Calmodulin-Androgen Receptor (AR) Interaction: Calcium-Dependent, Calpain-Mediated Breakdown of AR in LNCaP Prostate Cancer Cells

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Abstract
Chemotherapy of prostate cancer targets androgen receptor (AR) by androgen ablation or antiandrogens, but unfortunately, it is not curative. Our attack on prostate cancer envisions the proteolytic elimination of AR, which requires a fuller understanding of AR turnover. We showed previously that calmodulin (CaM) binds to AR with important consequences for AR stability and function. To examine the involvement of Ca2+/CaM in the proteolytic breakdown of AR, we analyzed LNCaP cell extracts that bind to a CaM affinity column for the presence of low molecular weight forms of AR (intact AR size, ~114 kDa). Using an antibody directed against the NH2-terminal domain (ATD) of AR on Western blots, we identified ~76-kDa, ~50-kDa, and 34/31-kDa polypeptides in eluates of CaM affinity columns, suggesting the presence of CaM-binding sites within the 31/34-kDa ATD of AR. Under cell-free conditions in the presence of phenylmethylsulfonyl fluoride, AR underwent Ca2+-dependent degradation. AR degradation was inhibited by N-acetyl-leu-leu-norleu, an inhibitor of thiol proteases, suggesting the involvement of calpain. In intact cells, AR breakdown was accelerated by raising intracellular Ca2+ using calcimycin, and increased AR breakdown was reversed with the cell-permeable Ca2+-chelator bis-(o-aminophenoxo)-ethane-N,N,N',N'-tetraacetic acid tetra-acetoxyethyl-ester. In CaM affinity chromatography studies, the Ca2+-dependent protease calpain was bound to and eluted from the CaM-agarose column along with AR. Caspase-3, which plays a role in AR turnover under stress conditions, did not bind to the CaM column and was present in the proenzyme form. Similarly, AR immunoprecipitates prepared from whole-cell extracts of exponentially growing LNCaP cells contained both calpain and calpastatin. Nuclear levels of calpain and calpastatin (its endogenous inhibitor) changed in a reciprocal fashion as synchronized LNCaP cells progressed from G1 to S phase. These reciprocal changes correlated with changes in AR level, which increased in late G1 phase and decreased as S phase progressed. Taken together, these observations suggest potential involvement of AR-bound CaM in calcium-controlled, calpain-mediated breakdown of AR in prostate cancer cells.

Introduction
Adenocarcinoma of the prostate is the most frequently diagnosed cancer and second leading cause of cancer deaths in American men (1). Although androgen ablation is the most common therapy for disseminated prostate cancer, it is palliative in nature and most patients eventually succumb to hormone-refractory disease resistant to chemotherapy. Whether normal or mutated, androgen receptor (AR) is required for growth in both androgen-sensitive and androgen-insensitive prostate cancer (2). Therefore, it is of paramount importance to dissect the various ways in which AR is regulated to not simply inactivate but to physically eliminate AR (3). This requires understanding the cellular mechanisms of AR breakdown.

Numerous studies have examined the proteolytic destruction of AR, and our knowledge can at present be divided into three areas. First, AR is degraded to small (~10 residues) peptides by the threonine proteases of the ubiquitin-proteasome system (4–9). Second, AR can be cleaved by serine proteases (10–16). These studies established that, structurally, AR consists of at least three domains, the ligand-binding domain (LBD), the DNA-binding domain (DBD), and the NH2-terminal domain (ATD), separated by protease-sensitive linker regions. Serine proteases cleave AR to yield a 34-kDa COOH-terminal LBD (12, 17) and the remaining ~76 kDa of AR comprising the ATD and DBD. Other studies (11) established that proteolysis can cleave AR into two fragments: one of ~50 kDa containing both the LBD and the DBD and another of approximately the same size, presumably the ATD (13). Third, the caspase family of thiol proteases is capable of cleaving AR, particularly those forms of AR with expanded polyglutamine repeats (18–22). To date, the cleavage of AR by the Ca2+-activated, thiol protease calpain has not been described.

The amount, localization, physiologic functions, and degradation of AR are influenced by the following factors. First, AR is transcribed, translated (23, 24), and post-translationally phosphorylated (25, 26). Androgen binds to the LBD, inducing conformational change critical to the transcription activity of AR as well as resistance to degradation (17, 27). Following androgen binding, AR is ready to act as a transcription factor (23), as the DBD binds to the androgen response element (ARE). The activity of the DNA-bound complex is governed by binding of coactivator and corepressor proteins to the LBD and the ATD. Finally, AR activity is also controlled by AR-calmodulin (CaM) interaction (28). It is the effect of AR interaction with CaM on the breakdown of AR that we examine herein.

