Control of Calcium Homeostasis by Angiotensin II in Adrenal Glomerulosa Cells through Activation of p38 MAPK

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Angiotensin II-induced activation of aldosterone secretion in adrenal glomerulosa cells is mediated by an increase of intracellular calcium. We describe here a new Ca\(^{2+}\)-regulatory pathway involving the inhibition by angiotensin II of calcium extrusion through the Na\(^+\)/Ca\(^{2+}\) exchanger. Caffeine reduced both the angiotensin II-induced calcium signal and aldosterone production in bovine glomerulosa cells. These effects were independent of cAMP or calcium release from intracellular stores. The calcium response to angiotensin II was more sensitive to caffeine than the response to potassium, suggesting that the drug interacts with a pathway specifically elicited by the hormone. In calcium-free medium, calcium returned more rapidly to basal levels after angiotensin II stimulation in the presence of caffeine. Thapsigargin had no effect on these kinetics, but diltiazem, which inhibits the Na\(^+\)/Ca\(^{2+}\) exchanger, markedly reduced the rate of calcium decrease and abolished caffeine action. The involvement of this exchanger was supported by the effect of cell depolarization and a reduction of extracellular sodium on the rate of calcium extrusion. We also determined the mechanism of angiotensin II action on the exchanger. Phorbol esters reduced the rate of calcium extrusion, which was increased by baicalein, an inhibitor of lipoxygenases, and by SB 203580, an inhibitor of the p38 MAPK. Finally, we showed that angiotensin II acutely activates, in a caffeine-sensitive manner, p38 MAPK in glomerulosa cells. In conclusion, in bovine glomerulosa cells, the Na\(^+\)/Ca\(^{2+}\) exchanger plays a crucial role in extruding calcium, and, by reducing its activity, angiotensin II influences the amplitude of the calcium signal. The hormone exerts its action on the exchanger through a caffeine-sensitive pathway involving the p38 MAPK and lipoxygenase products.

It has been firmly established that cellular calcium signaling in bovine adrenal glomerulosa cells plays a crucial role upon stimulation of aldosterone production by angiotensin II (AngII) \(^1\) or extracellular potassium, and the mechanisms of calcium mobilization by these agonists have been reviewed in detail (1–3). Whereas K\(^+\) principally activates voltage-operated calcium channels of both L and T type through direct membrane depolarization (4), the Ca\(^{2+}\) response to AngII appears biphasic; a first acute elevation of \([Ca\(^{2+}\)]\) due to Ca\(^{2+}\) release from intracellular stores is followed by a sustained response reflecting the activation of Ca\(^{2+}\) influx through channels of the plasma membrane. This second phase is maintained by the activity of both voltage-gated and store-operated Ca\(^{2+}\) channels.

Beside these basic pathways of Ca\(^{2+}\) mobilization, additional mechanisms have been proposed to participate or to modulate the Ca\(^{2+}\) signal elicited by AngII in bovine adrenal glomerulosa cells. For example, the participation of the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) in Ca\(^{2+}\) transport (in one direction or the other) during cell exposure to AngII has been suggested (2, 5). Moreover, the protein kinase C (PKC) wing of AngII signaling probably also regulates, directly or indirectly, the Ca\(^{2+}\) messenger system. Although it was proposed earlier that activation of this enzyme is essential for the steroidogenic response to Ca\(^{2+}\)-mobilizing hormones (6), the same authors reported an inhibition of the phospholipase C by the phorbol ester phorbol 12-myristate 13-acetate (PMA) (7), and the activation of PKC has been also shown to be responsible for acute inhibition of T type Ca\(^{2+}\) channels (8).

In addition to the phospholipase C-mediated formation of diacylglycerol, AngII also stimulates the production of arachidonic acid by activation of a phospholipase A2 (9) or from diacylglycerol through a diacylglycerol lipase (10). However, there is scant information concerning the exact relationship between PKC, phospholipase A2, and diacylglycerol lipase in steroidogenic cells. Nevertheless, arachidonic acid serves as a substrate for various enzymes that will further convert it into leukotrienes, prostaglandins, or hydroxyeicosatetraenoic acids (HETEs). The latter, and more specifically the 12-lipoxygenase products, have been proposed for many years to play an important role in aldosterone synthesis (11, 12). Interestingly, 12-HETE appears to be directly involved in the generation of the AngII-elicited Ca\(^{2+}\) signal, because this response is prevented by lipoxygenase blockers such as baicalein and restored by the addition of 12-HETE (13).

Mitogen-activated protein kinases (MAPKs) are a family of ubiquitous and highly conserved serine-threonine protein kinases activated by diverse stimuli ranging from cytokines, growth factors, neurotransmitters, hormones, cellular stress, and other stimuli. Currently, the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK are well characterized. The roles of these kinases in the control of aldosterone production are not well understood (10).

This paper is available on line at http://www.jbc.org

\^1 The abbreviations used are: AngII, angiotensin II; Bu2cAMP, dibutyryl cyclic AMP; TEMED, N,N,N,N′-tetramethylethylenediamine; Ins(1,4,5)P\(_3\), inositol 1,4,5-trisphosphate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; HETE, hydroxyeicosatetraenoic acid; NCX, Na\(^+\)/Ca\(^{2+}\) exchanger; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase.
and cell adherence (14). Mammalian MAPKs have been classified into three groups, which differ in their activation pathway and include the MAPK extracellular signal-regulated kinase (also referred to as p42/44 MAPK), the MAPK c-Jun N-terminal kinase, and the MAPK p38. Angiostatin II, through its AT1 receptor, has been shown to activate p42/44 MAPK in bovine adrenal fasciculata cells (15) as well as in bovine and rat glomerulosa cells (16, 17), an effect probably linked to the action of this hormone on cell proliferation (18). This activation, easily detected by measuring the phosphorylated forms of the enzymes, is maximal after a few minutes and appears mediated by various effectors including PKC and the Ras/Raf-1 kinase pathway (19).

