Janus Kinase 2 and Calcium are Required for Angiotensin II-dependent Activation of Steroidogenic Acute Regulatory Protein (StAR) Transcription in H295R Human Adrenocortical Cells.*

Abbreviated title: Transcriptional regulation of StAR by Ang II

Jianghong Li, Rhona E. Feltzer, Kevin L. Dawson, Elizabeth A. Hudson, and Barbara J. Clark#

Department of Biochemistry & Molecular Biology, School of Medicine, University of Louisville, Louisville, KY 40292.

# To whom correspondence should be addressed:
Dr. Barbara J. Clark
Department of Biochemistry & Molecular Biology
University of Louisville School of Medicine
Louisville, KY 40292
Phone: (502)-852-2814
Fax: (502)-852-6222
E-mail: bjclark@louisville.edu

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SUMMARY

Angiotensin II- and K⁺-stimulated aldosterone production in the adrenocortical glomerulosa cells requires induction of the steroidogenic acute regulatory protein (StAR). While both agents activate Ca²⁺ signaling, the mechanisms leading to aldosterone synthesis are distinct and the angiotensin II response cannot be mimicked by K⁺. We previously reported that StAR mRNA levels and promoter-reporter gene activity in transiently transfected H295R human adrenocortical cells were stimulated by angiotensin II but not by K⁺ treatment. The current study focused on identifying signaling pathways activated by angiotensin II that contribute to StAR transcriptional activation. We show that the angiotensin II-stimulated transcriptional activation of StAR is dependent upon influx of external calcium and requires protein kinase C activation. Furthermore, we describe for the first time that the Janus tyrosine kinase family member, JAK2, is activated by angiotensin II treatment of H295R cells. Treatment of the cells with AG490, a selective inhibitor of JAK2, blocks JAK2 activation and StAR reporter gene activity and inhibits steroid production. Taken together these studies describe a novel pathway controlling StAR expression and steroidogenesis in adrenocortical cells.

INTRODUCTION

The two major physiological regulators of aldosterone synthesis in the adrenal zona glomerulosa are angiotensin II (Ang II)¹ and extracellular K⁺ (1). Ang II binds to AT-1 G-protein-coupled cell surface receptors that activate phospholipase-C and results in the release of diacylglycerol and inositol 1,4,5-trisphosphate (IP₃) from plasma membrane...
lipid, phosphatidylinositol-4,5-bisphosphate (2). Diacylglycerol stimulates protein kinase C (PKC) while IP₃ stimulates the release of Ca²⁺ from internal stores and increases intracellular calcium levels ([Ca²⁺]ᵢ). Depletion of the intracellular calcium stores activates capacitative calcium entry, thus Ang II mediates an influx of external Ca²⁺ via calcium release activated calcium (CRAC) channels (3,4). Although Ang II has been shown to activate voltage-gated calcium channels, this pathway is not considered a major contributor to the steroidogenic response (5). K⁺ activation of aldosterone synthesis, on the other hand, is associated with an influx of external Ca²⁺ via voltage-gated Ca²⁺ channels (6). For both of these regulators it has been well established that aldosterone synthesis requires influx of external Ca²⁺ (2,6). However, while both agents activate Ca²⁺ signaling, the mechanisms leading to aldosterone synthesis are distinct and the Ang II response cannot be mimicked by K⁺ depolarization of the plasma membrane (5,7,8).

The Ca²⁺-dependent mechanisms that mediate the Ang II-stimulated increase in steroidogenesis are required for both the chronic and acute response (9-18). The chronic response is defined by increased transcription of the steroidogenic cytochrome P450 enzymes required for cortisol and mineralocorticoid biosynthesis (19). The acute response is characterized by the delivery of cholesterol, the steroid precursor, to the mitochondrial inner membrane where the first enzymatic reaction catalyzed by the cytochrome P450 side chain cleavage enzyme (P450scc) occurs (20). This response is dependent upon the increased de novo synthesis of the steroidogenic acute regulatory protein (StAR) that functions to facilitate cholesterol transport across the mitochondrial membranes (21-23). Our previous studies demonstrated that increased aldosterone
synthesis by Ang II and K⁺ treatment of H295R human adrenocortical cells is paralleled by an increase in the StAR protein (13). StAR mRNA and protein expression have also been shown to be increased by Ang II in both bovine and rat adrenal glomerulosa cells (16,17,24). Furthermore, an increase in StAR transcription was observed in bovine glomerulosa cells after high intracellular calcium levels were maintained by ionomycin treatment, suggesting calcium signaling is sufficient for StAR gene regulation (17). In contrast, we have shown that Ang II treatment of H295R cells increased both StAR mRNA and protein levels while K⁺ treatment increased only StAR protein levels (14). Since both Ang II and K⁺ increase [Ca²⁺], the current studies were performed to test the premise that StAR transcriptional regulation by Ang II requires more than a general increase in intracellular calcium. Alternatively, it may be that calcium localization or threshold levels of calcium are required for the Ang II-dependent StAR transcriptional response (25).

