Calcium influx and intracellular stores in angiotensin II stimulation of normal and hyperplastic pituitary cells

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ANGIOTENSIN II (ANG II) releases prolactin through a Ca

stimulation of normal and hyperplastic pituitary cells. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E455–E463, 1999.—In rat pituitary cells from estrogen-induced hyperplasia, angiotensin II (ANG II) does not evoke a clear spike elevation of intracellular Ca

channels (Ca

in the absence of extracellular Ca

channels in cells depolarized with 25 mM K

or in the presence of blockers of L-type voltage-sensitive Ca

channels (VSCC). These treatments did not alter spike elevation in [Ca

in controls but reduced plateau levels in hyperplastic cells. Intracellular Ca

stores were similar in both groups, as assessed by thapsigargin treatment, but this drug abolished spike increase in controls and scarcely modified plateau levels in hyperplastic cells. Finally, inositol trisphosphate (InsP

production in response to ANG II was significantly higher in control cells. We conclude that the observed plateau rise in hyperplastic cells results mainly from Ca

influx through VSCC. In contrast, in control cells, the ANG II-induced spike increase in [Ca

results from mobilization of Ca

from thapsigargin-sensitive internal channels, activated by higher inositol 1,4,5-trisphosphate generation.

We examined the state of Ca

release from the endoplasmic Ca

stores in both groups of cells after stimulation with ANG II. Finally, to determine whether the absence of an [Ca

spike in ANG II-stimulated hyperplastic pituitary cells was due to an alteration in inositol trisphosphate [InsP

production, we determined the increase in inositol phosphate species comparatively in both groups of cells after stimulation with ANG II.

MATERIALS AND METHODS

Animals

Female 60-day-old Sprague-Dawley rats were housed in an air-conditioned room with lights-on at 0700 and lights-off at 1900. They had free access to laboratory chow and tap water. Pituitary tumors were induced by subcutaneous implantation of 20 mg of diethylstilbestrol (Sigma). Rats in diestrous were used as controls. Pituitary weights at death were 14.3 ± 0.4 and 44.9 ± 3.0 mg, and serum prolactin levels were 5.2 ± 2.1.

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and 522.9 ± 77.3 ng/ml for control and hyperplastic groups, respectively.

**Cell Dispersion**

Unless specified, all drugs were purchased at Sigma.

Rats were killed by decapitation at 0900, and normal or tumoral pituitaries were removed on ice, separated from the neurointermediate lobe, and placed in chambers containing freshly prepared Krebs-Ringer bicarbonate buffer without Ca$^{2+}$ or Mg$^{2+}$. Buffer contained 14 mM glucose, 1% BSA, modified Eagle's medium (MEM) amino acids (2% Gibco), and phenol red (0.025%) and was previously gassed for 15 min with 95% O$_2$-5% CO$_2$ and adjusted to pH 7.35–7.40. Buffer was filtered through a membrane (Nalgene), the pore diameter of which was 0.45 µm. Hypophyses were washed three times with Krebs-Ringer bicarbonate buffer and then cut into 1-mm pieces. Obtained fragments were washed and incubated in the same buffer containing 0.5% trypsin for 30 min at 37 °C, 95% O$_2$-5% CO$_2$. They were treated for two additional minutes with DNase I (1 mg/ml), and digestion was ended by adding 0.2% newborn calf serum (Gibco). 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 with the use of a Neubauer chamber. Viability of cells determined by trypan blue was always >90%. Cells were used fresh for intracellular Ca$^{2+}$ measurements or plated in sterile cell culture plates (Corning P24; 750,000 cells/well) in Dulbecco's MEM (DMEM) supplemented with 10% horse serum, 2.5% FCS, 1% MEM nonessential amino acids, 10,000 U/ml of penicillin, 10,000 µl gentamicin for inositol phosphate determinations.