In contrast, the activation of the p38 MAPK by AngII in glomerulosa cells is poorly documented, despite the fact that the hormone has been shown to induce an increase in p38 activity in vascular smooth muscle cells, particularly under high glucose conditions (20). Recently, it has been reported that AngII leads to a dose-dependent increase in p38 MAPK activity in H295R adrenocortical cells, an effect mimicked by the addition of 12-18HETE (21). In contrast, no action of the hormone was observed on the activity-stimulated MAPK c-Jun N-terminal kinase. The authors also indicate that AngII-induced aldosterone stimulation was significantly attenuated by a specific p38 MAPK inhibitor but not by an inhibitor of the MEK/ERK pathway.

In the present study, we have analyzed the mechanisms by which caffeine, a drug known to interfere with multiple targets involved in cell signaling, exerts its action on the intracellular Ca\textsuperscript{2+} response elicited by AngII in bovine adrenal glomerulosa cells. After excluding multiple cellular mechanisms, we found that Ca\textsuperscript{2+} extrusion out of the cytosol through the plasma membrane NCX is negatively modulated by p38 MAPK and demonstrate that caffeine, a potent inhibitor of this regulatory pathway, maintains a high rate of calcium extrusion during hormonal stimulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antimycin, bovine serum albumin, Bu\textsubscript{2}cAMP, caffeine, chelerythrine chloride, creatine phosphokinase, DNase, EGTa, forskolin, Hepes, horseradish peroxidase-coupled anti-goat antibody, 3-isobutyryl-1-methylxanthine, ionomycin, insulin/transferrin/selenium, LaCl\textsubscript{3}, ATP, nicardipine, oligomycin, oxalate, phosphocreatine disodium salt, phenylmethylsulfonyl fluoride, polyvinyl alcohol, pyruvate, rotenone, succinate, theophylline, and Triton X-100 were obtained from Sigma. D-glucose, EDTa, TEMED, and Tween 20 were purchased from Merck. P-32, sodium selenite, 5\textsuperscript{14}S/H\textsubscript{9262}O\textsubscript{2}, /H\textsubscript{9262}O\textsubscript{2}50 mCi/ml 45Ca\textsuperscript{2+}, and EGTA was from Amersham Biosciences; fura-2/AM, fluo-3/AM, fluo-4/AM, fura 2 acid, and K\textsubscript{BAPTA were from Molecular Probes, Inc. (Eugene, OR); and Dulbecco’s medium, supplemented with insulin/transferrin/selenium (2 \mu M glucose, 2 \mu M transferrin, 2 ng/ml sodium selenite), 5 \mu M metyrapone, 2 mM glutamine, 6 units/ml nystatin, 2% (v/v) fetal calf serum, and 10% (v/v) horse serum. The cells were plated on 6- or 24-well culture plates (2 or 0.7 x 10\textsuperscript{4} cells/well) and incubated overnight at 37 °C in 5% CO\textsubscript{2}. The next day, the medium was removed and replaced with serum-free Dulbecco’s modified Eagle’s medium.

**Measurement of Calcium Concentration in Intact Cells**—Cytosolic calcium concentrations ([Ca\textsuperscript{2+}]c) were determined with fluorescent probes in freshly prepared cell populations as previously described (8). For this purpose, freshly purified cells were washed three times with 50 ml of Krebs-Ringer buffer and maintained in this medium for at least 60 min at 37 °C. Cells were then centrifuged, diluted at a concentration of 4 x 10\textsuperscript{8} cells/ml, and incubated in the presence of 2 \mu M fura-2/AM, 5 \mu M fluo-3/AM, 5 \mu M fluo-4/AM, and 5 \mu M fura-2 for 15 min. The dye excess was then washed away, and cells were maintained in the same medium. Immediately before aliquots of 2 x 10\textsuperscript{5} cells were washed and diluted in appropriate experimental medium.

The fluorescent signals (excitation at 340/380 nm and emission at 505 nm for fura-2, and excitation at 488 nm and emission at 540 nm for fluo-3 and fluo-4) were recorded with a Jasco (Hachioji City, Japan) CAF-110 fluorescence spectrometer. Calcium concentrations were calculated as described elsewhere (23), using a K\textsubscript{d} value of 224 nm for fura-2 and 325 nm for fluo-3 and fluo-4.

**Mathematical Analysis of Calcium Extrusion Rate**—Calcium traces, recorded in Ca\textsuperscript{2+}-free, EGTa (0.2 mM)-supplemented Krebs-Ringer medium, were digitized at a frequency of 2 Hz and analyzed with a mathematical analysis program (Origin version 5.0). The increasing phase of the response to AngII (Fig. 4) during the extrusion of calcium out of the cytosol was fitted over a 2-min period to a decreasing exponential function of the first order: \( y(t) = \gamma_o + A \times \exp(-t-t_o)/\tau_0 ) \), where \( y(t) \) is the value of [Ca\textsuperscript{2+}]c, as a function of time, \( \gamma_o \) is the final calcium level reached after calcium extrusion from the cytosol, \( A \) is the amplitude of the calcium release peak, \( t_o \) is the beginning of the analysis interval (15 s after the calcium release peak), and \( \tau_0 \) is the half-life corresponding to the half-life of calcium in the cytosol or more precisely the time taken by the cells to reach a cytosolic calcium level corresponding to 36.9% (1/e) of the initial concentration (at \( t_o \)). This latter parameter \( (\tau_0) \), determined after optimizing the fitting curve, reflects the rate of calcium extrusion out of the cytosol and was used to assess the action of various agents on calcium homeostasis.