In addition to the classical Ang II-activated calcium and PKC signaling pathways, mitogen activated protein kinase (MAPK/ERK1/2) and tyrosine kinase pathways can be activated by Ang II in adrenocortical cells (26-31). Ang II has also been shown to activate ERK1/2 in H295R cells (32,33). However, inhibition of ERK1/2 activation did not inhibit the Ang II-stimulated increase in aldosterone synthesis, suggesting this pathway is not involved in the Ang II-dependent steroidogenic response in this cell line (33). The evidence that tyrosine kinase signaling pathway(s) may be part of the Ang II response in adrenocortical cells was suggested by studies that reported genistein to be an inhibitor of aldosterone production, calcium influx via CRAC channels in bovine glomerulosa cells, and 3-β-hydroxysteroid dehydrogenase activity in H295R cells (29-
More specifically, inhibition of Src kinase with the inhibitor PP2 was effective in inhibiting Ang II-stimulated aldosterone production in H295R cells. However, the inhibition was present when 22R-hydroxycholesterol was used as a substrate, indicating that the target was a later step in the pathway, beyond StAR (31). Activation of the Janus tyrosine kinase (JAK) pathway mediated by Ang II binding to the AT-1 receptor has been well-documented for vascular smooth muscle cells (34-36), but to date there is no report of JAK activation by Ang II in adrenocortical cells.

The purpose for this study was to further define the mechanisms required for Ang II-stimulated increase in StAR transcription. The approach was two-fold; first, to test the contribution of release of intracellular calcium stores vs. influx of extracellular calcium on Ang II-dependent transcriptional activation of StAR and second, to determine whether Ang II activates JAK and/or ERK1/2 signaling and increases StAR transcription in H295R cells.

EXPERIMENTAL PROCEDURES

Materials: H295R cells were a gift from Dr. William E. Rainey, University of Texas Southwestern Medical Center (Dallas, TX). [Val<sup>5</sup>]Ang II acetate salt, thapsigargin, dibutyryl-cAMP, SKF96365, GF109203X, PD098059, and progesterone antibody were purchased from Sigma Chemical Co. (St. Louis, MO). AG490 was from Calbiochem-Novabiochem Corporation (La Jolla, CA). DMEM/F12 medium, lipofectamine PLUS reagent, and penicillin/ streptomycin antibiotics were obtained from Life Technologies, Inc. (Gaithersburg, MD). NuSerum type I and ITS culture supplements were supplied by Collaborative Biomed Products (Bedford, MA). The luciferase assay system was
from Promega Corp. (Madison, WI). $^3$H-progesterone was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

**Transient transfection:** H295R cells were maintained in culture using DMEM/F-12 medium containing 2.5% NuSerum, 0.5% antibiotic-antimycotic, and 1% ITS culture supplement. The cells were plated in a 24 well plate at 350,000 cells/well the day before cotransfection with −235bp human StAR-luciferase reporter gene constructs (−235StAR-luc) (1µg/ml) and either pCMV-β-galactosidase plasmids (1µg/ml) using lipofectamine reagent or phRL-null Renilla plasmids (0.5µg/ml) using lipofectamine PLUS reagent. After six hours, the lipid-plasmid complex was removed and fresh DMEM/F-12 medium was placed on the cells for 24 hours. The cells were then placed in serum-free medium for 16 hours then treated for 6 hours in serum-free medium as indicated. After six hours, the medium was removed and steroids were measured directly in the medium by radioimmunoassay described below. For cells cotransfected with StAR-luc and pCMV-β-galactosidase plasmids, luciferase activity was determined in cell lysates using Luciferase Reporter Assay System and measurement with an EG&G Berthold Lumat LB 9507 luminometer. β-galactosidase enzymatic activity was determined using β-D-galactopyranoside as substrate and measuring absorbance at 595nm. For cells cotransfected with StAR-luc and phRL-null Renilla plasmids, luciferase and Renilla activities were determined in cell lysates using Dual-Luciferase Reporter Assay System. Cell treatments were performed in triplicate within each independent transfection experiment and the mean values (+/- s.d.) for luciferase/β-galactosidase (Luc/β-gal) activity or luciferase/Renilla (Luc/Ren) activity were determined. The data were then normalized to the untreated control value that was set
to 1.0. For the inhibitor studies, the cells were treated with inhibitor alone (no agonist) to determine the effect on basal activity. An increase in basal activity is shown for PD098059 while GFX and AG490 had no effect on basal activity and the data are shown normalized to the untreated control value.

**Intracellular calcium measurements:** H295R cells were harvested from 75-mm cell culture flasks by trypsin-digestion, washed twice with Krebs-Ringer buffer, and resuspended in the same buffer to a final concentration of $10^7$ cells/ml. Fura-2 AM (Molecular Probes, Eugene, OR) was added to the cell suspension to a final concentration of 5 $\mu$M and the cells were incubated at 37 °C for 45 min. The cells were washed twice with either regular Krebs-Ringer buffer or with calcium-free Krebs-Ringer buffer for measurements without external calcium, and resuspended to a final concentration of $10^6$ cells/ml. Fura-2 AM fluorescence was detected using a LS50B spectrofluorometer (Perkin Elmer) connected to a computer for data collection with FL-WinLab software (Perkin Elmer). 2 ml of cells were placed into a quartz cuvette and all measurements were recorded at 37 °C under constant stirring. Excitation wavelengths of 340 and 380 nm and emission at 510 nm were used to collect the data and calcium concentrations were determined from fluorescence ratios using software provided with FL-WinLab.

**Radioimmunoassay:** Progesterone level was determined using $^3$H-progesterone (Amersham Pharmacia Biotech, Piscataway, NJ) and progesterone antibody (Sigma Laboratories; St. Louis, MO). Unbound progesterone was removed by charcoal extraction and bound progesterone was measured by scintillation counting. The concentrations of steroid in the medium were determined by extrapolation from a
standard curve. The mean values for ng steroid/ml medium (+/ s.d.) were determined and the data normalized to the control value that was set to 1.0.