**Intracellular Ca$^{2+}$ Measurements**

The acetoxyethyl ester of fura 2 (fura-2 AM) was used as a fluorescent indicator. The pellet of adenohypophysial cells of each experimental group was resuspended and incubated in a buffered saline solution (BSS; 140 mM NaCl, 3.9 mM KCl, 0.7 mM KH$_2$PO$_4$, 0.5 mM Na$_2$HPO$_4$·12H$_2$O, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$, and 20 mM HEPES, pH 7.5) 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 5% CO$_2$, during which time fura 2 was 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 × 10$^6$ cells/ml BSS. Fluorescence was measured in a spectrofluorometer (Jasco) provided with the accessory CA-261 to measure Ca$^{2+}$ with continuous stirring, with the 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 (F340/F380) were followed. Drugs were injected (5 µl) into the chamber as a 100-fold concentrated solution without interruption of recording. The preparation was calibrated by measuring maximal fluorescence induced by 0.1% Triton X-100 and minimal fluorescence in the presence of 6 mM EGTA (pH adjusted to over 8.3). [Ca$^{2+}$]i was calculated according to Grynkiewicz et al. (9). Basal values were considered those measured during the 20 s before the addition of ANG II, unless otherwise specified. Values were corrected for dye leakage, as described previously (8, 9), and for autofluorescence, using unlabeled cells. Both dye leakage and autofluorescence were minimal.

**Experiment 1. Responses to 10 nM ANG II in buffer with or without Ca$^{2+}$ were compared. In this second case, after incubation with fura 2-AM, cells were washed and resuspended in BSS without CaCl$_2$ and 1 min before the experiment, 60 µM EGTA was added. These cells did not respond to KCl, confirming that no extracellular Ca$^{2+}$ was present (data not shown).**

Where spike and plateau increments are reported, these are defined as the increase over basal levels achieved 12 s after stimulation with ANG II (spike) and the average increase between 18 and 36 s after ANG II (plateau).

**Experiment 2. BSS or 25 mM KCl was injected in the chamber at min 1, and 10 nM ANG II was applied 2 min thereafter.**

**Experiment 3. Responses to 10 nM ANG II in the presence and absence of 1 µM nifedipine or methoxyverapamil hydrochloride (2 and 5 µM; antagonists of L-type VSCC) were compared. Nifedipine solutions were protected from light. ANG II was tested 1 min after verapamil or nifedipine treatment. Two or three minutes after ANG II addition, cells were challenged with 25 mM K$^+$.**

**Experiment 4. The interaction of thapsigargin with ANG II was tested in the presence and absence of extracellular Ca$^{2+}$. A value of 0.5 or 2 µM thapsigargin was administered to cell suspension 23 min before ANG II to allow for complete inhibition of endoplasmatic reticulum ATPases and Ca$^{2+}$ depletion from this pool (35). This was verified by the inability of 0.1 µM thyrotropin-releasing hormone to increase [Ca$^{2+}$]i 23 min after thapsigargin treatment (data not shown).**

Time schedules and concentrations of drugs were chosen according to previous experiments (7).

**Determination of Cellular Inositol Phosphate Accumulation**

Inositol phosphates were measured as previously described (3), with minor modifications. Briefly, 1 day after plating, the medium in the wells was changed to fresh medium containing 4 µCi/ml myo-[2-3H(N)]inositol (specific activity 20 Ci/mm; NEN, Boston, MA) and incubated for 48 h before the experiment. At the end of the labeling period, the cells were washed twice with DMEM-Ham's F-12 solution with 2.2 g/l sodium bicarbonate containing 0.1% BSA (buffer 1). Cells were then incubated in buffer 1 with 20 mM LiCl for 15 min. Thereafter, stimuli (10 µl) were added (final concentrations in the well: 0.1 and 10 nM ANG II), and cells were further incubated for 30 min. After the incubation, cells were placed on ice, treated with 0.5 M HClO$_4$, and scraped. Well contents were transferred to tubes and centrifuged. Pellets were kept for DNA measurement. The neutralized supernatants (0.72 M KOH and 0.6 M HCO$_3$) were chromatographed on Dowex columns (AG-1-X8, 200–400 mesh, formate form; Bio-Rad) to elute inositol 4-monophosphate [Ins(4)P], inositol 1,4-bisphosphate [Ins(1,4)P$_2$], and Ins(1,4,5)P$_3$. 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 [3H]inositol), 0.1 M formic acid and 0.2 M ammonium formate (for Ins(4)P), 0.1 M formic acid and 0.4 M ammonium formate (for Ins(1,4)P$_2$), and 0.1 M formic acid and 1.0 M ammonium formate (for Ins(1,4,5)P$_3$). Two-milliliter aliquots of each wash were mixed with 6 ml Optiphase “Hisafe” 3 (Wallac Oy, Turku, Finland) and counted in a liquid scintillation counter. Experiments were repeated five times.

