Calcium Regulation of Androgen Receptor Expression in the Human Prostate Cancer Cell Line LNCaP*

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ABSTRACT
Elevation of intracellular calcium levels in the presence of normal androgen levels has been implicated in apoptotic prostate cell death. Since the androgen receptor (AR) plays a critical role in the regulation of growth and differentiation of the prostate, it was of interest to determine whether Ca²⁺ would affect the expression of androgen receptor messenger RNA (mRNA) and protein, thus affecting the ability of androgens to control prostate function. AR-positive human prostate cancer cells, LNCaP, were incubated with either the calcium ionophore A23187 or the intracellular endoplasmic reticulum Ca²⁺-ATPase inhibitor thapsigargin. Subsequently, AR mRNA and protein levels were assessed by Northern and Western blot analysis. Both A23187 and thapsigargin were found to down-regulate steady state AR mRNA levels in a time- and dose-dependent manner. AR mRNA began to decrease after 6-8 h of incubation with 10⁻⁶ M A23187 or 10⁻⁷ M thapsigargin, reaching a nadir at 16 and 10 h of incubation, respectively. In contrast, control mRNA (glyceraldehyde 3-phosphate dehydrogenase) did not change significantly during the treatments with either A23187 or thapsigargin. AR protein levels were found to be decreased after 12 h of incubation with either 10⁻⁶ M A23187 or 10⁻⁷ M thapsigargin. The decrease in AR mRNA and protein seemed to precede apoptosis, since neither A23187 (24 h) nor thapsigargin (30 h) was found to alter cell morphology within the treatment time. Cycloheximide and actinomycin D were unable to change the calcium-mediated decrease in AR mRNA, ruling out the necessity for de novo protein synthesis or a change in mRNA stability. Moreover, the decrease in AR mRNA induced by calcium does not seem to involve protein kinase C- or calmodulin-dependent pathways, since inhibitors of these cellular components had no effect. Nuclear run-on assays demonstrated little or no effects of either A23187 or thapsigargin treatment on AR gene transcription (8 h and 10 h). In conclusion, these studies show that intracellular calcium seems to be a potent regulator of AR gene expression in LNCaP cells. (Endocrinology 136: 2172-2178, 1995)

The androgen receptor (AR) belongs to the steroid and thyroid hormone receptor superfamily (1). It mediates the effects of testosterone and dihydrotestosterone on growth and differentiation of male urogenital organs (2). Factors that can modulate the expression of AR may play an integral role in these androgen-dependent processes (3).

It has been shown that several hormones and growth factors can regulate the expression of AR. In human cell lines and animal models, androgens can autoregulate the expression of AR (4, 5). For example, it has been shown that androgens decrease AR messenger RNA (mRNA) and protein levels in the rat prostate and several human cell lines (4-7). Conversely, it has been reported that AR mRNA and protein levels are increased by the administration of androgens in the rat and mouse prostate, as well as in human genital skin fibroblasts (8-10). More recently, Krongrad et al. (11) have shown that androgen down-regulation of AR mRNA is associated with a transient up-regulation of AR protein in LNCaP cells. It is not clear which intracellular mechanism mediates this autoregulation process, although transcriptional down-regulation of AR by androgens in LNCaP cells has been reported recently (12, 13).

At least three distinct second messenger pathways exist within the cell: 1) the protein kinase A (PKA)-dependent pathway; 2) the PKC-dependent pathway; and 3) the calcium-calmodulin kinase-dependent pathway. All three pathways have been implicated in the regulation of the AR. For example, FSH, which activates the PKA pathway, can stimulate the expression of AR mRNA and protein in the rat Sertoli cell (14) (characterization of the mouse AR and human AR (hAR) 5'-flanking regions indicated that sequences contained within the 5'-flanking region can mediate some of the effects of the PKA pathway on AR expression; 15, 16); epidermal growth factor, which activates the PKC pathway, can increase AR mRNA levels in the human prostate cancer cell line LNCaP (17); and calcitonic, which alters Ca²⁺ homeostasis, increases AR protein levels in rat Leydig cells (18).

