ca^{2+} mobilization 120 s later. Desensitization was observed in response to an ANG II concentration as low as 1×10^{-10} M (90.1 ± 4.4% of control), and it was significant at 1×10^{-9} M ANG II (34.2 ± 3.9% of control, $P = 0.011$; Fig. 2). Incubation of cells with 1×10^{-7} M ANG II completely abolished the $[Ca^{2+}]_i$ response to a second exposure to 1×10^{-7} M ANG II. A desensitizing concentration of 1×10^{-7} M was therefore used in subsequent experiments. A first-order (linear) regression model was applied to the transformed data [logit (%response) vs. log (ANG II)], yielding a square of the correlation coefficient (r^2) of 0.97 and a predicted value of 50% desensitization of 1.1 nM ANG II.

Incubation time required for desensitization. To determine the briefest ANG II exposure that would cause desensitization, 1×10^{-7} M ANG II was applied for varying periods of time (1, 5, 10, or 20 min) to pituitary cells in culture. Cells were washed repeatedly and subjected to a second 1×10^{-7} M ANG II pulse after 120 min. ANG II-induced $[Ca^{2+}]_i$ increase was already desensitized after 1 min of pretreatment (Fig. 3). The response obtained was 66.8 ± 2.1% of maximal response ($P = 0.044$), and this percentage did not change significantly with increasing incubation periods. For subsequent experiments, a 10-min pretreatment was chosen.

Recovery of Ca^{2+} response. In light of the susceptibility of cells to desensitization, we further assessed the ability of desensitized cells to recover. Cells were subjected to a 10-min pretreatment with 1×10^{-7} M ANG II and then washed with medium devoid of

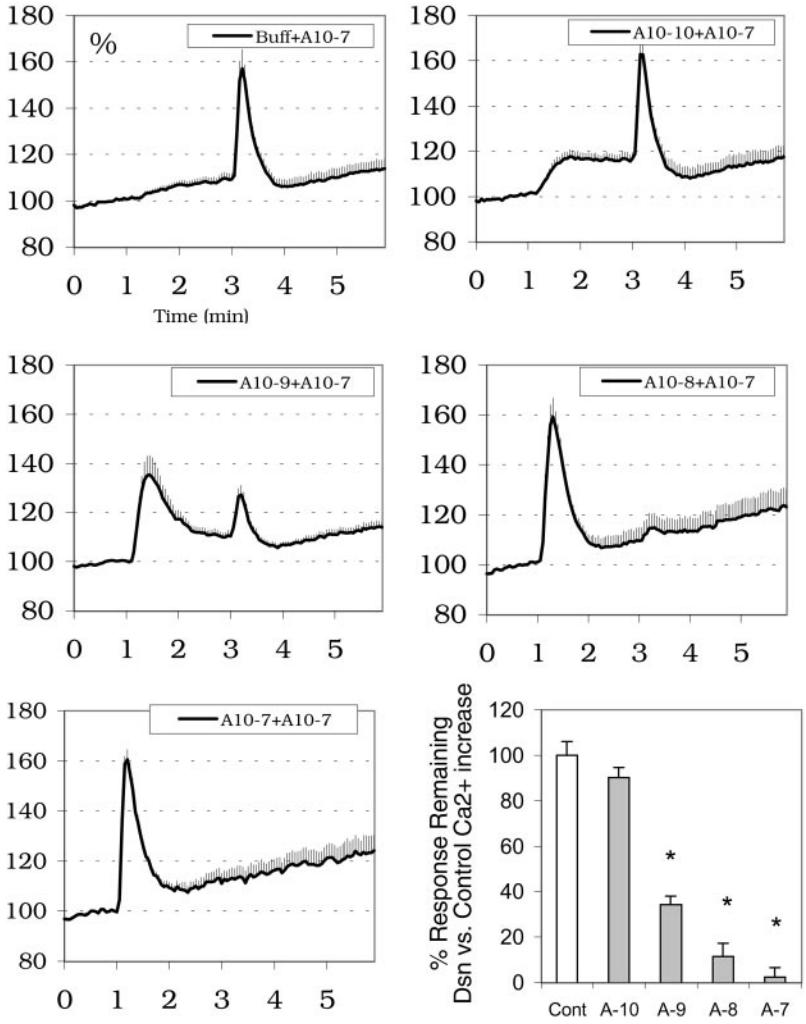

Fig. 2. Pretreatment with increasing concentrations of ANG II (A; *minute 1*, 1st arrow) on the ability of 1×10^{-7} M ANG II to stimulate intracellular Ca^{2+} mobilization 2 min later (second arrow). Results are expressed as %increase of $[Ca^{2+}]_i$ over basal levels. Keys express 1st and 2nd concentrations of ANG II used. *Bottom right*: $\%[Ca^{2+}]_i$ spike response remaining after a desensitizing (Dsn) stimulus of ANG II for each concentration. Spike increase in buffer-pretreated cells (Buff; *upper left*) was considered as 100%. $^*P < 0.011$ vs. control (buffer pretreatment); $n = 6$ experiments. The desensitizing effect of ANG II was concentration dependent with an EC_{50} of 1.1 nM.