**Immunoblot analysis:** H295R cells were grown in 60-mm tissue culture dishes to 80% confluence, then placed in serum-free medium for 16 hours before treatment. The cells were treated in either the absence or presence of the indicated inhibitor in either serum-free medium alone (control) or medium containing 10nM Ang II for the indicated time (0.5 hr – 2.0 hr.). Collection of cell lysate and immunoblot analysis were done as described previously (23), except that protein was separated on a 7.5% SDS-PAGE. Primary antibodies used were anti-phosphotyrosine-JAK2 (BioSource International, Inc., Camarillo, CA), anti-phospho-p44/42 ERK1/2 (Cell signaling Technology, Inc.), anti-JAK2 (Upstate Biotechnology, Lake Placid, NY), and anti-ERK2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Quantitation of the results was performed using Un-Scan-It, version 5.1 (Silk Scientific Corporation).

**Statistics:** One way ANOVA followed by a Dunnett post test using PRISM software (version 3.0, GraphPad Software, Inc., San Diego, CA) was used to determine statistical significance between treatments and between treatment and control. P < 0.05 was considered significant. The mean values for the normalized data (treated/untreated) from all independent experiments (n ≥3) were used for the reporter gene data analysis. Progesterone data analysis was performed using either the normalized data or the raw data (ng progesterone/ml) from all experiments and treatments; i.e. 3 experiments with triplicate treatment, n = 9.
RESULTS

*Extracellular calcium is required for StAR transcription and steroid production.*

Ang II or (Bu)$_2$cAMP treatment resulted in a 7- or 10-fold increase in StAR promoter activity, respectively, compared to untreated cells (Figure 1A)(14). Steroid production, as measured by both progesterone (Figure 1B) and aldosterone (Figure 1C) levels in the cell medium, was correspondingly increased by agonist treatment as well. Aldosterone was increased to a greater degree than progesterone by Ang II treatment and this is likely the result of the stimulatory actions of Ang II on aldosterone synthase expression (37,38). For the remainder of the studies, progesterone served as the indicator of increased (or decreased) steroidogenic activity. To determine whether calcium signaling is sufficient for the Ang II-dependent StAR transcriptional response, thapsigargin (Tg) treatment was used to mimic the Ang II stimulated calcium signaling in H295R cells. Tg is an inhibitor of the intracellular membranes calcium ATPase pump, therefore, calcium uptake by the intracellular stores is blocked and depletion of these stores results in activation of plasma membrane CRAC channels and influx of extracellular calcium (5,39-41). As shown in Figure 1, neither StAR promoter-reporter gene activity in transiently transfected H295R cells nor steroid production was significantly increased after Tg treatment. However, the Ang II-stimulated responses were inhibited when the experiments were repeated using calcium-free medium. (Bu)$_2$cAMP-stimulated StAR reporter gene activity in H295R cells, on the other hand, was less dependent upon calcium compared to Ang II with only a 30% decrease in the response in the absence of calcium in the medium (Figure 1A). These data indicate
that Tg cannot mimic the Ang II response in terms of StAR gene expression but do suggest an influx of extracellular calcium is required.

To confirm that Tg mediated an increase in intracellular calcium levels, H295R cells were loaded with Fura-2 AM and changes in calcium were measured. First, 10 nM Ang II treatment in the presence of external calcium (+Ca) caused a characteristic transient increase in \([\text{Ca}^{2+}]_i\), followed by a sustained increase in \([\text{Ca}^{2+}]_i\) (Figure 2A) (12). The transient rise in \([\text{Ca}^{2+}]_i\) has been attributed to the release of internal stores while the sustained levels are a result of influx of extracellular calcium to fill these depleted membrane stores (1,4,42). The maximal \([\text{Ca}^{2+}]_i\), measured is referred to as peak calcium while the sustained calcium level is reported as the calcium concentration 3-5 min. after agonist treatment. The difference between peak and resting or sustained and resting \([\text{Ca}^{2+}]_i\) was determined for each treatment and the data are presented in Table 1. 10 nM Ang II treatment resulted in a 2-fold increase in \([\text{Ca}^{2+}]_i\) over resting levels (\(\Delta\) peak \([\text{Ca}^{2+}]_i\); 239 nM) with sustained calcium levels maintained at 70% over resting levels (\(\Delta\) sustained \([\text{Ca}^{2+}]_i\); 72 nM). In the absence of extracellular calcium, the Ang II-stimulated increase in \([\text{Ca}^{2+}]_i\) was decreased 50% (\(\Delta\) peak \([\text{Ca}^{2+}]_i\); 126 nM) with the sustained calcium levels returning near baseline levels (\(\Delta\) sustained \([\text{Ca}^{2+}]_i\); 27 nM) (Table 1). Tg treatment increased peak calcium levels to 143 nM over resting levels and maintained sustained calcium levels at 103 nM above resting levels. Although these data verify that Tg activates calcium signaling in H295R cells, the peak calcium levels achieved with Tg were approximately was 40%-50% lower than the 10 nM Ang II response in the presence of calcium (Table 1). Therefore, we tested whether the lack of a steroidogenic response was due to less calcium released from internal stores. H295R
cells were treated with a lower concentration of Ang II (1 nM) and the resulting calcium transient was compared to the 10 nM Ang II and 50 nM Tg responses (Figures 2B & 2C). The peak over resting calcium level (Δ peak [Ca^{2+}]_i) with 1.0 nM Ang II treatment was 30% and 50% of the levels obtained with either 10 nM Ang II or 50 nM Tg level, respectively. The sustained calcium levels were also relatively decreased (Table 1). StAR reporter gene activity and progesterone synthesis, on the other hand, were significantly increased by 1 nM Ang II treatment (Figure 3A & 3B). Thus, StAR promoter activity is not dependent upon reaching a threshold level of [Ca^{2+}]_i as shown by the results with 1 nM Ang II treatment and increased [Ca^{2+}]_i alone is not sufficient for this response as shown by the Tg treatment. This premise is supported by the results with 16 mM K^+ treatment where the increased and sustained [Ca^{2+}]_i levels were 2-fold greater than that observed with 1nM Ang II but K^+ treatment failed to similarly stimulate StAR reporter gene activity (Table 1 and Figure 3A).