**Measurement of Calcium Release from the Organelles in Permeabilized Cells**—For measuring Ins(1,4,5)P\textsubscript{3}- and caffeine-induced calcium release from the organelles, glomerulosa cells were first permeabilized, as previously described (24). Freshly prepared cells were washed twice with M199 (2.66 g/liter modified Hanks’ medium, 15 mM NaHCO\textsubscript{3}, 3.5 mM Na\textsubscript{2}HPO\textsubscript{4}, 100 \mu M Mg\textsubscript{2+}, 10 \mu M EGTA, 25 mM Hepes, 2 mM KH\textsubscript{2}PO\textsubscript{4}, 5 mM MgCl\textsubscript{2}, pH 7.4) and then incubated for 10 min with a Ca\textsuperscript{2+}-free Krebs-Ringer solution supplemented with 50 \mu M EGTA. After incubation, cells were washed twice in 50 ml of permeabilization buffer (250 mM sucrose and 5 mM Hepes, pH 7.2). Cells were then permeabilized in a minimal volume by repeated (8-12 times) brief (100 \mu M) exposure to an intense electric field (1400 V/cm). Under these conditions, ~90% of treated cells were stained with trypan blue.

In experiments assessing \([\text{Ca}^{2+}]_o\) incorporation into the organelles, permeabilized cells were maintained at 4 °C until adding the reaction buffer (100 mM KCl, 25 mM Hepes, 2 mM KH\textsubscript{2}PO\textsubscript{4}, 5 mM Mg\textsubscript{2+}, pH 7.4) that contained 1 \mu M/Cl \textsuperscript{2+} \text{Ca}^{2+}, an ATP-regenerating system (3 mM Mg\textsubscript{2+}ATP, 10 mM phosphocreatine disodium salt, 8 mM/cell creatine phosphokinase (8 IU/ml), succinate (5 mM), pyruvate (5 mM), and oligomycin (1 \mu M) to initiate calcium incorporation into the organelles. After reaching a low steady state calcium level, detected agents were added directly into the ambient medium, and calcium fluctuations were followed by recording fura-2 fluorescent signal, as in intact cells. Calcium responses were calibrated by the addition of known amounts of Ca\textsubscript{2+} into the medium after inhibition of calcium incorporation into the organelles with theophylline and ionomycin.

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measured in a Packard 1900 TR β counter after adding 10 ml of UltimaGold.

Aldosterone Production—Aldosterone production was determined in the medium of cells in primary culture. Two days after replacing culture medium by serum-free Dulbecco's modified Eagle's medium, plated cells were washed three times with Krebs-Ringer buffer and incubated for 30 min at 37°C in the same solution containing different experimental drugs. After this first incubation, the medium was discarded and replaced by a fresh one of the same composition, and the cells were incubated for an additional 60-min period. At the end, the media were collected and centrifuged for 5 min at 1500 × g. The supernatants were frozen until aldosterone determination by direct radioimmunoassay using a commercially available kit (Diagnostic Systems Laboratories, Webster, TX). Aldosterone production was expressed per mg of cell protein, itself measured in each dish with the Coomassie Blue method (Bio-Rad).

Determination of p38 MAPK Activation in Cultured Glomerulosa Cells—The relative activity of the p38 MAPK was assessed by measuring the enzyme-phosphorylated form by immunoblotting. For this purpose, primary cultured cells were used 24–60 h after having been deprived of serum. At a 30-min preincubation at 37°C with various agents tested, cells were stimulated for the indicated periods of time with AngII (100 nm). At the end of the stimulation period, cells were washed with cold phosphate buffer (137 mM NaCl, 1.47 mM KH₂PO₄, 10 mM HEPES, 2.7 mM KCL, pH 7.2) and scraped into 75 μl of lysis buffer (50 mM Tris, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM EDTA, 2 mM EGTA, 40 μM β-glycerol phosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 200 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 100 mM okadaic acid, pH 7.4). The homogenates were centrifuged at 4°C for 10 min at 50,000 × g, and supernatants collected for protein assay and Western blot analysis. Cell lysates (20 μg) were analyzed by SDS-PAGE (electrophoretic migration was performed during 40 min at 150 V and protein transfer during 1.5 h at 110 V). After transfer, the nitrocellulose membranes were incubated in a blocking buffer (50 mM Tris/HCl, 200 mM NaCl, 0.2% Tween 20, and 5% nonfat dried milk) for 1 h at room temperature. For total p38 MAPK detection, dried milk was replaced by polyvinyl alcohol (1%) in the blocking buffer. Membranes were then incubated for 2 h in the same buffer containing 1% nonfat dried milk or polyvinyl alcohol with polyclonal antibodies raised against phosphorylated p38 MAPK (New England Biolabs, Beverly, MA) or total p38 MAPK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membranes were washed with the same buffer without milk or polyvinyl alcohol and then incubated for 1 h with horseradish peroxidase-labeled goat anti-rabbit (CovAlb, Oullins, France) or rabbit anti-goat (Sigma) antibodies. After washing six times for 10 min, the immunoreactive bands were visualized by ECL detection reagent (Amersham Biosciences) and quantified by densitometry (Molecular Dynamics, Inc., Sunnyvale, CA).

Statistics—Statistical significance of differences was assessed by the Student’s t test. Probability values of p < 0.05 were considered as being statistically significant.

RESULTS

Effect of Caffeine on Calcium Signaling and Steroidogenesis in Adrenal Glomerulosa Cells—Caffeine was initially used for investigating the presence and the role of various types of intracellular Ca²⁺ stores in the response of bovine adrenal glomerulosa cells to challenge with AngII. Surprisingly, we observed that, when added during the sustained phase of the Ca²⁺ response to AngII, caffeine (2 mM) markedly reduced [Ca²⁺], in freshly isolated glomerulosa cells (Fig. 1A). After inhibition by caffeine, nicardipine, a dihydropyridine antagonist, had only a slight effect on calcium levels. Interestingly, the same concentration of caffeine only marginally affected the calcium response to KCl (Fig. 1B) that remained sensitive to nicardipine. Moreover, no additional effect of caffeine was observed when maximal capacitative Ca²⁺ influx was quantitatively triggered by 200 nM thapsigargin (not shown). The analysis of concentration dependence of the caffeine-induced inhibition of the Ca²⁺ signal (Fig. 1C) revealed that caffeine is much more efficient at reducing the response to AngII (IC₅₀ = 1.3 mM) than the response to potassium (IC₅₀ > 10 mM), suggesting that the drug interacts with a mechanism specifically generated by AngII.