Extending our observation that CaM binds to AR directly (28), we now map the CaM-binding site(s) to the ATD of AR. We show for the first time that Ca2+ stimulates AR breakdown and that CaM bound to AR may play an important role in regulating AR levels by...
Calpain Cleavage of Androgen Receptor in LNCaP Cells

Materials and Methods

Cell culture. LNCaP cells (obtained from the American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10 mmol/L testosteronate (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS (Atlanta Biologicals, Grand Island, NY) containing 10 nmol/L testosterone (Sigma Chemical Co.) and 1 mmol/L penicillin. Cells were maintained in a humidified incubator with 5% CO2 and 95% air at 37°C.

Preparation of nuclear and cytoplasmic fractions. LNCaP cells were treated with the Ca2+ ionophore A23187 (calcimycin) or the cell-permeable calcium chelator 1,2-bis-(O-aminophenoxy)-ethane-N,N',N''-tetraacetic acid tetra-(acetoxymethyl)-ester (AM-BAPTA; a Sorvall RT7 centrifuge and resuspended in buffer A [0.16 mol/L sucrose, 50 mmol/L Tris-HCl (pH 7.6), 25 mmol/L KCl, 10 mmol/L MgCl2, 70 mmol/L HEPES (pH 7.6), 0.025 mmol/L CaCl2, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2 mmol/L DTT, 1 mmol/L EDTA] at a density of 2 × 107/mL. The cell suspension was then homogenized in a top-driven Wheaton homogenizer (Wheaton Co., Wheaton, IL) until ~90% of the cells were stained with trypan blue dye (usually three strokes at a rotation setting of 3). The cytosolic supernatant was then separated from the nuclear pellet by centrifugation at 4°C in a Beckman Microfuge B at 6,000 rpm for 5 minutes (Beckman Instruments, Palo Alto, CA). A volume of buffer A equal to that used for homogenization was added to the microfuge tube, and the nuclei were then subjected twice to 30 pulses of sonication with a Branson Sonifier 250 (Branson Co., Danbury, CT) set at an output control of 2 and a duty cycle of 20, with intermittent cooling on ice. The sonicated nuclear extract was cleared by centrifugation in the microfuge as described above. Protein concentration in cytosolic and nuclear extracts was assessed with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) using the Bradford method of Subramanyam et al. (28). Briefly, LNCaP cells were suspended at a density of 2 × 107/mL in buffer A and sonicated and the extracts were clarified as above.

UVB irradiation. UV irradiation was administered (31) using a single FS40 lamp (National Biological, Twinsburg, OH). FS40 lamps produce 0.3% UVC (260–280 nm), 62% UVB (280–320 nm), and 38% UVA (320–400 nm) with a peak emission at 313 nm. The average irradiance of the source was approximately 1.58 × 10−3 W/cm2 (17 cm distance as measured by an IL1700 Research Radiometer (International Light, Newburyport, MA) with a UVB-1 filter (#25628) and 300 detector. LNCaP cells were grown to 70% confluence in P100 plates. Immediately before UV radiation treatment, the medium was removed from each culture, the cells were washed once with PBS, and fresh PBS was added. Cultures were exposed to one of two doses of UV radiation (300 J/m2 for 40 seconds or 600 J/m2 for 80 seconds) under sterile conditions with the cover of the culture dish off. Following UV exposure, the PBS was removed and replaced with serum-containing culture medium and cultured for an additional 12 or 24 hours. Control cultures were identically treated but were not UV irradiated. The cells were then harvested by pipetting the culture medium up and down on the cells with a Pasteur pipette until all cells were detached. The cells were washed once in PBS/EDTA before sonicating to prepare extract cells as usual.