The withdrawal of androgens from the prostate results in massive programmed cell death. It has been indicated that calcium ion influx into the cells may play a role in the pathway leading to androgen-induced apoptosis (19). Moreover, Martikainen and Issacs (20) have shown that in a rat ventral prostate organ culture system, an elevation of intracellular calcium in the presence of normal androgen levels could increase apoptosis to a similar level as caused by androgen
ablation. Given these data, it seemed worthwhile to investigate whether increased intracellular calcium levels would affect AR mRNA and protein levels, and thereby affect the ability of androgens to control prostate function. Therefore, we have examined the role of calcium in the regulation of AR mRNA and protein expression in the human prostate cancer cell line LNCaP, using Northern and Western blotting analyses.

**Materials and Methods**

**Materials and cell culture**

A23187, thapsigargin, staurosporine, and trifluoperazine were dissolved in dimethyl sulfoxide (DMSO). Cycloheximide and actinomycin D were dissolved in water. All were purchased from Sigma Chemical Co. (St Louis, MO), except A23187, which was a gift from Dr. C. Y.-F Young (Mayo Clinic). The synthetic androgen mibolerone (Upjohn, Kalamazoo, MI) was dissolved in ethanol. The final concentration of DMSO or ethanol in the culture medium was 0.1%. RPMI 1640 culture medium was purchased from CELOR Co. (Hopkins, MN). Trypsin-EDTA, antibiotics, and fungizone were purchased from GibCO (Gaithersburg, MD).

The human prostate cancer cell LNCaP (21) was obtained from the American Type Culture Collection (Rockville, MD). LNCaP cells (30–60 passages) were maintained in RPMI 1640 as a monolayer in the presence of 5% fetal calf serum (FCS) and 2 mM L-glutamine. The medium was changed every 3–4 days. Cells used in the experiment were first grown to 70–80% confluence in 5% FCS RPMI 1640, then cultured in serum-free media for 24 h, after which the medium was replaced by 1% charcoal-stripped FCS RPMI 1640 with added hormones. For the actinomycin D experiment, cells were incubated for 8 h with DMSO or thapsigargin before incubating in fresh medium for the indicated period with 5 µg/ml actinomycin D.

3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

To assess the growth rate of cells, a modification of the MTT assay was used (22). Cells were trypsinized, resuspended in RPMI 1640 medium with 1% charcoal-stripped FCS, and 0.1-ml aliquots (10,000 cells) were plated into 96-well plates and cultured for 7 days. Aliquots of culture medium containing the calcium ionophore or thapsigargin (50 µM) were added to reach the final concentrations indicated in the experiment. At the end of the incubation period, the medium was carefully removed by inverting the plates on a paper towel, and 50 µl of a 0.5 mg/ml solution of MTT at 37 °C, 0.15 ml DMSO was added to each well. The plates were placed on a shaker for 5 min, and the absorbance at 550 nm was read using a multwell plate reader (SLT Lab Instruments, model EL4001A1). Six wells were used for each test point. Wells containing medium, but no cells, served as blank controls.

**RNA extraction and Northern blot analysis**

Total RNA was obtained by lysing the cells in LiCl (3 M) and urea (6 M) (23). Samples were homogenized on ice for 1 min at 24,000 rpm with a Tissuemizer (Kunkel, Staufen, Germany) and incubated at −20 °C for 30 min. Samples were centrifuged for 20 min at 80,000 × g. Pellets were resuspended in ES solution [0.1% sodium dodecyl sulfate (SDS) and 0.2 mM EDTA], extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol, and precipitated with 1:9 vol of 3 M sodium acetate (pH 5.2) and 2.5 vol ethanol. The RNA pellet was resuspended in water and was quantified by reading the absorbance at 260 and 280 nm. RNA was separated by electrophoresis on a 1% agarose gel, which contained 2% formaldehyde, using 1 X MOPS buffer (20 mM 3-N-morpholino propane sulfonic acid, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA). The RNA was transferred to a Hybond-N or Hybond-N+ membrane (Amersham, Arlington Heights, IL) and was UV cross-linked to the membrane using a stratalinker (Stratagene, San Diego, CA).