**Statistical Analyses**

Results are expressed as means ± SE. Peak values or plateau levels at determined time periods were analyzed by
two-way ANOVA for repeated measures for the effects of drug (ANG II or buffer) and pretreatment (i.e., with or without Ca²⁺, thapsigargin, K⁺, nifedipine, or verapamil). If F of interaction was found significant, individual means were compared by Scheffe’s test if it was not significant, groups of means were analyzed by the same test. Basal [Ca²⁺] was analyzed by Student’s t-test. Inositol phosphate production was analyzed by two-way ANOVA for the effects of drug and cell group. P < 0.05 was considered significant.

RESULTS

In pituitary cells from female diestrous rats, 10 nM ANG II induced an [Ca²⁺] spike response (Fig. 1A). It consisted of a 2- to 6-s delay, a rise from 180.5 ± 2.6 to a peak of 253.1 ± 5.7 nM after 12 ± 1 s, and a subsequent decay to resting levels after 49 ± 3 s. From min 1 to min 2 after ANG II stimulus, [Ca²⁺] remained slightly below resting levels (−2.0%).

In cells derived from an estrogen-induced pituitary tumor, the spike phase was practically absent (Fig. 1B). A value of 10 nM ANG II increased [Ca²⁺] from 192.5 ± 8.6 to a plateau concentration of 222.7 ± 8.9 nM after 23 ± 2 s, and levels remained elevated for at least 3 min.

To ascertain the participation of plasma membrane Ca²⁺ channels in mediating ANG II action in control and in hyperplastic cells, we tested the effect of 10 nM ANG II on [Ca²⁺] in the absence of extracellular Ca²⁺, 2) in the presence of a depolarizing agent (25 mM K⁺), or 3) in the presence of blockers of L-type VSCC (nifedipine and verapamil).

Removal of extracellular Ca²⁺ consistently reduced [Ca²⁺], indicating that Ca²⁺ entry is coupled to basal [Ca²⁺]. Levels were reduced to 148.4 ± 8.2 and 145.4 ± 9.5 nM in control and hyperplastic cells, respectively (Fig. 1A and B, respectively).

Spike-phase response to 10 nM ANG II in control cells was slightly attenuated in absolute values [increments with and without Ca²⁺ were 69.6 ± 7.0 and 55.0 ± 7.5 nM, respectively; not significant (NS); Fig. 1A]; however, percent increment over basal levels was unaltered (39.5 ± 6.4 and 38.7 ± 3.1%, with and without extracellular Ca²⁺, respectively).

In hyperplastic cells, Ca²⁺ removal led to a significant decrease of plateau Ca²⁺ response in absolute and in percent values (Fig. 1B). Plateau-phase increment over basal levels in response to ANG II decreased from 32.1 ± 3.3 in Ca²⁺-containing medium to 7.8 ± 2.7 nM in Ca²⁺-free medium (P < 0.05).

A high extracellular K⁺ concentration depolarizes the cells and stimulates Ca²⁺ influx through VSCC. In both groups, when extracellular K⁺ was increased to 25 mM, there was an immediate, significant, and transient increase in [Ca²⁺] that reached a peak at 16 ± 1 s. The [Ca²⁺] subsequently dropped, at first rapidly and then gradually.

In control cells, the addition of K⁺ did not modify the posterior spike Ca²⁺ response evoked by 10 nM ANG II (Fig. 2A), suggesting that the principal component of the spike rise in [Ca²⁺], induced by ANG II was independent of VSCC activation. Absolute increments achieved by ANG II in the presence and absence of K⁺ were 62.5 ± 5.1 and 71.7 ± 15.4 nM, respectively (NS). On the other hand, in hyperplastic cells, plateau response to ANG II was markedly reduced in the presence of 25 mM K⁺ (Fig. 2B). ANG II induced a plateau increment of 15.3 ± 2.3 nM in these cells, and previous depolarization with K⁺ reduced the effect to 2.2 ± 2.4 nM (P < 0.05).