ANG II and incubated for varying periods before the second pulse of 1×10^{-7} M ANG II. The Ca^{2+} response recovered partially after 15 min but remained at only 41.4 ± 2.6% of the control response ($P =$ 0.00016 vs. control). The response to ANG II was still different from the control value 4 h after the initial ANG II stimulus (77.6 ± 2.4%, $P = 0.00022$; Fig. 4).

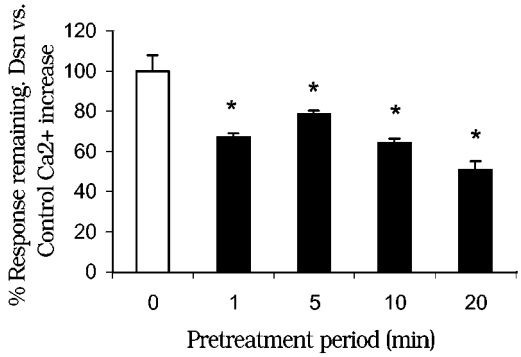

Fig. 3. Effect of duration of 1×10^{-7} M ANG II pretreatment (1, 5, 10, or 20 min) on intracellular Ca^{2+} response to a second 1×10^{-7} M ANG II stimulus applied 2 h later. Percent desensitized (Dsn) response remaining was calculated as spike $[Ca^{2+}]_i$ increase in ANG II-pretreated cells in relation to spike increase in buffer-pretreated cells, which was considered as 100%. Values are means ± SE of 4 experiments. $^*P < 0.05$ vs. buffer pretreatment (open bar); $n = 5$ experiments.

Recovery of intracellular Ca^{2+} stores after a desensitizing stimulus of ANG II. To test the possible involvement of intracellular Ca^{2+} pool depletion, we compared the effects of the Ca^{2+} ionophore ionomycin (5×10^{-7} M) on $[Ca^{2+}]_i$ mobilization in control cells and in cells desensitized with a 10-min pretreatment of 1×10^{-7} M ANG II 2 h earlier. We found that the increase in $[Ca^{2+}]_i$ in response to ionomycin was not different in control and desensitized cells (Fig. 5), suggesting that intracellular Ca^{2+} stores had already recovered by the time desensitization was still significant.

Heterologous desensitization of intracellular Ca^{2+} mobilization. To investigate whether a pretreatment with 1×10^{-7} M ANG II produced heterologous desensitization, 1×10^{-7} M TRH was applied 2 h after a 10-min pretreatment with buffer or 1×10^{-7} M ANG II, and $[Ca^{2+}]_i$ was monitored. As observed in Fig. 6, the TRH-induced Ca^{2+} peak was attenuated in the ANG II-pretreated group and reached only 60.3% of levels attained in the control group ($P = 0.001$).

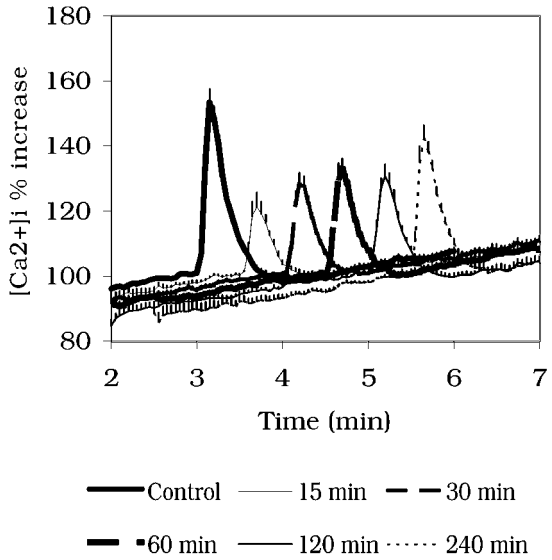

Fig. 4. Recovery from desensitization: cells were pretreated for 10 min with buffer (control) or 1×10^{-7} M ANG II and then washed extensively and maintained without ANG II for the indicated period (see keys) before a second stimulus of 1×10^{-7} M ANG II. Traces were arbitrarily off-set in time for clarity. Values are means ± SE of 5 separate experiments.

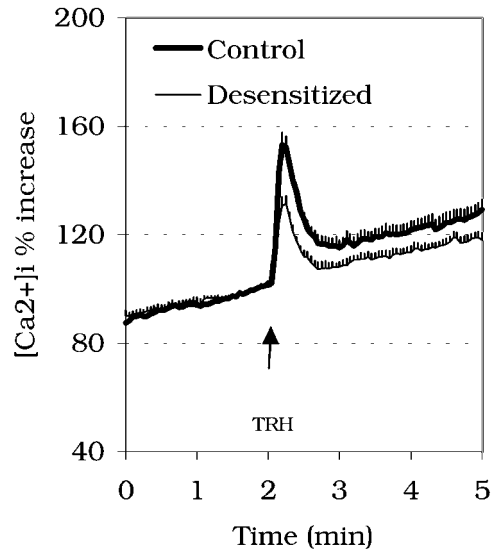

Fig. 6. Effect of thyrotropin-releasing hormone (TRH) on $[Ca^{2+}]_i$ mobilization in control and desensitized cells. Cells were pretreated for 10 min with buffer (control) or 1×10^{-7} M ANG II (Desensitized) and then washed extensively and maintained without ANG II for 2 h before being stimulated with 1×10^{-7} M TRH (arrow). Results are expressed as %increase of $[Ca^{2+}]_i$ over basal levels (the average of $[Ca^{2+}]_i$ 20 s before TRH stimulus); $n = 4$ experiments.