The consequence of calcium inhibition by caffeine on the steroidogenic response to AngII was investigated in primary cultured bovine glomerulosa cells (Fig. 1D). Whereas basal aldosterone production appeared unaffected by caffeine, AngII-stimulated aldosterone was significantly reduced by 56 ± 5% in the presence of 10 mM caffeine. Aldosterone synthesis was not sensitive to lower concentrations of the drug, despite the marked decrease of the calcium signal observed previously, suggesting that AngII-induced steroidogenesis is also controlled by additional pathways, compensating for the partial decrease of calcium, or that the sensitivity of glomerulosa cells to caffeine is different in freshly prepared cells, used for calcium measurements, and in primary culture.

Lack of Caffeine Effect on Calcium Release from Intracellular Stores—To determine the site and mechanism of caffeine action on AngII-induced calcium signaling, we first measured whether caffeine affects intracellular calcium pools. Preliminary experiments performed with intact cells showed that caffeine (10 mM) had no effect on [Ca²⁺] in unstimulated cells and did not significantly reduce the amplitude of the transient [Ca²⁺] response to AngII (data not shown). Additional experiments directly assessed the action of caffeine on Ins(1,4,5)P₃-sensitive and -insensitive stores in permeabilized glomerulosa cells (Fig. 2). Whereas the addition of micromolar concentrations of Ins(1,4,5)P₃ induced an immediate and transient release of calcium into the ambient medium, low millimolar concentrations of caffeine were inefficient and did not prevent a subsequent response to Ins(1,4,5)P₃ (Fig. 2A). The integrity of the pools was finally tested by sequential addition of 400 nM thapsigargin, an inhibitor of intracellular Ca²⁺-ATPases (SERCA), and 2 μM ionomycin, a Ca²⁺ ionophore. The lack of caffeine-sensitive intracellular Ca²⁺ pools in bovine glomerulosa cells was confirmed by adding increasing concentrations of ryynodine, a drug acting on the same type of Ca²⁺ stores as caffeine and which could not induce calcium release in permeabilized (Fig. 2B) as well as in intact glomerulosa cells (data not shown). Moreover, the presence of 10 mM caffeine in the medium did not affect the functional response to Ins(1,4,5)P₃ (Fig. 2C). Indeed, EC₅₀ values determined in the absence and in the presence of 10 mM caffeine (0.59 and 0.45 μM, respectively) were not significantly different, a finding in agreement with the previous observation that the peak response to AngII was unaffected by caffeine in intact cells.

Caffeine Action Is Not Mediated by cAMP—To exclude the possibility that caffeine could modulate calcium levels through an inhibition of the phosphodiesterases and a subsequent elevation of cAMP concentration, we measured the effect of various agents affecting cAMP on Ca²⁺ homeostasis. As shown in Fig. 3, forskolin (25 μM), a pharmacological activator of adenyl cyclases, was unable to reduce the AngII-induced [Ca²⁺], plateau and did not prevent caffeine action. The efficacy of forskolin was indirectly assessed in parallel experiments by measuring aldosterone secretion (Fig. 3, inset). At the same concentration (25 μM), forskolin appeared as potent as AngII (100 nM) to stimulate steroidogenesis, most probably through activation of the cAMP pathway, which, like calcium, is a well recognized modulator of aldosterone biosynthesis. The same results have been obtained with either 3-isobutyl-1-methylxanthine (100 μM), an inhibitor of the cAMP phosphodiesterase, or the cell-permeant analog Bu₄CAMP (1 mM). Thus, it appears that caffeine action on [Ca²⁺] is not mediated by a change in cAMP levels.

Caffeine Accelerates Calcium Extrusion Out of the Cytosol—A reduction of [Ca²⁺] as illustrated in Fig. 1A, can result either from a decrease of calcium influx into the cytosol or from an activation of calcium extrusion. This second possibility was
tested by exposing fura-2-loaded cells to AngII in the absence of extracellular calcium. We observed, after a transient response exclusively due to calcium release from intracellular stores, a dramatic acceleration of calcium lowering back to basal levels in the presence of 10 mM caffeine as compared with control cells (Fig. 4). A systematic analysis of the rate of calcium extrusion was performed by fitting the decreasing phase of the calcium signal by caffeine (C) as a percentage of the maximal inhibition induced by caffeine from traces similar to those presented in A and B. Data are mean values ± S.E. from nine independent experiments. D, bovine glomerulosa cells in primary culture were incubated for 1 h in the presence or absence of 100 nM AngII and with increasing concentrations of caffeine. Aldosterone was measured in the medium by direct radioimmunoassay and normalized to the amount of cell protein. Data (mean ± S.E. from nine independent experiments) are expressed as a percentage of the aldosterone induced by AngII in control cells (no caffeine), and that amounted to 106 ± 10 ng aldosterone/mg of protein. *, p < 0.0001.