To evaluate the contribution of L-type VSCC in mediating Ca²⁺ entry induced by ANG II, we tested its interaction with inhibitors of L-type VSCC: methoxyverapamil (a phenylalkylamide) and nifedipine (a dihydropyridine).

When cells were incubated with 1 µM nifedipine, baseline [Ca²⁺] decreased significantly by 11.0 ± 2.4% in control cells and by 6.0 ± 2.4% in hyperplastic cells (Fig. 3). Nifedipine did not reduce percent or absolute Ca²⁺ mobilization induced by ANG II in control cells (Fig. 3A) but in hyperplastic cells reduced the plateau response to 47.5% of maximal response (Fig. 3B). Absolute increments for control cells were 68.7 ± 6.6 and 73.7 ± 5.8 nM (without and with nifedipine, respectively; NS) and for hyperplastic cells were 25.8 ± 3.1 and 12.2 ± 1.6 nM, respectively (P < 0.05).
The addition of 2 or 5 µM methoxyverapamil to cells 1 min before 10 nM ANG II also decreased resting $[\text{Ca}^{2+}]_{i}$ by 11.4 ± 2.3 and 13.0 ± 3.1%, respectively, in control cells and by 3.3 ± 1.6 and 2.3 ± 1.1%, respectively, in hyperplastic cells. Nevertheless, verapamil did not reduce peak height of $\text{Ca}^{2+}$ response to ANG II in control cells (Fig. 4B). In hyperplastic cells, 5 µM verapamil reduced the plateau response to 49.6% of maximal response (Fig. 4B) and to 61.2% at the concentration of 2 µM. Absolute increments for control cells were 86.9 ± 10.8, 82.4 ± 8.3, and 89.0 ± 8.9 nM (without and with verapamil at 2 and 5 µM, respectively; NS); values for hyperplastic cell plateau increments were 43.2 ± 5.6, 24.7 ± 3.1, and 21.4 ± 2.7 nM, respectively (P < 0.05, both concentrations vs. without verapamil).

Conversely, an increase in $[\text{Ca}^{2+}]_{i}$ after subsequent addition of K$^{+}$ was reduced but not abolished in nifedipine- and verapamil-treated cells of both groups (Figs. 3 and 4).

The relative $\text{Ca}^{2+}$ content of the intracellular $\text{Ca}^{2+}$ pools was estimated by mobilization of stored $\text{Ca}^{2+}$ to the cytosol in $\text{Ca}^{2+}$-free medium to prevent $\text{Ca}^{2+}$ entry. Under these conditions, the size of the transient $\text{Ca}^{2+}$
increase is a good index of the relative degree of filling of intracellular stores (36). To that end we used thapsigargin, the specific inhibitor of the endoplasmic reticulum Ca^{2+} pumps. A concentration of 2 µM thapsigargin elicited a peak in \([\text{Ca}^{2+}]_i\) followed by a decrease to subbasal level. The response was similar in control and hyperplastic cells: absolute increments were 67.2 ± 6.4 and 70.4 ± 10.9 nM, respectively (NS; Fig. 5A). In Ca^{2+}-containing medium, absolute increments after treatment with 2 µM thapsigargin were also similar (71.2 ± 9.0 and 68.0 ± 5.8 nM, respectively; NS; Fig. 5B), but there was a sustained \([\text{Ca}^{2+}]_i\) increase that was due to \(\text{Ca}^{2+}\) influx, since it was prevented by \(\text{Ca}^{2+}\) removal. A concentration of 0.5 µM thapsigargin produced a similar response in both groups (data not shown). This \(\text{Ca}^{2+}\) influx probably represents "capacitative \(\text{Ca}^{2+}\) entry," which is triggered by the emptying of intracellular stores. Levels rose well above those found before treatment with thapsigargin.