The hAR probe for Northern analysis [542-base pair puill-puill complementary DNA (cDNA) fragment] was generated by digesting the full-length hAR cDNA (24) with puill. The housekeeping enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 740-base pair ps11-ps11 cDNA fragment) was generated by cutting the plasmid, which contained GAPDH, with ps11. The cDNA was labeled with [α-32P]dideoxy-ATP using a random-primed labeling kit (Boehringer Mannheim, Indianapolis, IN). The reaction was incubated at 22 °C for 1 h, and the labeled cDNA probe was separated from free nucleotides by passage through Sephadex G-50 packed in a 5-ml pipette and added directly into the hybridization buffer [50% formamide, 0.5% SDS, 6× SSC (1× SSC = 150 mM sodium chloride and 15 mM sodium citrate), 5× Denhardt's solution, 10 mM phosphate buffer, and 100 µg/ml salmon sperm DNA].

Northern blots were prehybridized for 1 h at 42 °C and then hybridized with labeled probes overnight at 42 °C. Blots were washed twice in 2× SSC and 0.1% SDS at room temperature for 15 min and once in 0.1× SSC and 0.1% SDS at 65 °C for 15 min, quantified with a PhosphorImager 425 (Molecular Dynamics, Sunnyvale, CA), and exposed to x-ray film.

**Immunoblotting**

Cellular proteins were isolated by homogenizing the cells in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 10 mM MgCl2, 1% SDS, 0.25 M sucrose, 5 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 5 µg/ml bacitracin for five strokes. The lysates were dialyzed against water overnight, lyophilized, and reconstituted in water. Equal amounts of protein (10 µg/lane) were passed through a 10% precast gel (NOVEX, San Diego, CA) at 150 V for 2 h at 4 °C. Resolved proteins were transferred electrophoretically to nitrocellulose (Bio-Rad, Hercules, CA) at 4 °C for 3 h at 30 mA in a buffer containing 25 mM glycine, 192 mM Tris, and 10% methanol. Nonspecific binding of the antibodies to the membranes was diminished by preincubating the blots in TBS (50 mM Tris·HCl and 150 mM NaCl, pH 7.4) in the presence of 3% dry milk for 1 h at room temperature. Blots were incubated with rabbit anti-AR antibody (0.5 µg/ml PG-21, kindly provided by Dr. Gail Prins, University of Illinois School of Medicine, Chicago, IL) for 1 h in the presence of a 10-fold molar excess of AR21 peptide (0.1 µg/ml) in TBS containing 1% dry milk and 0.2% Tween-20 (Bio-Rad) at 4 °C for 24–48 h and then with a peroxidase-labeled antirabbit antibody (1:1000 dilution; Amersham) in the same buffer at room temperature for 1 h. After each incubation, blots were washed twice with TBS containing 0.2% Tween-20 for 10 min. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Amersham).

**Nuclear run-on**

The nuclear run-on assay was performed as described by Blok et al. (14). Cloned cDNAs (5 µg/spot) were used to detect the mRNA transcription rate of AR and GAPDH genes were: Bluescript II SK− (Stratagene, La Jolla, CA) linearized with EcoRI as a background control, PGp21 (26) linearized with HindIII for AR, and pGAPDH (Clontech, Palo Alto, CA) linearized with Psll for GAPDH. Culture conditions were as described previously; the time points at which the cells were harvested for nuclei isolation for the transcription elongation reaction were 8 and 10 h after A23187 or thapsigargin was administered to the LNCaP cells. Hybridization was performed at 65 °C for 72 h; washing of the blots was performed in 1× SSC and 0.5% SDS at 55 °C for 30 min. The film was exposed for 72 h.

**Results**

To determine whether intracellular levels of calcium affected the expression of the AR, the AR-containing cell line LNCaP was treated with the calcium ionophores A23187 (10−6 M) or ionomycin (10−4 M), or the intracellular Ca2+ ATPase inhibitor thapsigargin (10−7 M), for 16 h. RNA was analyzed on Northern blots incubated with an hAR cDNA probe. Three major AR mRNAs of 11, 8, and 4.7 kilobases
were detected (Fig. 1), which were of similar sizes to those reported previously for AR mRNA in LNCaP cells (27). The expression of all three AR transcripts decreased after treatment with A23187, ionomycin, or thapsigargin.

The time dependencies of A23187 and thapsigargin downregulation of AR were examined next. Levels of AR transcripts in LNCaP cells began to decrease after 6-8 h of incubation with A23187 (10^{-6} M), reached a nadir after 16 h of incubation (29% of control), and increased to 59% of the control level at 24 h (Fig. 2). Thapsigargin (10^{-7} M) treatment also resulted in a reduction in AR transcripts, decreasing after 6-8 h of incubation, reaching 23% of the control level after 10 h, and increasing to 68% of control by 24 h (Fig. 2).