FIG. 1. Caffeine-induced inhibition of the calcium and steroidogenic responses to angiotensin II in bovine adrenal glomerulosa cells. Fura-2-loaded freshly isolated bovine glomerulosa cells were challenged, in the presence of extracellular calcium (1.2 mM), with 100 nM AngII (A) or 9 mM KCl (B) before being exposed to caffeine (caff) and nicardipine (nic). Calcium levels during the plateau phase (before the addition of caffeine) and after the inhibition of the signal with nicardipine were determined as described under “Experimental Procedures.” The concentration-dependent inhibition of the sustained calcium signal by caffeine (C) was calculated as a percentage of the maximal inhibition induced by nicardipine from traces similar to those presented in A and B. Data are mean values ± S.E. from 13 experiments. D, bovine glomerulosa cells in primary culture were incubated for 1 h in the presence or absence of 100 nM AngII and with increasing concentrations of caffeine. Aldosterone was measured in the medium by direct radioimmunoassay and normalized to the amount of cell protein. Data (mean ± S.E. from nine independent experiments) are expressed as a percentage of the aldosterone induced by AngII in control cells (no caffeine), and that amounted to 106 ± 10 ng aldosterone/mg of protein. *, p < 0.0001.

Fig. 2. Intracellular calcium stores are insensitive to caffeine in bovine glomerulosa cells. Freshly prepared glomerulosa cells were permeabilized by using high voltage electric field discharges, as described under “Experimental Procedures,” and ambient calcium concentration fluctuations were recorded at 37 °C with free acid fura-2. After the addition of ATP and accumulation of ambient calcium into the organelles (not shown), cells were exposed to various concentrations of Ins(1,4,5)P$_3$ (IP$_3$, caffeine (caff), or ryanodine. At the end of each trace, Ca$^{2+}$ was totally released from the organelle by the successive addition of 400 nM thapsigargin (thapsi) and 2 μM ionomycin (iono), and calcium responses were normalized by the addition of known amounts of CaCl$_2$ in the medium (not shown).

because Ca$^{2+}$ is absent from the medium and because the addition of nifedipine, another blocker of these channels, did not change the kinetics of calcium extrusion (data not shown). Diltiazem treatment did not affect the size of the peak due to the Ca$^{2+}$ release phase. Moreover, after inhibition of the NCX with diltiazem, caffeine was not any more efficient, indicating that it probably interferes with this pathway of Ca$^{2+}$ extrusion.

The role of the NCX in bringing Ca$^{2+}$ back to basal levels...
Fig. 3. Caffeine-induced inhibition of the calcium signal does not involve the cAMP pathway. Intact fura-2-loaded cells were stimulated with 100 nM AngII, as in Fig. 1A, but 25 μM forskolin was added before the inhibition by caffeine (caff). This trace is representative of five independent experiments giving similar results. Inset, aldosterone production by cells exposed to 25 μM forskolin was determined as in Fig. 1D and compared with aldosterone secreted by control or AngII-stimulated cells. nic, nicardipine.

after release from the stores was confirmed 1) by decreasing the concentration of extracellular Na⁺ or 2) by depolarizing the cells with KCl. Indeed, when [Na⁺]o was reduced from 140 down to 15 mM, Ca²⁺ extrusion after release by AngII was markedly impaired (Fig. 6, inset). Actually, at low [Na⁺]o, cells were unable to bring [Ca²⁺]i back to basal levels, even in the absence of extracellular Ca²⁺, a fact probably reflecting the establishment of a new steady state and impairing analysis of kinetics. We therefore calculated the ratio between the [Ca²⁺]i peak during the release phase as an inverse index for the efficacy of calcium extrusion. The value of this ratio significantly increased from 17 to 33% when [Na⁺]o was reduced from 140 to 65 mM and even more at lower [Na⁺]o (Fig. 6). The osmolarity of the medium was maintained at 325 mosM/liter in these experiments by adding glucose. The reduced ability of the cells to extrude Ca²⁺ in low [Na⁺]o strongly supports a crucial role for the NCX in this process.

Because the NCX exchanges three Na⁺ for one Ca²⁺, it is electrogenic, and its activity therefore decreases upon plasma membrane depolarization. Indeed, we observed that upon the addition of 15 mM potassium, which depolarizes the cells from their resting potential, ~80 mV, to approximately ~50 mV, the rate of calcium extrusion was significantly reduced (Table 1) but remained sensitive to caffeine.

Mechanism of Angiotensin II Action on the Sodium/Calcium Exchanger—Because caffeine accelerates Ca²⁺ extrusion more efficiently upon stimulation with AngII, we hypothesized that the hormone inhibits the exchanger and that the drug interferes with this regulatory pathway. We therefore dissected the possible mechanisms of action employed by AngII to modulate NCX activity.

PKC is known to be activated upon glomerulosa cell stimulation by AngII but not by extracellular potassium (25). Therefore, this pathway appeared first as a good candidate for playing a role in AngII action on NCX. Indeed, we found that a pharmacological activation of PKC with the phorbol ester PMA (500 nM) markedly slowed the Ca²⁺ decrease phase after AngII (Table 1), whereas, under these conditions, the mechanism remained sensitive to caffeine, which probably acts downstream of PKC. However, the results obtained with a series of PKC inhibitors, used in order to mimic caffeine action, were not entirely consistent with a major role of PKC in the regulation of the NCX activity. While the PKC inhibitor CGP41 (26), as expected, significantly accelerated Ca²⁺ extrusion, other PKC inhibitors such as chelerythrine chloride (27) and calphostin C (28) had no significant effect (Table 1).

These results suggest that PKC could participate in the negative regulation of the exchanger, but it appears not to be absolutely required, and other mechanisms probably coexist. We therefore investigated alternative pathways activated by AngII and modulated by PKC, such as the 12-lipoxygenase and p38 MAPK action on calcium extrusion.
The action was not any more significant, suggesting that caffeine induced acceleration of Ca$^{2+}$/H$^{+}$ addition was reduced from 24 to 0.04 to 0.51 $\pm$ 0.02 mm (p $< 0.001$, n = 17). Most interestingly, in the presence of this inhibitor, caffeine action was not any more significant, suggesting that caffeine and SB 203580 act on a common target.