Pretreatment with 0.5 or 2 µM thapsigargin prevented the ANG II-induced Ca^{2+} peak in control cells. The ANG II-induced transient spike in \([\text{Ca}^{2+}]_i\) was 83.9 ± 5.6 nM over resting levels and 12.6 ± 2.2 nM (P < 0.05) after treatment of control cells with thapsigargin (Fig. 6A). In hyperplastic cells, 2 µM thapsigargin nonly slightly reduced the ANG II-induced plateau in \([\text{Ca}^{2+}]_i\) from 34.4 ± 4.6 to 28.8 ± 2.5 nM (NS; Fig. 6B). At the concentration of 0.5 µM thapsigargin, \([\text{Ca}^{2+}]_i\) in response to ANG II rose only 11.4 ± 1.9 in control cells (P < 0.05) and 27.6 ± 3.1 nM in hyperplastic cells (NS).

In thapsigargin-pretreated cells incubated in Ca^{2+}-free medium, the ANG II response was abolished in both groups of cells (data not shown).

We finally evaluated inositol phosphate species production in cultured cells from both groups after treatment with 0.1 and 10 nM ANG II. There were no differences in \(\text{Ins}(4)\text{P}_1\) or \(\text{Ins}(1,4)\text{P}_2\) percent increment induced by ANG II between both groups of cells (Fig. 7). In control cells, \(\text{InsP}_3\) production was significantly higher than in hyperplastic cells at both concentrations tested (absolute values ± SE for buffer: 0.1 and 10 nM ANG II = 363 ± 44, 1,129 ± 190, and 1,806 ± 175 in control cells and 239 ± 140, 565 ± 39, and 723 ± 102 counts/min in hyperplastic cells; P < 0.05 for 0.1 and 10 nM ANG II, control vs. hyperplastic).

**DISCUSSION**

In a previous work (5) we described the way in which chronic in vivo treatment with estrogens modifies the...
response of pituitary cells to ANG II in vitro. There is an alteration in intracellular Ca\(^{2+}\) mobilization induced by the octapeptide in correlation with a decreased sensitivity to its prolactin-releasing effect. We showed that changes in pituitary cell function acquired in vivo persisted in culture, suggesting that there had been a profound alteration in cellular mechanisms controlling Ca\(^{2+}\) homeostasis in estrogen-treated cells.

In many cells, Ins (1,4,5)P\(_3\)-induced Ca\(^{2+}\) release from intracellular stores is commonly associated with extracellular Ca\(^{2+}\) influx. ANG II induces Ca\(^{2+}\) mobilization from the endoplasmic reticulum, evoking an early spike response in [Ca\(^{2+}\)]\(_i\) and producing a temporary hyperpolarization of the plasma membrane. Thereafter, it generates an increase in the steady-state firing of action potentials, facilitating extracellular Ca\(^{2+}\) influx. Operation of these two Ca\(^{2+}\)-signaling pathways may mediate ANG II-induced prolactin secretion from rat pituitary lactotrophs. As in hyperplastic cells derived from an estrogen-induced tumor, the initial spike-phase mobilization of intracellular Ca\(^{2+}\) is lost or greatly reduced; we attempted to elucidate the participation of Ca\(^{2+}\) channels of the endoplasmic reticulum and of plasma membrane Ca\(^{2+}\) channels in ANG II-induced Ca\(^{2+}\) signaling in these cells in comparison with pituitary cells from rats in diestrus.

We first evaluated the involvement of plasma membrane Ca\(^{2+}\) channels in mediating ANG II action. Removal of external Ca\(^{2+}\) decreased resting [Ca\(^{2+}\)]\(_i\) levels, indicating that Ca\(^{2+}\) influx maintains basal [Ca\(^{2+}\)]\(_i\) in both types of cells. To this effect, it has been shown that basal influx in pituitary cells is not linked to action potentials and that it has a substantial impact on the steady-state basal [Ca\(^{2+}\)]\(_i\). The fact that nifedipine and verapamil also reduced basal [Ca\(^{2+}\)]\(_i\) suggests that this influx is partly through L-type VSCC.

The ANG II-induced [Ca\(^{2+}\)]\(_i\) spike elevation in normal cells is predominantly dependent on the release of Ca\(^{2+}\) from the intracellular stores and independent of VSCC activation. This has been described in Chinese Hamster ovary CHO-K1 cells expressing recombinant AT\(_1\) receptors (31) and in bovine adrenal glomerulosa cells (2). In the present experiments, results obtained from

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**Fig. 6.** Effect of depletion of intracellular Ca\(^{2+}\) stores with 2 µM thapsigargin (23 min of pretreatment) on ANG II-induced Ca\(^{2+}\) mobilization in dispersed anterior pituitary cells. A: control. B: hyperplastic cells. Pretreatment with buffer, thick line; pretreatment with thapsigargin, thin line. A concentration of 10 nM ANG II was administered at min 3, and 25 mM K\(^+\) was administered at min 6; n = 8 for each group.