Next, the dose response of AR mRNA to A23187 and thapsigargin was examined. An 8-h treatment with thapsigargin reduced AR mRNA levels at doses as low as 50 nM, whereas higher concentrations of A23187 (about 500 nM) were required to reduce AR mRNA levels (Fig. 3). These doses are similar to those reported previously for biological effects of these compounds (28). Next, AR protein levels were examined. As shown in Fig. 4, 10^{-6} M A23187 and 10^{-7} M thapsigargin effectively reduced AR protein levels after 12 h of incubation. However, at 24 h of incubation with either compound, the AR protein levels had almost returned to control levels.

Calcium has been reported to induce apoptosis in prostate epithelial cells (19, 20). To determine whether the observed effects on the AR were the result of apoptosis, LNCaP cells were incubated with different concentrations of A23187 or thapsigargin for various lengths of time (0-24 h). Total RNA was extracted and analyzed on Northern blots (20 μg/lane) with an hAR cDNA probe. The filter was rehybridized with GAPDH cDNA to correct for variations in the loading of RNA. The graph represents the mean of two independent experiments. This suggests that calcium ionophore A23187 and thapsigargin effectively reduced AR protein levels after 12 h of incubation. However, at 24 h of incubation with either compound, the AR protein levels had almost returned to control levels.

Calcium has been reported to induce apoptosis in prostate epithelial cells (19, 20). To determine whether the observed effects on the AR were the result of apoptosis, LNCaP cells were incubated with different concentrations of A23187 or thapsigargin for various lengths of time (Fig. 5). At the end of the experiments, no morphological differences were observed between control cells incubated with DMSO or cells treated with A23187 or thapsigargin (data not shown). Furthermore, A23187 had no effect on the growth rate of the LNCaP cells as measured by the MTT assay, indicating the viability of the cells. In the presence of thapsigargin, however, LNCaP cells became arrested in growth (Fig. 5). The first morphological indications for apoptosis were observed after 36 h of culture in the presence of A23187 and 12 h later for thapsigargin (48 h; data not shown). These results suggest
that calcium-induced apoptosis is preceded by a downregulation of AR mRNA levels in LNCaP cells.

To explore the mechanism of calcium regulation of AR mRNA expression in LNCaP cells, we measured the effects of various inhibitors on the regulatory pathways in LNCaP cells. The dosages of these drugs and the time frames of the experiments were based on our previous experiences using endometrial carcinoma cell lines (29). The 30-min pretreatment of the cells with either cycloheximide, staurosporine, or trifluoperazine was performed to allow these compounds to exert their actions before intracellular calcium became increased. Cycloheximide was used to inhibit protein synthesis (30); staurosporine was used to inhibit PKC (31); and trifluoperazine was used to inhibit calmodulin (32). None of these inhibitors affected the AR mRNA levels, nor did they prevent the calcium down-regulation of AR mRNA levels (Fig. 6). The lack of effect of staurosporine and trifluoperazine suggests that a pathway other than PKC or calmodulin might be involved.

Since cycloheximide had no effect, de novo protein synthesis did not seem to be involved in the calcium effects on AR mRNA expression. Thus, the effect of calcium might be due to a posttranscriptional change in the half-life of the message. Therefore, we measured the effects of thapsigargin on AR mRNA half-life. LNCaP cells were incubated with DMSO or thapsigargin for 8 h and then treated with the RNA synthesis inhibitor actinomycin D (5 µg/ml) for different periods of time (0–24 h). As shown in Fig. 7, the half-life of the AR mRNA in DMSO-treated LNCaP cells was 7.5 h. For thapsigargin-treated LNCaP cells, the half-life of AR mRNA was 8.1 h. This experiment showed that an accelerated turnover of AR mRNA in the presence of thapsigargin was not detectable in LNCaP cells, suggesting that a posttranscriptional mechanism of calcium down-regulation of AR mRNA in LNCaP cells was unlikely.