The lack of StAR reporter gene activity by Ang II treatment of H295R cells in calcium-free medium indicated that influx of external calcium was required for the Ang II response (Figure 1A). To independently test this premise, calcium influx was blocked with SKF96365 (SKF), an inhibitor of both receptor-mediated and voltage-gated calcium entry (43). The increase in peak [Ca^{2+}]_i over resting levels with 10 nM Ang II treatment in the presence of SKF was unchanged while the sustained [Ca^{2+}]_i levels were decreased 75% (Table 1). SKF treatment alone had no effect on basal StAR reporter gene activity and decreased the Ang II-stimulated response 60% (Figure 3A). Surprisingly, progesterone synthesis was increased 2-fold by SKF treatment alone. Nevertheless, the 10 nM Ang II response in the presence of SKF was diminished from a
4- to 5- fold induction to a 1.5- to 2- fold induction. Together our data indicate Ang II-stimulated StAR promoter activity requires influx of extracellular calcium. However, [Ca^{2+}]_{i} signaling alone is not sufficient for StAR transcription, therefore, we investigated other potential signaling pathways activated by Ang II.

**Ang II activation of JAK2 in H295R cells is required for StAR promoter activity.**

As a first approach to test the contribution of known Ang II-activated pathways on StAR transcriptional activation, we measured the effects of inhibitors of PKC, tyrosine kinase, and mitogen activated protein kinase, MAPK (ERK1/2) on Ang II-stimulated StAR reporter gene activity in transiently transfected H295R cells. Treatment of the cells with the general PKC inhibitor, GF109203X (GFX), decreased Ang II-stimulated StAR promoter activity in a concentration-dependent manner (Figure 4A). Maximal inhibition (50%) was reached with 1.0 μM GFX treatment. This effect was specific since (Bu)_2-cAMP-stimulated StAR promoter activity was unaffected by GFX treatment. GFX also inhibited Ang II-stimulated progesterone production, although to a lesser extent, with 25% inhibition at 1.0 μM GFX (Figure 4B).

To investigate the potential involvement of a tyrosine kinase in Ang II-stimulated StAR expression, we treated H295R cells with genistein and measured the Ang II-stimulated StAR reporter gene activity. Genistein treatment resulted in a concentration-dependent decrease in the Ang II-stimulated StAR promoter activity and progesterone production reaching a maximum inhibition of 60%-70%. However, genistein has many effects within the cell, including inhibition of capacitative calcium influx in bovine adrenal cells (30). Therefore, we tested Src and JAK2 kinases using the more selective inhibitors PP2 and AG490, respectively. PP2 had no effect on Ang II-stimulated StAR
reporter gene activity but had a small stimulatory effect on basal StAR promoter activity as well as progesterone production\textsuperscript{2}. On the other hand, 50 µM AG490 blocked Ang II-stimulated StAR reporter gene activity and progesterone production without having a significant effect on the (Bu)\textsubscript{2}-cAMP stimulated responses (Figures 5A – 5C).

Immunoblot analysis for phosphotyrosine-JAK2 in cell lysates from control (untreated) and Ang II-stimulated H295R cells demonstrated that JAK2 tyrosine phosphorylation was increased 3.5-fold by Ang II treatment within 30 minutes and remained elevated over 2 hours (Figure 6). 50 µM AG490 inhibited the Ang II-dependent JAK2 activation ~50% within 30 minutes and blocked the phosphorylation after 2 hour treatment (Figure 6).

Together these data demonstrate that Ang II activates JAK2 signaling in H295R cells and suggests that activation of this tyrosine kinase pathway is a critical part of the StAR transcriptional response. Activation of the PKC pathway, on the other hand, does not appear to be a major contributor to the Ang II response but may be required for maximal activation.

**Ang II activates a JAK2-ERK signaling pathway in H295R cells.**

Ang II has been shown to activate ERK1/2 in H295R cells and activated ERK1/2 has been linked to both positive and negative effects on steroidogenesis and StAR expression depending on the agonist or cell type studied (33,44-48). To determine whether the MAPK pathway has a positive or negative effect on StAR in H295R cells, we treated the cells with the MEK-1 inhibitor, PD098059, and measured StAR reporter gene activity. As shown in Figure 7, treatment with 10 µM and 25 µM of PD098059 resulted in an increase in basal StAR promoter activity. However, the Ang II-stimulated
activity was not correspondingly increased in the presence of PD098059, therefore, the fold-induction (Ang II-stimulated/basal) was reduced 50%-60%. The (Bu)2cAMP-stimulated/basal activity remained the same either in the absence or presence of PD098059, indicating the inhibitory effect was specific for Ang II response (Figure 7).