Similarly, in Ca$^{2+}$-containing medium, SB 203580 (20 nm) induced a rapid decrease of AngII-induced Ca$^{2+}$ plateau levels, as previously observed in response to caffeine (data not shown). Moreover, when SB was added 15–30 min before AngII, the sustained Ca$^{2+}$ phase (measured 3 min after the hormone addition) was reduced from 24 to 7 (in control cells) down to 6 ± 2 mm (mean ± S.E., n = 3). In the presence of SB, the caffeine (3 mm) addition had no more effect on [Ca$^{2+}$]c levels, once again strongly suggesting the existence of a common mechanism for caffeine and SB 203580 action.

In contrast, when both capacitative influx and Ca$^{2+}$ entry through voltage-operated Ca$^{2+}$ channels were triggered in the absence of AngII (by the simultaneous addition of 200 mm theophylline and 9 mm KCl), no effect of SB was observed on [Ca$^{2+}$]c levels, therefore excluding a direct effect of this drug on Ca$^{2+}$ channel activity.

The lipoxigenases have been shown to be activated by AngII in adrenal glomerulosa cells (18), and more specifically the product 12-HETE appeared to be required for an optimal Ca$^{2+}$ response (15). In addition, p38 MAPK has been proposed to be regulated by the lipoxigenase product 12-HETE (14). We therefore tested a possible role of this pathway on the rate of Ca$^{2+}$ extrusion. As shown in Table III, the inhibitor of the 12-lipoxygenase, baicalein, also accelerated Ca$^{2+}$ decrease. However, baicalein did not reduce caffeine action, which presumably acts downstream of lipoxigenase.

Finally, the time course of p38 MAPK activation by AngII and its inhibition by caffeine and SB 203580 have been determined by measuring the phosphorylated form of the enzyme with a specific antibody (Fig. 7). The hormone increased the active form of the kinase by 22-fold within 2 min, without modifying the amount of total p38 MAPK (Fig. 7A). The time course analysis of this activation was transient, with a maximal response between 2 and 5 min and a decrease to basal values after 15 min (Fig. 7B).

The ability of caffeine to prevent p38 MAPK activation was compared with that of SB 203580 by adding the drugs in the medium at different times before or during a 4-min stimulation with AngII (Fig. 7C). The addition of 10 mm SB into the medium resulted in a rapid reduction of p38 MAPK phosphorylation levels by −50% within 30 s. The inhibitory effect of SB tended to diminish upon prolonged (> 20 min) treatment, but inhibition was further reinforced (by 15%) when increasing the concentration of the drug up to 100 mm (not shown).

Although slightly slower for inducing its effect, caffeine (10 mm), at 5 min, was as efficient as the SB compound (10 mm) at reducing AngII-induced p38 activation, and no additional effect was observed when combining optimal concentrations of caffeine and SB 203580 (not shown).

Moreover, in two independent experiments, baicalein (10 mm) also slightly reduced p38 activation (by 20%), reinforcing the hypothesis that the 12-HETE products could act as physiological activators of the kinase.

We have also determined the effect of medium osmolality on p38 MAPK activity. As illustrated in Fig. 7D, increasing medium osmolality from 225 up to 425 mosM/liter led to a more than 50% reduction of basal p38 activity. Although the activation by AngII was particularly weak under these conditions (at low extracellular sodium concentration), the general tendency to obtain a higher p38 activity in hypo-osmotic medium was maintained in the presence of the hormone.

DISCUSSION

In the present study, we have unraveled a new cellular pathway involved in the regulation of calcium homeostasis upon stimulation of adrenal glomerulosa cells by AngII. While the presence of this pathway was initially suggested by its sensitivity to caffeine, a largely nonspecific drug, further investigation showed the involvement of well characterized effectors, such as PKC, lipoxigenase, p38 MAPK, and the NCX.

Caffeine action on the calcium signal elicited by AngII was robust and reproducible enough to use this effect as a sort of marker for analyzing the mechanisms involved in the hormone action. Moreover, caffeine acted much more efficiently on the Ca$^{2+}$ response to AngII than on the response to extracellular potassium (Fig. 1C) or the Ca$^{2+}$ ionophore, ionomycin (data not shown), suggesting that the drug interfered with a pathway specifically induced by AngII and not simply activated by an elevation of the cytosolic calcium concentration. Finally, caffeine-induced inhibition of the Ca$^{2+}$ response to the hormone was translated in a concomitant reduction of aldosterone production, highlighting the physiological relevance of this observation.

Caffeine is known for its multiple actions on various targets involved in cell signaling. In this context, we first showed that caffeine does not influence Ca$^{2+}$ through modulation of cAMP levels or Ins(1,4,5)P$_3$-induced Ca$^{2+}$ release. Indeed, a cross-talk between cAMP and the Ca$^{2+}$ messenger system has been described in many cell types with either potentiating or antagonistic properties. For example, by inhibiting the cAMP-specific phosphodiesterases (35) caffeine could induce an elevation of basal cytosolic cAMP concentration and subsequently activate phospholamban-regulated Ca$^{2+}$/Mg$^{2+}$-ATPases (36). This mechanism could have been responsible for the decrease of [Ca$^{2+}$], observed upon the addition of caffeine. To exclude this
TABLE I

Effect of potassium and modulators of protein kinase C on the rate of calcium extrusion

| Agent          | Basal t_{1/2} min | Basal p (versus control) | Basal t_{1/2} min | Basal p (versus control) |
|----------------|-------------------|--------------------------|-------------------|--------------------------|
| Control        | 0.67 ± 0.04       | <0.05                    | 0.35 ± 0.03       | <0.05                    |
| KCl            | 0.88 ± 0.07       | <0.005                   | 0.62 ± 0.07       | <0.05                    |
| PMI            | 1.30 ± 0.14       | <0.0005                  | 0.74 ± 0.05       | <0.01                    |
| CGP 41         | 0.53 ± 0.05       | ND                       | ND                | ND                       |
| Chelerythrine  | 0.67 ± 0.03       | NS                       | ND                | NS                       |
| Calphostin C   | 0.79 ± 0.08       | NS                       | ND                | NS                       |