ANG II-mediated homologous and heterologous desensitization of inositol phosphate generation. To ascertain whether homologous and heterologous desensitization of PLC-β function occurred, we determined the effect of a 10-min pretreatment with 1×10^{-7} M ANG II on the ability of ANG II or TRH to generate inositol phosphates 2 h later. Basal levels of the three inositol phosphate species were increased in cells previously treated with ANG II. Basal levels in buffer-pretreated cells were 452.3 ± 37.3, 95.4 ± 8.7, and 32.63 ± 2.79 counts·min^{-1} (cpm)·μg DNA^{-1} ± SE for InsP, $InsP_2$, and $InsP_3$, respectively, and in ANG II-pretreated cells, levels were 802.2 ± 57.3, 171.6 ± 12.8, and 52.65 ± 1.67 cpm/μg DNA ($P = 0.038$ control vs. desensitized for the three inositol phosphate species). Such increment was probably related to the effect of ANG II pretreatment, which was prolonged by addition of LiCl, which prevents inositol phosphate breakdown. When we evaluated absolute increments after the second stimulus, similar patterns of response were found for the three inositol phosphate species. $InsP_3$ production was lower in desensitized cells (response to 1×10^{-9} and 1×10^{-7} M ANG II was 69.8 ± 3.5 and 72.8 ± 3.5% of control cells, $P = 0.045$; Fig. 7, *left*), and both $InsP_2$ and InsP production was also lower in these cells ($P = 0.05$ and 0.046, respectively; Fig. 7, *middle* and *right*). Interestingly, the response of inositol phosphates to 1×10^{-7} M TRH was also desensitized by previous treatment with ANG II (response was 69.6 ± 6.1% that of control response for $InsP_3$, $P = 0.045$).

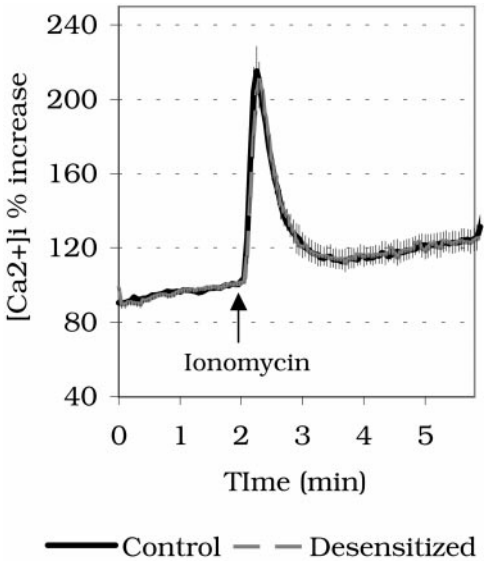

Fig. 5. Effect of ionomycin on intracellular Ca^{2+} mobilization in control and desensitized cells. Cells were pretreated for 10 min with buffer (control) or 1×10^{-7} M ANG II (Desensitized) and then washed extensively and maintained without ANG II for 2 h before being stimulated with 5×10^{-7} M ionomycin (arrow). Results are expressed as %increase of $[Ca^{2+}]_i$ over basal levels (basal levels being the average of $[Ca^{2+}]_i$ 20 s before ionomycin stimulus); $n = 4$ experiments.

ANG II-mediated homologous and heterologous desensitization of prolactin secretion. Response to the secretory activity of ANG II was also desensitized, because a pretreatment with 1×10^{-7} M ANG II for 10 min significantly reduced ANG II-induced prolactin secretion 2 h later (Fig. 8). The response was only 64.3 ± 9.9 and 53.4 ± 2.3% of that achieved in the control group for the concentrations of 1×10^{-9} and 1×10^{-7} M ANG II, respectively ($P = 0.022$). Response of prolactin secretion to 1×10^{-7} M TRH was also decreased (65.1 ± 7.2% of control response, $P = 0.022$) by a desensitizing pretreatment with ANG II.