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**Fig. 7.** Percent inositol phosphate (IP\(_x\)) generation in response to 10\(^{-10}\) M ANG II (10) and 10\(^{-8}\) M ANG II (8). * P < 0.05 vs. respective control cells. A: inositol 4-monophosphate. B: inositol 1,4-bisphosphate. C: inositol trisphosphate.
VSCC activation by K⁺ or inhibition by blockers and in extracellular Ca²⁺-free cells indicate a similar behavior in normal pituitary cells.

Removal of external Ca²⁺, however, decreased the plateau response of ANG II in hyperplastic cells, suggesting that the increase in [Ca²⁺]i, was caused by Ca²⁺ entry. Furthermore, depolarization with K⁺, which activates voltage-dependent Ca²⁺ influx, significantly reduced the ANG II-induced plateau in [Ca²⁺]i in these cells, suggesting a common entry pathway.

To define the transmembrane pathway through which extracellular Ca²⁺ enters the cytoplasm during ANG II stimulation in hyperplastic cells, nifedipine and verapamil (selective L-type Ca²⁺ blockers) were tested. Results indicate that L-type VSCC are essential for ANG II-induced Ca²⁺ entry in these cells. Because inhibition of plateau phase by these blockers was only partial, another mechanism could contribute to the ANG II-induced Ca²⁺ increase. To this effect, it has been shown that L-type 1,4-dihydropyridine-sensitive channels are the major pathway for voltage-dependent Ca²⁺ entry, controlling hormone release (34); however, ω-conotoxin-sensitive P- and T-type channels have also been described in pituitary cells (13, 27).

Failure of ANG II to evoke a consistent spike phase in hyperplastic cells could be related to 1) reduced InsP₃ production in response to ANG II, 2) altered expression of the InsP₃ receptor on the endoplasmic reticulum, or 3) a decreased activity of the Ca²⁺-ATPase pump on the endoplasmic reticulum with a concomitant decrease in the Ca²⁺ content of intracellular stores.

In this respect, both estradiol and progesterone have been implicated in the regulated activity of Ca²⁺-ATPase in myometrium (20) and in ANG II-induced inositol phosphate production in donal pituitary cells (12). Therefore, we attempted to define whether Ca²⁺ stores of the endoplasmic reticulum were altered in hyperplastic cells. The relative Ca²⁺ content of the intracellular Ca²⁺ pools was estimated with the use of thapsigargin. This drug blocks the ATPase of the endoplasmic reticulum that normally sequesters Ca²⁺, which leaks out of this compartment. Thapsigargin administration in the absence of extracellular Ca²⁺ indicated that the relative degree of filling of stores was not altered.

Treatment with thapsigargin in Ca²⁺-containing medium resulted in a sustained increase in [Ca²⁺]i that was similar in both groups. There was no return to prestimulatory or subprestimulatory levels in this medium. Such a Ca²⁺ influx response was characterized by its latency in being activated and occurred only after stored Ca²⁺ had been discharged. Mechanisms and mediators of this Ca²⁺-influx pathway (termed capacitative entry) remain largely unknown (32).

Thapsigargin pretreatment inhibited the action of ANG II on Ca²⁺ mobilization in control cells, confirming that the majority of the Ca²⁺ of the initial spike is released from intracellular thapsigargin-sensitive stores, which are known to be activated by Ins(1,4,5)P₃. The same result for ANG II was found in astroglia cultured from rat hypothalamus (37). A very small percentage of the response could not be abolished. Therefore, our results do not exclude the fact that ANG II is able to mobilize Ca²⁺ from intracellular stores that are thapsigargin insensitive. In this respect, it has been shown that GH4C1 cells contain at least two functionally distinct intracellular Ca²⁺ stores (11). One is sensitive to Ins(1,4,5)P₃ and thapsigargin. The second store is not and is regulated through a Ca²⁺-release mechanism (36). Extracellular Ca²⁺ is needed to keep this pool in a functional state. Furthermore, a nonacidic Ins(1,4,5)P₃- and thapsigargin-insensitive Ca²⁺ pool has been characterized in GH3 cells (23). This pool was also affected by Ca²⁺ influx. In agreement, we found that if thapsigargin was administered in Ca²⁺-free medium, the residual effect of ANG II on [Ca²⁺]i in control cells was completely abolished.