Immunoblot analysis for phospho-ERK1/2 in cell lysates from control (untreated) and Ang II-stimulated H295R cells demonstrated that ERK1/2 phosphorylation was significantly increased (~50-fold) by Ang II treatment (Figure 8). In the presence of 10 µM and 25 µM PD098059 the Ang II-dependent increase in ERK1/2 phosphorylation was blocked by 80% and 90%, respectively (Figure 8).

Since the JAK signaling pathway has been shown to activate ERK in vascular smooth muscle cells (35,49), we tested whether JAK2 activates ERK1/2 in H295R cells. Immunoblot analysis of phospho-ERK1/2 in cell lysates from control (untreated) and Ang II-stimulated H295R cells in the absence and presence of the JAK2 inhibitor, AG490, demonstrated that 50 µM AG490 inhibited ERK1/2 phosphorylation 50% within 30 minutes (Figure 9). These data indicate that Ang II activates JAK2 and ERK1/2 is a downstream target of JAK signaling in H295R cells. However, ERK1/2 does not appear to be the only downstream target of JAK2 for Ang II activated StAR gene expression.

**DISCUSSION**

The importance of extracellular Ca\(^{2+}\) in mediating aldosterone production is well established and we now report a direct link between Ang II-stimulated influx of extracellular Ca\(^{2+}\) and StAR gene expression. Previously it was shown that increasing [Ca\(^{2+}\)] to 600-700 nM with ionomycin, a calcium ionophore, activated StAR transcription
in bovine glomerulosa cells (17). The increase in [Ca$^{2+}$]$_i$ by ionomycin was correlated with increased aldosterone production with an EC50 of 303nM, while an intracellular calcium level of 200nM did not result in significant increase of aldosterone production (50). We now demonstrate that StAR promoter activity and steroid production were increased significantly by 1nM Ang II, an agonist concentration that resulted in relatively low calcium signaling with [Ca$^{2+}$]$_i$ increased to 162 ± 27 nM and 139 ± 16 nM for the peak and sustained response, respectively. Tg treatment, on the other hand, was unable to stimulate either StAR promoter activity or steroid production in H295R cells despite causing an increase in [Ca$^{2+}$]$_i$ (258 ± 30 nM and 218 ± 32 nM for the peak and sustained response, respectively) sufficient to elicit a steroidogenic response by Ang II. Together these data indicate that Ang II-stimulated StAR promoter activity is not dependent upon reaching a threshold level of [Ca$^{2+}$]$_i$. Previous studies reported Tg increased aldosterone production in both primary cultures of bovine and rat adrenal glomerulosa cells (5,39,40). However, a significant Tg response in bovine adrenal cells required 8 mM K$^+$ in the medium and our culture conditions have 4mM K$^+$ that may account for the difference. It is also possible that there are species- or cell culture-specific differences in the Tg response as previously suggested for this inhibitor (41).

Our data indicate that inhibiting the influx of extracellular calcium inhibited the Ang II-dependent increase in StAR transcription. It is known that Ang II stimulates both CRAC channels and voltage-gated calcium channels in glomerulosa cells (2). However, Ang II-stimulated StAR promoter activity was unaffected by the voltage-gated calcium channel inhibitor, nifedipine, which blocked the influx of calcium stimulated by K$^+$ (2). Therefore, we conclude that Ang II-dependent activation of the CRAC channel is
required for StAR promoter activity. Potential downstream target(s) for calcium include
calcium-dependent activation of calmodulin and calmodulin-dependent protein kinase II.
These targets have been shown to be important for the Ang II-stimulated increase in
aldosterone synthase (CYP11B2) transcriptional response (11,51,52); however, we
have shown that inhibitors of these pathways have no effect on the Ang II-stimulated
increase in StAR protein levels in H295R cells (51). Therefore, the calcium-dependent
targets in terms of StAR expression remain to be determined.

We previously reported that StAR mRNA levels or reporter gene activity were not
increased by direct activation of PKC by phorbol ester treatment of H295R cells,
suggesting PKC activation alone was not sufficient for StAR transcriptional response
(13,14). Similar results for a lack of an effect of phorbol ester treatment on CYP11B2
expression in H295R cells have been reported (38). We now have addressed the
question of whether Ang II-dependent PKC activation contributes to StAR expression.
Inhibition of PKC by GFX treatment resulted in 50% inhibition of StAR reporter gene
activity, suggesting that PKC in combination with other Ang II-mediated activators is part
of the Ang II signaling pathway for StAR transcription. PKC isoforms α, −ε, -θ, -ζ, and λ
were detected by Western blot analysis in NCI-H295 cells (53). Of these, PKCα is
calcium-dependent and activated by diacylglycerol. Although we did not directly
demonstrate PKC activity was inhibited by GFX or identify the isoform-specific PKC
required for Ang II-stimulated StAR expression, it is possible that the lack of an effect of
phorbol ester treatment can be attributed to a lack of a Ca²⁺ activator. We also noted
that GFX had less of an inhibitory effect on progesterone synthesis compared to StAR
promoter activity. This result suggests that StAR protein expression may be less dependent on PKC.