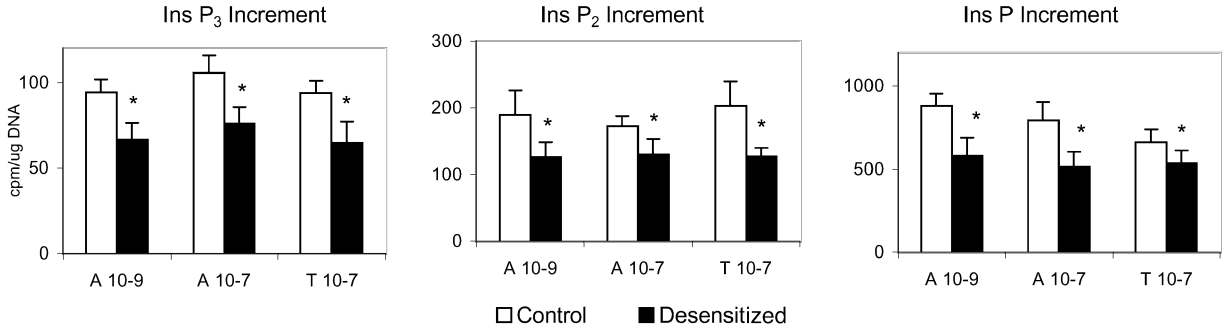

Fig. 7. Desensitization of inositol phosphate (inositol monophosphate, InsP; inositol biphosphate, $InsP_2$; inositol triphosphate, $InsP_3$) production elicited by ANG II in cultured pituitary cells. Cells were pretreated with buffer (control) or with 1×10^{-7} M ANG II (Desensitized) for 10 min, washed, and incubated for 2 h in agonist-free medium. They were next stimulated with 1×10^{-9} M ANG II, 1×10^{-7} M ANG II, or 1×10^{-7} M TRH, and inositol phosphates were measured 30 min thereafter. Results are expressed as absolute increment in counts·min^{-1} (cpm)·μg DNA^{-1} over basal levels; they are means ± SE of 4 independent experiments performed in duplicate. *$P < 0.05$ vs. respective control cells.

Involvement of PKC on ANG II-mediated heterologous desensitization of prolactin secretion and Ca^{2+} mobilization. To assess the role of PKC in the agonist-induced desensitization of the ANG II receptor, we investigated the effect of PMA pretreatment on the ANG II-mediated desensitization of the prolactin and intracellular Ca^{2+} response. PMA pretreatment (1 μM, for 24 h) did not modify basal prolactin levels [control: 901.8 ± 73.4, PMA: 807.6 ± 63.0 ng/ml, not significant (NS)]. Prolactin increment in response to ANG II and TRH was not significantly different in buffer or PMA-pretreated cells. As expected, prolactin response to ANG II and TRH was lower in ANG II-desensitized cells (Fig. 9; $P = 0.043$). In PMA-pretreated cells, ANG II-induced homologous and heterologous desensitization was still observed.

We also tested ANG II-induced heterologous desensitization of the Ca^{2+} response to TRH in PMA-pretreated and untreated cells. We found that the degree of desensitization was similar in both groups (remaining response to TRH in untreated and PMA-pretreated cells was 70.0 ± 8.0 and 78.1 ± 5.2%, respectively, NS).

DISCUSSION

The octapeptide ANG II binds and activates receptors in the plasma membrane of target cells, thereby

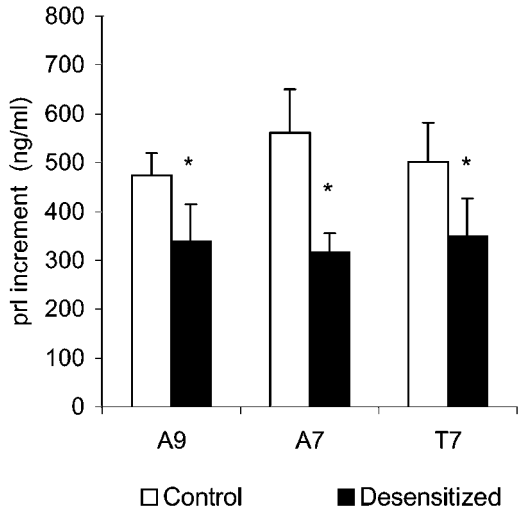

Fig. 8. Desensitization of prolactin (prl; PRL) secretion induced by ANG II in cultured pituitary cells. Cells were pretreated with buffer (control) or with 1×10^{-7} M ANG II (Desensitized) for 10 min, washed, and incubated for 2 h in agonist-free medium. A second stimulus of ANG II or TRH was then added, and samples were taken 30 min later. Results are expressed as absolute increment over basal levels; they represent means ± SE of 4 independent experiments performed in duplicate. Basal levels of PRL release in control and desensitized cells were 874 ± 55 and 990 ± 59 ng/ml ($P = 0.038$). *$P = 0.022$ vs. respective undesensitized cells.