TABLE II

Effect of medium osmolarity on the rate of calcium extrusion

| Osmolarity (mOsm/l) | Δ plateau/Δ peak (%) | n | p (versus control) |
|---------------------|----------------------|---|------------------|
| 225                 | 54.8 ± 3.2           | 4 | <0.01            |
| 325 (control)       | 32.9 ± 4.1           | 7 |                 |
| 425                 | 35.8 ± 4.9           | 4 | NS               |

Bovine glomerulosa cells were incubated a few minutes in a calcium-free medium in the presence of various agents (18 mM KCl, 500 mM PMA, 2 μM CGP 41, 1–10 μM chelerythrine chloride, or 0.2–1 μM calphostin C) and with or without 10 mM caffeine before being stimulated with 100 nM AngII. Cytosolic calcium responses were recorded with fura-2, or, in the case of chelerythrine, with fluo-3 or fluo-4. The rate of calcium extrusion was then determined as described in the legend to Fig. 4. Values obtained with untreated (control) cells are indicated for comparison. Data are the mean ± S.E. from 5–37 determinations. NS, not significantly different; ND, not determined.

Although diltiazem does not affect NCX, the absence of extracellular Ca^{2+}, the contribution of diltiazem-sensitive Ca^{2+} channels is negligible. Actually, the rate of Ca^{2+} extrusion was profoundly affected after inhibition of the exchanger, not only pharmacologically with diltiazem but also after reducing the driving force for this transport either by decreasing the extracellular Na⁺ concentration or by depolarizing the cells with potassium. Altogether, these observations indicate that the NCX plays a crucial role in extruding cytosolic Ca^{2+} and that its activity is rate-limiting. Moreover, this mechanism is probably in great part responsible for the increase in cytosolic Na⁺ concentration we previously observed in bovine glomerulosa cells upon stimulation with AngII (5). More importantly, after NCX inhibition, caffeine was unable to accelerate Ca^{2+} extrusion. In this case, the involvement of PKC was possible. We have verified that neither forskolin, a potent activator of adenyl cyclase; 3-isobutyl-1-methylxanthine, an inhibitor of the phosphodiesterases; nor a cell-permeant analog of cAMP, Bu₂cAMP, could mimic or prevent caffeine action.

Another relevant candidate for controlling Ca^{2+} removal from the cytosol was the diltiazem-sensitive plasma membrane sodium/calcium exchanger. Although diltiazem does not affect NCX, the contribution of diltiazem-sensitive Ca^{2+} channels is negligible. Actually, the rate of Ca^{2+} extrusion was profoundly affected after inhibition of the exchanger, not only pharmacologically with diltiazem but also after reducing the driving force for this transport either by decreasing the extracellular Na⁺ concentration or by depolarizing the cells with potassium. Altogether, these observations indicate that the NCX plays a crucial role in extruding cytosolic Ca^{2+} and that its activity is rate-limiting. Moreover, this mechanism is probably in great part responsible for the increase in cytosolic Na⁺ concentration we previously observed in bovine glomerulosa cells upon stimulation with AngII (5). More importantly, after NCX inhibition, caffeine was unable to accelerate Ca^{2+} extrusion. Thus, we propose that the NCX is the ultimate effector affected by caffeine.

The activity of this exchanger is directly dependent on the levels of [Ca^{2+}][i], but caffeine affects more efficiently the response to AngII than the response to extracellular potassium. We therefore postulated that caffeine does not act directly on the exchanger but on a regulatory step specifically elicited by AngII. In fact, the acceleration of Ca^{2+} extrusion by caffeine is believed to result from the relief of an inhibitory signal on the NCX induced by the hormone itself. Indeed, calcium signaling upon stimulation by AngII results from the opening of various Ca^{2+} channels that will lead to an increase in [Ca^{2+}][i]. If no other mechanism is involved by the hormone, this Ca^{2+} elevation will trigger extrusion mechanisms, like Ca^{2+} ATPases and the NCX, that will immediately oppose this Ca^{2+} increase. The consequence will be the creation of futile cycles of Ca^{2+} across the membrane and therefore a waste of energy for the cell. In contrast, if the hormone simultaneously stimulates Ca^{2+} entry and reduces Ca^{2+} extrusion, the Ca^{2+} response is optimized.

Therefore, we investigated which pathway, sensitive to caffeine and elicited by the hormone, could be responsible for the NCX inhibition. In this regard, the involvement of PKC was
Bovine glomerulosa cells were incubated for 5–30 min in the presence of various agents (50 μM PD 98059, an inhibitor of p42/44 MAPK, 10 μM SB 203580, an inhibitor of p38 MAPK, or 10 μM baicalein, an inhibitor of lipoxygenases) and with or without 10 mM caffeine before being stimulated, in the absence of extracellular calcium, with 100 nM AngII. Cytosolic calcium responses were recorded with fluo-3 (PD and SB compounds) or fura-2 (baicalein). The rate of calcium extrusion was then determined as described in the legend to Fig. 4. Values obtained with untreated (control) cells in the same experiments are also indicated for comparison. Data are the mean ± S.E. from 5–17 determinations. NS, not significantly different.