In hyperplastic cells, thapsigargin pretreatment did not modify the ANG II-induced plateau in [Ca²⁺]i, indicating that the plateau increase in [Ca²⁺]i can be accounted for mainly by Ca²⁺ influx and not by Ca²⁺ derived from intracellular thapsigargin-sensitive stores.

Our results suggest that the size of the InsP₃-sensitive Ca²⁺ pool was not affected in hyperplastic cells, but ANG II was unable to evoke a spike increase in [Ca²⁺]i because it failed to mobilize the extent intracellular pool. We therefore evaluated the production of inositol phosphates by ANG II. Ins(1,4,5)P₃ binds to Ca²⁺-channel receptors located in the endoplasmic reticulum, leading to channel opening and release of stored Ca²⁺ to the cytoplasm.

In hyperplastic cells, there was a reduction in ANG II-induced intracellular InsP₃ generation. Therefore, the concentration of InsP₃ may not be sufficient to mobilize intracellular Ca²⁺ stores to achieve a spike response. Moreover, it has been shown that the mobilization of Ca²⁺ from intracellular pools by Ins(1,4,5)P₃ was an "all-or-nothing" phenomenon: a critical concentration of Ins(1,4,5)P₃ must be reached to mobilize intracellular Ca²⁺ (26).

We have therefore demonstrated that there are differences in the subcellular pathways and sources of Ca²⁺ involved in the increase in [Ca²⁺]i, evoked in normal and hyperplastic cells. Whereas in control cells, ANG II induces a spike response in Ca²⁺ that is the result of mobilization of internal stores by InsP₃ in hyperplastic cells, the principal component of the response is a plateau phase in [Ca²⁺]i, resulting mainly from Ca²⁺ influx through plasma membrane channels. It has been proposed that pathways of Ca²⁺ mobilization diverge at the level of receptor and G protein coupling. For example, in bovine adrenal chromaffin cells, it has been suggested that Ca²⁺-entry and Ca²⁺-mobilization responses to ANG II can be dissociated (29). The present studies indicate that the same may be true in the case of pituitary cells.

On the other hand, it has been shown that administration of estradiol to intact female rats results in increased PKC activity in pituitary cells (16). Not only PKC activity, but also diacylglycerol and PKC-α, −β₂, and -β₃ protein expression are increased in the rat
hyperplastic pituitary (6, 17, 19). Because PKC can activate nifedipine- and verapamil-sensitive Ca2+ influx (1), enhanced PKC expression and activity might be related to the ANG II plateau response in hyperplastic cells. It has also been shown that estrogen increases low voltage-activated Ca2+-current density, increasing the number of functional channels in the membrane of pituitary cells (25). Moreover, the low production of InsP3 generation, coupled to a lack of spike response, might be related to the decrease in AT1 receptors induced by estrogen, which we and others (10, 28) described in pituitary cells. However, we cannot discard the possibility that estrogen treatment could favor the proliferation of cells unresponsive to ANG II.

In conclusion, this is the first demonstration that long-term exposure to estrogens in vivo modulates participation of Ca2+ channels in response to ANG II in vitro in anterior pituitary cells. Although Ca2+ influx through membrane channels accounts for most of ANG II action in hyperplastic cells, thapsigargin-sensitive intracellular stores are preferentially mobilized by ANG II in control cells. Definition of the specific action of estradiol on Ca2+ homeostasis in lactotrophs will be of considerable importance to our understanding of the control of estrogen-induced hyperplasia and prolactin secretion.

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REFERENCES


27. Roussel, J. P., E. Grazzini, R. Zumbihl, E. Rodriguez, and H. Astier. Tridol-o-l-thyronine enhances TRH-induced TSH release from perfused rat pituitary and intracellular Ca2+ levels...