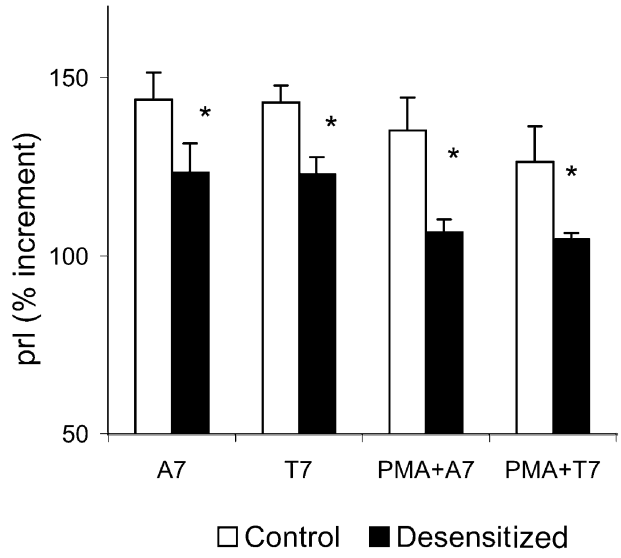

Fig. 9. Effect of 12-myristate 13-acetate (PMA) pretreatment (1 μM, 24 h) on ANG II-induced homologous and heterologous desensitization of PRL secretion in cultured pituitary cells. Cells were treated with buffer (control) or with 1×10^{-7} M ANG II (Desensitized) for 10 min, washed, and incubated for 2 h in agonist-free medium. A second stimulus of 1×10^{-7} M ANG II (A7) or 1×10^{-7} M TRH (T7) was then added, and samples were taken 30 min later. Results are expressed as %increment (means ± SE) over basal levels of PRL secretion (basal levels for control: 901.8 ± 73.4 and for PMA: 807.5 ± 63.0 ng/ml, not significant). *$P = 0.043$ vs. respective undesensitized cells; $n = 4$ experiments.

mediating a variety of important cardiovascular, homeostatic, and neuroendocrine functions. A brain-renin-ANG II, as well as a pituitary-renin-ANG II, system has been well characterized, and there are particularly high levels of ANG II in the hypothalamus. ANG II receptors are present in both the hypothalamus and the anterior pituitary of several species. Studies from numerous laboratories have shown that ANG II affects pituitary prolactin, growth hormone, adrenocorticotropin, and luteinizing hormone acting at the hypothalamus and/or the pituitary, and receptor number varies in physiologically diverse conditions (29). We have demonstrated that ANG II activates AT_1 pituitary receptors, and that inositol phosphate generation, $[Ca^{2+}]_i$ increase, and prolactin stimulation ensue (10, 16).

In many cells or tissues, exposure of ANG II receptors to ANG II often leads to a rapid loss of receptor responsiveness, or receptor desensitization. This has been described in cells from the kidney, heart, adrenal gland, and brain (1, 2, 14, 21, 28, 34). In the present study, we describe for the first time ANG II-induced homologous and heterologous desensitization of intracellular Ca^{2+} mobilization, $InsP_3$ generation, and prolactin release in dispersed anterior pituitary cells.

It has been documented that desensitization is an event associated with receptor structure as well as with the cell type in which the receptor is expressed (12). It is therefore understandable that different temporal and functional aspects of ANG II receptor desensitization have been described.

The desensitization of the ANG II-induced Ca^{2+} response was a concentration-related phenomenon reaching an apparent maximum at 1×10^{-7} M, and desensitization could not be overcome by using higher concentrations of ANG II (data not shown). The EC_{50} that induced homologous desensitization (1.1 nM) was in the range of the dissociation constant K_d for the AT_1 receptor in the pituitary (8). It was also similar to that obtained for ANG II-induced desensitization of $Ins(1,4,5)P_3$ production in CHO cells (33).

In contrast to the present results, in CHO-K1 cells expressing the AT_1 receptor (34, 35) after an initial stimulus with a subsaturating concentration (1×10^{-9} M) of ANG II, no response in $[Ca^{2+}]_i$ was observed to a second ANG II challenge added 2 min later. We observed that 1×10^{-9} M ANG II reduced the subsequent response to 34.2%, which was still a significant response. In human embryonal kidney, 293 cells stably expressing AT_{1A} receptors, cells initially stimulated with 1×10^{-7} M ANG II were refractory to a second ANG II stimulus 4 min later, as in the present work (18). Transfected cell systems represent a useful tool to study cellular mechanisms, but they may not reliably reflect the actual mechanism present in cells endogenously expressing AT_1 receptors. Particularly when desensitization is considered, discrepant results may be related to different amounts of expressed receptors or differential cell expression of elements involved in the mechanism of desensitization.

We next evaluated the extent of desensitization in relation to the duration of the initial stimulus. When cells were stimulated by ANG II and then washed and tested for desensitization 2 h later, an initial stimulus as short as 1 min was effective in producing maximal desensitization. This time course resembles desensitization of the AT_{1A} receptor in rat cardiomyocytes and in CHO cells, where rapid desensitization can occur even if receptor internalization is inhibited (1, 32). This suggests that receptor desensitization and internalization are distinct events, even though they can occur concurrently. In CHO cells expressing the AT_{1B} receptor, there was also a rapid (few minutes) and dose-dependent homologous desensitization of receptor-mediated production of second messengers (25, 33). Nevertheless, it has been shown that in bovine adrenal glomerulosa cells, AT_1 receptors are resistant to short-term desensitization and that long-term pretreatments with high concentrations of ANG II are needed to desensitize AT_1-mediated cellular responses (28). This may reflect the influence of cell type on the AT_1 receptor sensitivity to desensitization.

When ANG II was washed, the cells recovered the ability to increase $[Ca^{2+}]_i$ in response to a second ANG II stimulus already at 15 min, even though maximal response could not be obtained at that time. Full recovery from the desensitized state required more than 4 h. In CHO-K1 cells, a small response could be obtained 30 min after the initial stimulus, and an incomplete resensitization was observed 60 min after washing the initial stimulus. In contrast, in bovine adrenal glomerulosa cells, desensitization of the AT_{1B} receptor was a reversible phenomenon, because most of the binding capacity was recovered after 60 min (28).