**Table III**

| Agent       | Basal t_{50} min | Basal p (versus control) | With caffeine t_{50} min | With caffeine p' (versus basal) |
|-------------|------------------|--------------------------|--------------------------|--------------------------------|
| Control     | 0.72 ± 0.04      | NS                       | 0.41 ± 0.06              | <0.005                         |
| PD 98059    | 0.73 ± 0.02      | NS                       | 0.36 ± 0.05              | <0.005                         |
| SB 203580   | 0.51 ± 0.02      | <0.001                   | 0.47 ± 0.03              | NS                             |
| Baicalein   | 0.44 ± 0.02      | <0.005                   | 0.20 ± 0.01              | <0.005                         |

strongly suggested by the negative effect of PMA as well as the positive action of the PKC inhibitor, CPG41, on the rate of Ca^{2+} extrusion. However, two other pharmacological antagonists of the enzyme, chelerythrine chloride and calphostin C, had no effect on this parameter. This apparent contradiction could be explained by the fact that PKC could act as a simple modulator of a signaling step that is directly activated by AngII, independently of this enzyme. This step could be mediated, for example, by a phospholipase A_2 or a diacylglycerol lipase, leading to the formation of arachidonic acid. Alternatively, only specific isoforms of PKC, with various sensitivities to pharmacological inhibitors, could be involved in this modulation. In this context, it is noteworthy that, in rat glomerulosa cells, the lipoxygenase products 12- and 15-HETE have been shown to specifically activate PKC-ε, whereas AngII stimulates both the α and ε PKC isoforms (45).

The implication of the p38 MAPK in the modulation of the NCX activity was first suggested by the observation that a low osmolarity in the medium reduces, in an Na^+ -independent manner, the ability of the cells to extrude calcium (Table II). Indeed, this kinase has been shown to be involved in the response to various cellular stresses, including osmotic shock (14). We therefore verified that AngII effectively activates p38 MAPK, with kinetics in agreement with an effect on the early Ca^{2+} response to the hormone. A more than 10-fold increase in the activity of p38 within 1 min is perfectly compatible with a role of this kinase in the regulation of Ca^{2+} extrusion occurring immediately after the Ca^{2+} release phase induced by AngII. Moreover, the presence of a specific inhibitor of p38 MAPK, SB 203580, that reduces the AngII-induced activation of the kinase by 50% within 30 s, significantly accelerated Ca^{2+} extrusion (Table III), mimicking and preventing caffeine action. These results suggest that the SB compound and caffeine could act on the same target, a hypothesis supported by the fact that caffeine prevents the activation of p38 by AngII as efficiently as SB, although with slightly slower kinetics.

Although p38 MAPK is classically activated in most of the cell types by an increase in medium osmolarity (hyperosmotic shock), the opposite is apparently happening in bovine glomerulosa cells, as already described in renal epithelial A6 cells by Niisato et al. (29). The reason for this discrepancy between cell types is still unclear, but our results are consistent with the different rates of Ca^{2+} extrusion we have measured in the present study in hypo- and hyperosmotic conditions and with the observation by Makara et al. (46) that lowering osmolality leads to increased potassium-induced calcium response and aldosterone production in rat adrenal glomerulosa cells.

From a teleological point of view, the inhibition of the NCX by a kinase activated upon cell exposure to a low osmolar medium is certainly an advantage for these cells. Indeed, the stoichiometry of the exchanger allows the entry of 3 mol of Na^+ when extruding 1 mol of Ca^{2+}, and this activity could be harmful for the cell in a situation where it should rather expel osmotoles to the exterior.

In contrast to SB 203580, the inhibitor specifically blocking the p42/44 MAPK, PD 98059, did not affect the kinetics of Ca^{2+} extrusion and did not prevent caffeine action, showing the specificity of the pathway involved in the control of the NCX.

In summary, our hypothesis that caffeine reduces AngII-evoked Ca^{2+} signaling through an inhibition of p38 MAPK is strongly supported by the fact that SB 203580 mimics caffeine action on Ca^{2+} responses, that caffeine mimics the SB inhibitory action on p38, and, most importantly, that none of these caffeine and SB actions are additive when combining the two drugs.

The molecular link between p38 MAPK and NCX activity remains to be demonstrated. The large increase in cytosolic sodium observed upon bovine glomerulosa cell challenging with AngII (5), if not secondary to NCX activation, could be responsible for reducing the activity of this exchanger. In this regard,
Kusuhara et al. (47) have reported a modulation of the Na⁺/H⁺ exchanger type 1 by MAPKs in vascular smooth muscle cells. However, whereas Na⁺/H⁺ exchanger type 1 was activated by p42/p44 MAPK, leading to an increase of intracellular Na⁺, it was inhibited upon stimulation of p38 MAPK. It therefore appears that a major modulation of the NCX by AngII through MAPK-dependent increase in sodium influx is unlikely in bovine glomerulosa cells.

Finally, because the 12-HETE compound has been reported to activate p38 MAPK in H295R cells (21), we tested whether inhibition of lipoxygenases with baicalein interferes with Ca²⁺ homeostasis. The acceleration of Ca²⁺ extrusion by baicalein (Table III) speaks in favor of the involvement of lipoxygenase products in the modulation of the exchanger. Interestingly, caffeine remained efficient after lipoxygenase inhibition, probably because caffeine acts downstream of baicalein, a suggestion supported by the fact that the latter slightly reduces the p38 MAPK activity.

In conclusion, our data strongly suggest that AngII, in parallel to the activation of Ca²⁺ entry into adrenal glomerulosa cells, also reduces Ca²⁺ extrusion from the cytosol through the Na⁺/Ca²⁺ exchanger in order to prevent futile cycling of Ca²⁺ across the plasma membrane. This modulation of the exchanger activity involves lipoxygenases, the p38 MAPK, and possibly some specific isofoms of PKC. The sensitivity of p38 MAPK inhibition to caffeine explains the action of this drug on the Ca²⁺ homeostasis previously observed upon stimulation with AngII. Because of the ubiquity of this mechanism, possibly resulting from an ancestral mean of the cells to protect themselves against osmotic shocks, similar pathways are probably present in a variety of cell types and could be elicited by various Ca²⁺-mobilizing hormones.

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