The evidence that a tyrosine kinase signaling pathway(s) is part of the Ang II response in steroidogenic cells was suggested by studies that reported genistein inhibited aldosterone production, calcium influx via CRAC channels in bovine glomerulosa cells, and 3-β-hydroxysteroid dehydrogenase activity in H295R cells (29-31). Specific inhibition of Src kinase by PP2 inhibited Ang II-stimulated aldosterone production in H295R cells but the point of inhibition was determined to be at a post-StAR step in the Ang II pathway (31). Our data support this premise and indicate that the Src kinase inhibitor PP2 had no inhibitory effect on Ang II-stimulated StAR promoter activity. Furthermore, PP2 treatment alone was recently shown to increase DHEA production in H295R cells and an increase in StAR protein was part of this response (54). Our data support this finding in that basal StAR reporter gene activity and progesterone production were increased by PP2 alone.

We demonstrate for the first time that JAK2 is activated by Ang II in H295R cells and that inhibition of JAK2 with AG490 blocked the Ang II-stimulated increase in StAR reporter gene activity and inhibited progesterone production. JAK2 was shown to be activated by Ang II in vascular smooth muscle cells via the AT1 receptor (34,35). Further evidence that Ang II stimulates JAK2 signaling was demonstrated in rat aortic smooth muscle cells where an Ang II-dependent physical interaction between JAK2 and AT1 was demonstrated by both co-immunoprecipitation studies and in vitro “pull-down” of JAK2 from cell lysates by binding immobilized GST-AT1 (55). Although a direct demonstration of an Ang II receptor – JAK2 interaction in H295R cells remains to be
shown, AT1 is the Ang II receptor subtype in H295R cells that mediates the steroidogenic response (12). Thus, it is likely that a physical interaction between AT1 and JAK2 mediates the Ang II-dependent activation of JAK2 in this cell type as well.

Potential downstream targets for JAK2 signaling include the signal transducers and activators of transcription (STAT) and the MAPK pathways. We detected an increase in phospho-STAT3 with Ang II treatment of H295R cells. However, using a STAT3 responsive element-luciferase reporter gene construct we were unable to detect an Ang II response in transiently transfected H295R cells. Furthermore, the Ang II responsive region of the StAR promoter does not contain a STAT element (14). Therefore, we conclude that the JAK-STAT3 signaling pathway is not part of the Ang II response for StAR activation.

Although not the direct focus for the current studies, our data show that basal StAR reporter gene activity was increased in the presence of PD098059 and that the (Bu)2cAMP response was similarly increased. As stated previously, both positive and negative effects of ERK1/2 activation have been reported for cAMP-stimulated StAR expression and steroid production. In Y1 mouse adrenal cells PD098059 treatment decreased the forskolin-stimulated increase in StAR steady-state mRNA levels by approximately 50% (44). In primary cultures of rat granulosa the MEK-1 inhibitor U1026 inhibited FSH-stimulated increase in StAR mRNA (48) while in a transformed cell lines U1026 potentiated the stimulatory effects of FSH on StAR protein levels (47). These studies indicate that communication between MAPK pathway and cAMP signaling is cell-type dependent. Our data are consistent with the premise that ERK1/2
suppresses StAR basal expression independent of the cAMP activated pathway in H295R cells.

The observation that 10 μM PD098059 inhibited Ang II-stimulated ERK1/2 phosphorylation by 80% and decreased the Ang II-dependent StAR promoter activity by 50% suggests ERK1/2 activation is part of the Ang II response. However, the JAK2 inhibitor AG490 decreased Ang II stimulated ERK1/2 phosphorylation by 50% yet blocked StAR reporter gene activity. Although it is not possible to correlate the level of phosphorylation of target proteins, such as ERK, to the functional response in the cell with these studies, these data indicate that JAK2 activates ERK1/2 but that another downstream target of JAK2 is also important for StAR gene expression.

In summary, our data indicate that the influx of extracellular calcium and JAK2 activation are critical for Ang II-stimulated StAR transcriptional activation. The Ang II response is not dependent upon activation of the PKC or MAPK pathway although inhibition of either pathway attenuates the response. Recent data on Ang II action in vascular smooth muscle cells have supported a model for calcium and PKC functioning together to activate a phosphotyrosine kinase that activates JAK2 leading to activation of downstream targets (34). Our data are consistent with this hierarchy of Ang II responses, although further validation of this model requires establishment of a direct link between calcium influx and JAK2 activation and identification of JAK2 targets for StAR transcription.
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REFERENCES

FOOTNOTES

1 The abbreviations used are: Ang II, Angiotensin II; AT-1, Angiotensin II receptor subtype 1; β-gal, beta-galactosidase; (Bu)2cAMP, N6,2'-O-dibutyryl-adenosine 3':5' cyclic monophosphate; [Ca2+]i, intracellular calcium; CRAC, calcium release activated calcium channels; CYP17, gene encoding cytochrome P450 17α-hydroxylase-17,20 lyase enzyme; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-regulated kinase; GFX, GF109203X; IP3, inositol 1,4,5-trisphosphate; JAK, Janus kinase (soluble receptor-associated tyrosine kinase); P450scc, cytochrome P450 side chain cleavage enzyme encoded by the CYP11A gene; Luc, luciferase; MAPK, mitogen activated protein kinase; MEK, MAPK-ERK kinase; PKA, protein kinase A; PKC, protein kinase C; Ren, Renilla; SF-1, steroidogenic factor-1; StAR, steroidogenic acute regulatory protein; SKF, SKF96365; Tg, thapsigargin.