The lack of full recovery of Ca^{2+} response in desensitized pituitary cells could be related to a depleted intracellular Ca^{2+} pool. This was verified by using ionomycin 120 min after a desensitizing treatment with ANG II. We found that Ca^{2+} stores had fully recovered by that time, suggesting that Ca^{2+} pool depletion might not account for ANG II-induced desensitization. In contrast, it has been described that gonadotropin-releasing hormone-induced desensitization of calcium response in the pituitary rapidly recovers (37) in parallel with a rapid replenishment of Ca^{2+} stores (23).

Desensitization was accompanied by a decrease of PLC-β function, as assessed by measurement of $InsP_3$ and by a decrease in prolactin release in response to a second stimulus of ANG II. Desensitization in all cases was not complete, because a response of 54 to 72% could be obtained depending on the parameter measured. A similar degree of desensitization of $Ins(1,4,5)P_3$ response to ANG II has been described in cultured smooth muscle cells (20) and in CHO cells stably transfected with the AT_{1A} receptor (9). This suggests that the desensitized Ca^{2+} response could be due to impaired $InsP_3$ production. The fact that the prolactin response was also desensitized would indicate that lactotropes are target cells of ANG II-induced desensitization. Nevertheless, response of corticotropes could

also be involved, inasmuch as it has been described that AT_{1B} receptors are present in both lactotropes and corticotropes in the pituitary, lactotropes being the predominant cell type in the female pituitary (22).

ANG II pretreatment also desensitized TRH-induced Ca^{2+} mobilization, $InsP_3$ generation, and prolactin release. This would indicate that a common element in the signal transduction pathway of ANG II and TRH was desensitized by ANG II. In this regard, both ANG II and TRH receptors interact with the pertussis toxin-insensitive G_q/G_{11} class of G proteins (19, 29). It has been proposed that, within any given cell, there is a limited pool of G proteins shared by a variety of endogenous receptors. It is possible that ANG II receptor stimulation and subsequent receptor uncoupling, or receptor G protein internalization, limits the G protein pool available to couple to other endogenously expressed receptors, in this case the TRH receptor.

Current evidence suggests that the initial, most rapid phase of desensitization occurs with a time course of seconds to minutes after exposure to an agonist and involves agonist-induced phosphorylation of GPCRs, uncoupling of the receptors from G proteins, and a loss of subsequent downstream events (7, 13). GPCRs can be phosphorylated by two different types of kinases: *1*) second messenger-activated kinases, such as protein kinase A or PKC, which produce a negative feedback and a nonspecific mechanism of desensitization, and *2*) specific kinases that form the growing family of G protein-coupled receptor kinases, which phosphorylate only the activated, or agonist-occupied, forms of GPCRs (13). Phosphorylation and regulation of GPCRs by second messenger-activated protein kinases are commonly suggested to play a role in heterologous desensitization of GPCRs.

Because both ANG II and TRH activate PKC, it is possible that this kinase might play an important role in heterologous desensitization. Because PKC does not discriminate between agonist-occupied and unoccupied receptors (13), activation of PKC is believed to be associated with both homologous and heterologous desensitization.

When PKC activation was prevented by 24 h of prior exposure to PMA, homologous and heterologous desensitization induced by ANG II still occurred, suggesting that this process is independent of PKC activation. Similar results for ANG II have been described in cardiomyocytes (38), bovine adrenal glomerulosa cells (6), and CHO cells transfected with the AT_{1A} receptor (9). It has also been reported that PKC inhibition reduced agonist-induced phosphorylation of the AT_{1A} receptor but did not affect its desensitization (25). Nevertheless, the PKC independence has not been a universal finding, because inhibitors of PKC prevented ANG II-mediated desensitization in vascular smooth muscle cells (26), glomerular mesangial cells (24, 27), and *Xenopus* oocytes (30), or they partially reverted ANG II-induced heterologous desensitization in CHO cells (33). These results confirm that different mechanisms of desensitization occur for the same agonist in different cell types.

Another possible mechanism involved in heterologous desensitization would be downregulation of the $Ins(1,4,5)P_3$ receptor, which has been shown to participate in heterologous desensitization of responses to ANG II in a rat liver epithelial cell line (5). Nevertheless, that event was observed only after longer treatments. Results from the present study suggest that the observed reduction in $InsP_3$ formation per se could explain the decrease in intracellular Ca^{2+} mobilization.

Desensitization is a complex process that plays an important role in turning off receptor-mediated signal transduction pathways. Although rapid desensitization appears to be a feature common to PLC-linked receptors, the extent and persistence of desensitization have been reported to vary from receptor to receptor. We provide evidence that ANG II induces homologous and heterologous desensitization of $[Ca^{2+}]_i$ mobilization, $InsP_3$ formation, and prolactin release in pituitary cells by a mechanism that does not involve PKC activation. Desensitization was rapid in onset and long lasting, although it was not complete because partial responses could be obtained. Time and concentration patterns of desensitization presented similarities and differences with respect to endogenously expressed or transfected AT_1 receptors in different cell types. In light of the development of antihypertensive treatments that use selective AT_1 receptor or angiotensin converting enzyme antagonists, it is important to gain an insight into the physiology of ANG II in the different organs in which it might have an impact.