We next asked whether transcription of the AR gene was affected by A23187 or thapsigargin. Transcription of the AR gene in LNCaP cells, cultured in the presence of A23187 or thapsigargin, was measured using a nuclear run-on assay. As shown in Fig. 8, transcription of both the AR gene and the GAPDH gene was not affected by A23187 or thapsigargin.
The prostate is dependent on circulating concentrations of androgens and the presence of androgen receptors for normal growth, differentiation, and maintenance of differentiated functions (2, 3). Thus, factors that regulate expression of the AR presumably regulate the ability of androgens to control prostate function. For example, it has been demonstrated that retinoic acid can down-regulate both AR protein levels and androgenic induction of prostate-specific androgen and hK2 mRNAs in LNCaP cells (33).

The studies reported in this paper demonstrated that calcium can also modulate the expression of AR protein and mRNA in LNCaP cells. Thus, two distinct pathways, calcium and retinoic acid, are able to regulate AR expression and consequently androgen effects in the prostate.

In the current study two compounds, A23187 and thapsigargin, were used to increase intracellular calcium levels in LNCaP cells. A23187 is a Ca2+ ionophore, widely used to analyze the role of Ca2+ in the regulation of gene expression (34–36). However, several disadvantages in the use of A23187 have been reported; A23187 has pleiotropic effects (34, 36), and A23187 administration sometimes gives rise to a mixed Ca2+ and cAMP signal (35, 36). Therefore, we also used the specific endoplasmic reticulum Ca2+-ATPase inhibitor thapsigargin. Its effects are reported to be highly specific by initiating only one event: a rapid increase in cytosolic free Ca2+. Thapsigargin has been reported to be a potent tumor promoter, inducing growth- and transformation-related genes in NIH3T3 cells (36). Its role in LNCaP cells, however, seems to be directly opposite of these observations, as will be described below.

Since calcium has been reported to mediate apoptosis in the rat ventral prostate (19, 20), we tested the possibility of whether the down-regulation of AR by calcium could be due to the initiation of apoptosis. In our experiments, the calcium ionophore A23187 had no effect on cell growth or morphology, indicating that these cells were viable within the experimental setup. Thapsigargin treatment of the LNCaP cells, however, caused growth arrest. We do not think apoptosis is involved in thapsigargin-induced growth arrest, because the morphology of the cells remained unchanged during the 30-h incubation period (the first morphological changes were observed after 48 h; cells were rounding up and 24 h later were detaching from the plastic). Also, it has been demonstrated previously that cycloheximide can prevent calcium-induced apoptosis (37), suggesting that calcium-induced apoptosis in prostate cells requires new protein synthesis. However, in our studies, cycloheximide did not reverse the calcium effect on AR gene expression. Finally, expression of the housekeeping enzyme GAPDH was stable during treatment with either the calcium ionophore or thapsigargin. Our data suggest that the effect of calcium on AR mRNA occurs before apoptotic activation in LNCaP cells.

Several studies have shown that the calcium ionophore A23187 and thapsigargin can selectively activate gene expression (36, 38) or inactivate gene expression via increasing intracellular calcium levels (28, 39, 40). Recently, calcium-activated cis-acting elements have been found in the 5'-flanking region of a glucose- and calcium-regulated gpp78 gene (41, 42). The sequence of this element is distinct from the cAMP response element, which mediates A23187 activation of the c-fos promoter (43), and from tetradecanoylphorbol acetate- and serum-responsive elements that have been shown to mediate thapsigargin activation of the c-jun and c-fos promoters (36). Interestingly, sequences within the hAR

**Discussion**

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Calcium effects on gene expression are usually dependent on a rapid phosphorylation mechanism mediated by calmodulin or other calcium-regulated kinases. Calcium normally induces rapid expression of many genes such as c-jun and c-fos. However, inhibition of gene expression by calcium usually occurs with a pattern of relatively slow kinetics (39, 41). Calcium inhibition of the AR gene occurred after 6 h of incubation with A23187 and thapsigargin. This would argue against trans activation of AR via a rapid phosphorylation mechanism. The inability of staurosporine, a PKC inhibitor (31), and trifluoperazine, a calmodulin inhibitor (32), to reverse the calcium inhibition of AR gene expression also supports the argument that calcium down-regulation of AR gene expression occurs by a mechanism other than phosphorylation.

In conclusion, increases in intracellular calcium concentration by the calcium ionophore or thapsigargin effectively reduce AR expression in LNCaP cells. Calcium inhibition of AR expression seems to precede calcium-induced apoptotic cell death. Furthermore, the mechanism of this reduction seems to be different from a pathway in which the calcium signal is transduced via a phosphorylation mechanism.

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