2 unpublished data
FIGURE LEGENDS

Figure 1. Angiotensin II-stimulated StAR transcription in H295R cells is dependent upon external calcium. H295R cells were transiently transfected with the −235hStAR-luc reporter construct and pCMV-β-galactosidase control plasmid as described in the Experimental Procedures. 16 hours post-transfection, the cells were placed in serum-free medium for 24 hours then treated in triplicate with fresh serum-free medium alone (control) or containing either 1 mM (Bu)2cAMP, 10 nM Ang II, or 50 nM thapsigargin (Tg). In some experiments, serum-free, calcium-free medium (minus Ca2+) was used with the (Bu)2cAMP or Ang II treatment. After 6 hours the medium was removed for progesterone and aldosterone measurements by RIA. Luciferase (Luc) and β-galactosidase (β-gal) activities were determined in the cell lysates and Luc activity was normalized to β-gal as described in the Experimental Procedures. The mean Luc/β-gal values, ng progesterone/ml, or pg aldosterone/ml from triplicate wells were determined and the data were expressed as the fold increase in treated cells relative to untreated cells that was set to a value of 1.0. Shown are the mean values ± s.e.m. for normalized data from 3 independent transfection experiments for (A) Luc/β-gal activity (B) ng progesterone/ml and (C) pg aldosterone/ml. *, denotes significant increase compared to control value. StAR reporter gene activity, progesterone level, and aldosterone level was not affected, 50%, and 70% lower, respectively, in untreated calcium-free control (Minus Ca2+) compared to activity in untreated the calcium replete medium (Plus Ca2+).
**Figure 2. Changes in intracellular calcium in H295R cells after agonist treatment.**

H295R cells were loaded with Fura-2 AM and baseline or resting calcium concentrations were monitored for 2 minutes before addition of either Ang II or Tg, and fluorometric recordings were continued for an additional 6 minutes. The data were converted to nM calcium and shown are the trace recordings from representative experiments that were repeated 5-8 times for each agonist. (A) Calcium transients recorded with 10 nM Ang II in the presence (+Ca) or absence (-Ca) of external calcium and 50 nM Tg in the presence of calcium. For –Ca recording, the Fura-2 AM loaded cells were washed and resuspended in calcium-free Krebs-Ringer buffer prior to the fluorometric assay. (B) Calcium transients recorded with 1 nM and 10 nM Ang II. (C) Comparison of calcium transients between Tg and 1 nM Ang II treatments.

**Figure 3. Calcium signaling alone is not sufficient for StAR transcriptional activation in H295R cells.** H295R cells were transiently transfected with the –235hStAR-luc reporter construct and phRL-null Renilla control plasmid as described in the *Experimental Procedures*. 16 hours post-transfection, the cells were placed in serum-free medium for 24 hours then treated in triplicate with fresh serum-free medium alone (control) or containing the indicated agonist. After 6 hours the medium was removed for progesterone measurement by RIA. Luciferase (Luc) and Renilla (Ren) activities were determined in the cell lysates and Luc activity was normalized to Ren as described in the *Experimental Procedures*. The mean Luc/Ren values from triplicate wells were determined and the data were expressed as the fold increase in Luc/Ren activity in the treated cells relative to untreated cells that was set to a value of 1.0. (A) Shown are the mean values ± s.e.m. for relative Luc/Ren activity compared to control.
from 3 independent transfection experiments. (B) Shown are the mean values ± s.e.m. for ng progesterone/ml from 3 independent transfection experiments (n = 9). *, denotes significant increase compared to control value.

**Figure 4. Inhibition of PKC activity has minimal effect on Ang II-stimulated StAR promoter activity in H295R cells.** H295R cells were transiently transfected with –235hStAR-luciferase reporter gene and phRL-null Renilla vectors. 16 hours post-transfection the cells were placed in serum-free medium for 24 hours then treated in triplicate with fresh serum-free medium alone (0) or containing the indicated concentration of GFX. A second set of cells was treated with 10 nM Ang II in the absence (0) or presence of the indicated concentration of GFX. After 6 hours the medium was removed for progesterone measurement by RIA. Luciferase (Luc) and Renilla (Ren) activities were determined in the cell lysates and the mean Luc/Ren values from triplicate wells were determined as described in the Experimental Procedures. GFX treatment alone had no effect on StAR reporter gene activity or progesterone synthesis and the data were expressed as the fold increase in Luc/Ren activity in the treated cells relative to untreated control cells that was set to a value of 1.0. Shown are the mean values ± s.d. from 4 independent transfection experiments for control, Ang II, and 2 µM GFX or the average values ± range from 2 independent experiments for 0.1, 0.5, 1.0, and 5.0 µM GFX for (A) StAR reporter gene activity and (B) progesterone synthesis. #, denotes significant decrease compared to 10nM Ang II value.

**Figure 5. The JAK2 inhibitor, AG490, inhibits Ang II-stimulated StAR promoter activity and progesterone synthesis in H295R cells.** H295R cells were transiently
transfected with –235hStAR-luciferase reporter gene and phRL-null Renilla vectors. 16 hours post-transfection the cells were placed in serum-free medium for 24 hours then treated in triplicate for 6 hours with fresh serum-free medium alone (0) or containing the indicated concentration of AG490. A second set of cells was treated with either 1 mM (Bu)2cAMP or 10 nM Ang II in the absence (0) or presence of the indicated concentration of AG490. After 6 hours the medium was removed for progesterone measurement by RIA. Luciferase (Luc) and Renilla (Ren) activities were determined in the cell lysates and the mean Luc/Ren values from triplicate wells were determined as described in the Experimental Procedures. AG490 treatment alone had no effect on StAR reporter gene activity or progesterone synthesis and the data were expressed as the fold increase in Luc/Ren activity in the treated cells relative to untreated control cells that was set to a value of 1.0. (A & B) Shown are the mean values ± s.d. from 3-4 independent transfection experiments. (C) Shown are the mean values ± s.d. for ng progesterone/ml from 3 independent transfection experiments (n = 9). #, denotes significant decrease from Ang II value.