We thank the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases and Dr. A. F. Parlow for the RIA kits.

This work was supported by Consejo Nacional de Investigaciones Cientificas y Técnicas, Agencia Nacional de Promoción Científica y Técnica, Centro de Investigaciones Quimicas y Farmacológias, and Fundación Antorchas, Buenos Aires, Argentina.

REFERENCES

1. **Abdellatif MM, Neubauer FC, Lederer WJ, and Rogers TB.** Angiotensin-induced desensitization of the phosphoinositide pathway in cardiac cells occurs at the level of the receptor. *Circ Res* 69: 800–809, 1991.
2. **Aboulafia J, Oshiro MEE, Feres T, Shimuta SI, and Paiva AC.** Angiotensin II desensitization and Ca^{2+} and Na^+ fluxes in vascular smooth muscle cells. *Pflügers Arch* 415: 230–234, 1989.
3. **Ascoli M, Pignataro OP, and Segaloff DL.** The inositol phosphate/diacylglycerol pathway in MA-10 Leydig tumor cells. *J Biol Chem* 264: 6674–6681, 1989.
4. **Becú-Villalobos D, Lacau-Mengido IM, Thyssen SM, Díaz-Torga GS, and Libertun C.** Effects of LHRH and ANG II on prolactin stimulation are mediated by hypophysial AT_1 receptor subtype. *Am J Physiol Endocrinol Metab* 266: E274–E278, 1994.
5. **Bokkala S and Joseph SK.** Angiotensin II-induced down-regulation of inositol trisphosphate receptors in WB rat liver epithelial cells. *J Biol Chem* 272: 12454–12461, 1997.
6. **Boulay G, Chretien L, Richard DE, and Guillemette G.** Short-term desensitization of the angiotensin II receptor of bovine glomerulosa cells corresponds to a shift from a high to a low affinity state. *Endocrinology* 135: 2130–2136, 1994.
7. **Bünemann M, Lee KB, Pals-Rylaarsdam R, Roseberry AG, and Hosey MM.** Desensitization of G-protein-coupled receptors in the cardiovascular system. *Annu Rev Physiol* 61: 169–192, 1992.

8. **Chen FM and Printz MP.** Chronic estrogen treatment reduces angiotensin II receptors in the anterior pituitary. *Endocrinology* 113: 1503–1510, 1983.
9. **Conchon S, Peltier N, Corvol P, and Clauser E.** A noninternalized nondesensitized truncated AT_{1A} receptor transduces an amplified ANG II signal. *Am J Physiol Endocrinol Metab* 274: E336–E345, 1998.
10. **Diaz-Torga GS, Becu-Villalobos D, and Libertun C.** Ontogeny of angiotensin-induced prolactin release in vivo and in vitro in female and male rats. *Neuroendocrinology* 59: 57–62, 1994.
11. **Duvilanski BH, Velardez MO, Gonzalez Iglesias A, Theas S, Seilicovich A, and Becu-Villalobos D.** Nitric oxide donors modify intracellular calcium in rat anterior pituitary cells. *Mol Cell Endocrinol* 146: 19–26, 1998.
12. **Falck-Pedersen E, Heinflink M, Alvira M, Nussenzveig DR, and Gershengorn MC.** Expression of thyrotropin-releasing hormone receptors by adenovirus-mediated gene transfer reveals that thyrotropin-releasing hormone desensitization is cell specific. *Mol Pharmacol* 45: 684–689, 1994.
13. **Freedman NJ and Lefkowitz RJ.** Desensitization of G protein-coupled receptors. *Rec Prog Horm Res* 51: 319–351, 1996.
14. **Gebke E, Muller AR, Jurzak M, and Gerstberger R.** Angiotensin II-induced calcium signalling in neurons and astrocytes of rat circumventricular organs. *Neuroscience* 85: 509–520, 1998.
15. **Gobbe P and Herchuelz A.** Effects of verapamil and nifedipine on gliclazide-induced increase in cytosolic free Ca^{2+} in pancreatic islet cells. *J Endocrinol Invest* 12: 469–474, 1989.
16. **Gonzalez Iglesias A, Diaz-Torga G, Lux-Lantos V, Libertun C, and Becu-Villalobos D.** Calcium influx and intracellular stores in angiotensin II stimulation of normal and hyperplastic pituitary cells. *Am J Physiol Endocrinol Metab* 277: E455–E463, 1999.
17. **Grynkiewicz G, Poenie M, and Tsien RY.** A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440–3450, 1985.
18. **Hein L, Meinel L, Pratt RE, Dzau VJ, and Kobilka BK.** Intracellular trafficking of angiotensin II and its AT_1 and AT_2 receptors: evidence for selective sorting of receptor and ligand. *Mol Endocrinol* 11: 1266–1277, 1997.
19. **Hinkle PM, Nelson EJ, and Ashworth R.** Characterization of the calcium response to thyrotropin-releasing hormone in lactotrophs and GH cells. *Trends Endocrinol Metab* 7: 370–374, 1996.
20. **Kai H, Fukui T, Lassègue B, Shah A, Minieri CA, and Griendling KK.** Prolonged exposure to agonist results in a reduction in the levels of the $G_q/G_{11}\alpha$ subunits in cultured vascular smooth muscle cells. *Mol Pharmacol* 49: 96–104, 1996.
21. **Kornfeld M, Gutiérrez AM, Persson AEG, and Salomonsson M.** Angiotensin II induces a tachyphylactic calcium response in the rabbit afferent arteriole. *Acta Physiol Scand* 160: 165–173, 1997.
22. **Lenkei Z, Nuyt AM, Grouselle D, Corvol P, and Llorens-Cortes C.** Identification of endocrine cell population expressing the AT1B subtype of angiotensin II receptors in anterior pituitary. *Endocrinology* 140: 472–477, 1999.
23. **McArdle CA, Willars GB, Fowkes RC, Nahorski SR, Davidson JS, and Forrest-Owen W.** Desensitization of gonadotropin-releasing hormone action in alpha T3-1 cells due to uncoupling of inositol 1,4,5-triphosphate generation and Ca2+ mobilization. *J Biol Chem* 271: 23711–23717, 1996.
24. **Ochsner M, Huwiler A, Fleck T, and Pfeilshifter J.** Protein kinase C inhibitors potentiate angiotensin II-induced phosphoinositide hydrolysis and intracellular Ca^{2+} mobilization in renal mesangial cells. *Eur J Pharmacol* 245: 15–21, 1993.
25. **Oppermann M, Freedman NJ, Alexander RW, and Lefkowitz RJ.** Phosphorylation of the type 1A angiotensin II receptor by G-protein-coupled receptor kinases and protein kinase C. *J Biol Chem* 271: 13266–13272, 1996.
26. **Palaparti A and Anand-Srivastava MB.** Angiotensin II modulates ANP-R2/ANP-C receptor-mediated inhibition of adenylyl cyclase in vascular smooth muscle cells: role of protein kinase C. *J Mol Cell Cardiol* 30: 1471–1482, 1998.
27. **Pfeilshifter J, Fandrey J, Ochsner M, Whitebread S, and de Gasparo M.** Potentiation of angiotensin II-stimulated phosphoinositide hydrolysis, calcium mobilization and contraction of renal mesangial cells upon downregulation of protein kinase C. *FEBS Lett* 261: 307–311, 1990.
28. **Richard DE, Laporte SA, Bernier SG, Leduc R, and Guillemette G.** Desensitization of AT_1 receptor-mediated cellular responses requires long term receptor down regulation in bovine adrenal glomerulosa cells. *Endocrinology* 138: 3828–3835, 1997.
29. **Saavedra JM.** Brain and pituitary angiotensin. *Endocr Rev* 13: 329–380, 1992.
30. **Sakuta H, Sekiguchi M, Okamoto K, and Sakai Y.** Desensitization of endogenous angiotensin II receptors in *Xenopus oocytes*: a role of protein kinase C. *Eur J Pharmacol* 208: 41–47, 1991.
31. **Sibley DR and Lefkowitz RJ.** Molecular mechanisms of receptor desensitization using the β-adrenergic receptor-coupled adenylate cyclase system as a model. *Nature* 317: 124–129, 1985.
32. **Tang H, Guo DF, Porter JP, Wanaka Y, and Inagami T.** Role of cytoplasmic tail of the type 1A angiotensin II receptor in agonist- and phorbol ester-induced desensitization. *Circ Res* 82: 523–531, 1998.
33. **Tang H, Shirai H, and Inagami T.** Inhibition of protein kinase C prevents rapid desensitization of type 1B angiotensin receptor. *Circ Res* 77: 239–248, 1995.
34. **Thekkumkara TJ, Du J, Dostal DE, Motel TJ, Thomas WG, and Baker KM.** Stable expression of a functional rat angiotensin II (AT1A) receptor in CHO-K1 cells: rapid desensitization by angiotensin II. *Mol Cell Biochem* 146: 79–89, 1995.
35. **Thomas WG, Thekkumkara TJ, Motel TJ, and Baker KM.** Stable expression of truncated AT_{1A} receptor in CHO-K1 cells. The carboxyl-terminal region directs agonist-induced internalization but not receptor signaling or desensitization. *J Biol Chem* 270: 207–213, 1995.
36. **Tobin AB.** Phosphorylation of phospholipase C-coupled receptors. *Pharmacol Ther* 75: 135–151, 1998.
37. **Weiss J, Cote CR, Jameson JL, and Crowley WFJ.** Homologous desensitization of gonadotropin-releasing hormone (GnRH)-stimulated luteinizing hormone secretion in vitro occurs within the duration of an endogenous GnRH pulse. *Endocrinology* 136: 138–143, 1995.
38. **Zhang M, Turnbaugh D, Cofie D, Dogan S, Koshida H, Fugate R, and Kem DC.** Protein kinase C modulation of cardiomyocyte angiotensin II and vasopressin receptor desensitization. *Hypertension* 27: 269–275, 1996.