**Figure 6. The JAK2 inhibitor, AG490, inhibits Ang II-stimulated JAK2 activation.** H295R cells were incubated in serum-free medium for 24 hours then the medium was replaced with fresh serum-free medium containing the 50 µM of AG490. The cells were preincubated with the inhibitor for 30 minutes prior to the addition of 10 nM Ang II and the incubation was continued for an additional 30 minutes or 2 hours as indicated. Total cell lysates were collected and an equivalent amount of protein (50 µg) was separated by SDS-PAGE. Phosphotyrosine-JAK2 and total JAK2 were detected by immunoblot analyses as described in the Experimental Procedures. The membrane was first probed
using anti-phospho-JAK2, then stripped and reprobed for total JAK2 using anti-JAK2. Shown is a representative blot from one of two independent experiments. The signals were quantitated as described in Experimental Procedures and the values expressed as a percentage of the 30 min Ang II pJAK/JAK value that was set to 100. The numerical data from the two experiments are shown.

**Figure 7.** The MEK-1 inhibitor, PD098059, inhibits Ang II-stimulated StAR promoter activity in H295R cells. H295R cells were transiently transfected with –235hStAR-luciferase reporter gene and phRL-null Renilla vectors. 16 hours post-transfection the cells were placed in serum-free medium for 24 hours then treated for 6 hours in triplicate with fresh serum-free medium either alone (Basal) or containing 1 mM (Bu)2cAMP or 10 nM Ang II in the absence (0) or presence of the indicated concentrations (10 μM or 25 μM) of PD098059. After 6 hours the medium was removed for progesterone measurement by RIA. Luciferase (Luc) and Renilla (Ren) activities were determined in the cell lysates and Luc activity was normalized to Ren as described in the Experimental Procedures. The mean values from triplicate wells were determined and the data were then expressed as the fold increase in activity in the treated cells relative to untreated cells that was set to a value of 1.0. Shown are the mean values ± s.d. from 3-4 independent transfection experiments. *, denotes significant increase from control value. #, denotes significant decrease from Ang II.

**Figure 8.** The MEK-1 inhibitor, PD098059, inhibits Ang II-activated ERK1/2 in H295R cells. H295R cells were incubated in serum-free medium for 24 hours then the medium was replaced with fresh serum-free medium containing the indicated concentrations of PD098059. The cells were preincubated with the inhibitor PD098059
for 15 minutes prior to the addition of 10 nM Ang II and the incubation was continued for an additional 30 minutes. Total cell lysates were collected and an equivalent amount of protein (50 µg) was separated by SDS-PAGE and phospho-ERK and total ERK were detected by immunoblot analyses as described in Experimental Procedures. The membrane was first probed for phospho-ERK1/2 using anti-phospho-44/42, then stripped and reprobed for total ERK using anti-ERK2 antibody. The signals were quantitated as described in Experimental Procedures. Shown is a representative blot from one of two independent experiments.

Figure 9. The JAK2 inhibitor, AG490, inhibits Ang II-stimulated ERK1/2 activation. H295R cells were incubated in serum-free medium for 24 hours then the medium was replaced with fresh serum-free medium containing the 50 M of AG490. The cells were preincubated with the inhibitor for 30 minutes prior to the addition of 10 nM Ang II and the incubation was continued for an additional 30 minutes or 2 hours as indicated. Total cell lysates were collected and an equivalent amount of protein (50 µg) was separated by SDS-PAGE and phospho-ERK and total ERK were detected by immunoblot analyses as described in Figure 8. Shown is a blot representative of 3 independent experiments. The signals were quantitated as described in Experimental Procedures and the values expressed as a percentage of the 30 min Ang II pERK/ERK value that was set to 100. The numerical data are the avg. ± s.d. for the 3 experiments.
Table 1. Changes in intracellular calcium concentration in H295R cells by agonist treatment. Intracellular calcium levels were measured by Fura-2 AM detection as described in Experimental Procedures and all data are shown as nM calcium. Resting calcium levels were recorded for 2 minutes prior to agonist treatment. Peak Ca\(^{2+}\) is the maximal concentration of calcium while Δ peak Ca\(^{2+}\) is the difference in peak and resting calcium levels. The sustained calcium level is the calcium concentration 3-5 min. after agonist treatment and Δ sustained Ca\(^{2+}\) is the difference between sustained and resting calcium levels. Shown are the mean ± s.e.m. values from 5-8 independent experiments except for SKF that has a n = 3. - Ca2+, 10 nM Ang II treatment in calcium-free medium. SKF treatment was in the presence of 10 nM Ang II.
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Time (min.)

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Figure 6
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| pERK/ERK (%Ang II) | 2.3 ± 0.6 | 100 ± 28 | 52 ± 12 | 78 | 15 |

Figure 9
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Janus kinase 2 and calcium are required for angiotensin II-dependent activation of steroidogenic acute regulatory protein (StAR) transcription in H295R human adrenocortical cells

Jianghong Li, Rhona E. Feltzer, Kevin L. Dawson, Elizabeth A. Hudson and Barbara J. Clark

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