Treatment of cells with Ca2+ ionophore and intracellular Ca2+ chelator. Subconfluent P150 plates of exponentially growing cultures of LNCaP cells were treated with the Ca2+ ionophore A23187 (calcimycin) or the cell-permeable calcium chelator 1,2-bis-(O-aminophenoxy)-ethane-N,N',N''-tetraacetic acid tetra-(acetoxymethyl)-ester (AM-BAPTA; a Sorvall RT7 centrifuge and resuspended in buffer A [0.16 mol/L sucrose, 50 mmol/L Tris-HCl (pH 7.6), 25 mmol/L KCl, 10 mmol/L MgCl2, 70 mmol/L HEPES (pH 7.6), 0.025 mmol/L CaCl2, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2 mmol/L DTT, 1 mmol/L EDTA] at a density of 2 × 107/mL. The cell suspension was then homogenized in a top-driven Wheaton homogenizer (Wheaton Co., Wheaton, IL) until ~90% of the cells were stained with trypan blue dye (usually three strokes at a rotation setting of 3). The cytosolic supernatant was then separated from the nuclear pellet by centrifugation at 4°C in a Beckman Microfuge B at 6,000 rpm for 5 minutes (Beckman Instruments, Palo Alto, CA). A volume of buffer A equal to that used for homogenization was added to the microfuge tube, and the nuclei were then subjected twice to 30 pulses of sonication with a Branson Sonifier 250 (Branson Co., Danbury, CT) set at an output control of 2 and a duty cycle of 20, with intermittent cooling on ice. The sonicated nuclear extract was cleared by centrifugation in the microfuge as described above. Protein concentration in cytosolic and nuclear extracts was assessed with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) using the Bradford method of Subramanyam et al. (28). Briefly, LNCaP cells were suspended at a density of 2 × 107/mL in buffer A and sonicated and the extracts were clarified as above.

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Synchronization of LNCaP cells by isoleucine deprivation. Isoleucine deprivation was done by our previously published method (29). The cells were grown to 60% to 70% confluence, and then the medium was replaced with isoleucine-free RPMI 1640 supplemented with 6% dialyzed FCS (Life Technologies), 2.5 mmol/L glutamine, 100 μg/mL streptomycin, 100 units/mL penicillin, and 100 units/mL testosteronate. Cells were maintained in isoleucine-free medium for 36 hours in an incubator at 37°C. Cells were released from isoleucine block by replacing the medium with the corresponding complete medium containing 10% FCS.

Preparation of nuclear and cytoplasmic fractions. LNCaP cells were subjected to nuclear and cytoplasmic fractionation by slight modification of the method of Subramanyam et al. (30). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), except where otherwise noted. After harvest, cells were pelleted by centrifugation for 10 minutes at 4°C at 2,000 rpm in a Sorvall RT7 centrifuge and resuspended in buffer A [0.16 mol/L sucrose, 50 mmol/L Tris-HCl (pH 7.6), 25 mmol/L KCl, 10 mmol/L MgCl2, 70 mmol/L HEPES (pH 7.6), 0.025 mmol/L CaCl2, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2 mmol/L DTT, 1 mmol/L EDTA] at a density of 2 × 107/mL. The cell suspension was then homogenized in a top-driven Wheaton homogenizer (Wheaton Co., Wheaton, IL) until ~90% of the cells were stained with trypan blue dye (usually three strokes at a rotation setting of 3). The cytosolic supernatant was then separated from the nuclear pellet by centrifugation at 4°C in a Beckman Microfuge B at 6,000 rpm for 5 minutes (Beckman Instruments, Palo Alto, CA). A volume of buffer A equal to that used for homogenization was added to the microfuge tube, and the nuclei were then subjected twice to 30 pulses of sonication with a Branson Sonifier 250 (Branson Co., Danbury, CT) set at an output control of 2 and a duty cycle of 20, with intermittent cooling on ice. The sonicated nuclear extract was cleared by centrifugation in the microfuge as described above. Protein concentration in cytosolic and nuclear extracts was assessed with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) using the Bradford method of Subramanyam et al. (28). Briefly, LNCaP cells were suspended at a density of 2 × 107/mL in buffer A and sonicated and the extracts were clarified as above.

Table 1. Densitometric analysis of the Western blot in Fig. 1 to determine the degree of breakdown of AR in the initial sample and in the EGTA eluate

<table>
<thead>
<tr>
<th>Form of AR</th>
<th>Cell lysate, n (%)</th>
<th>Drop thru</th>
<th>EGTA eluate, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact AR</td>
<td>114,249 (38)</td>
<td>8,167</td>
<td>90,001 (9)</td>
</tr>
<tr>
<td>50-kDa frag</td>
<td>107,436 (36)</td>
<td>432,440 (43)</td>
<td></td>
</tr>
<tr>
<td>31/34-kDa frag</td>
<td>78,006 (26)</td>
<td>428,377 (43)</td>
<td></td>
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*On an arbitrary scale of 0 to 256 density units above background per pixel determined using the Stratagene (La Jolla, CA) Eagle Eye II system.
Molecular Probes, Eugene, OR) for 24 hours. BAPTA or control vehicle was added to cultured and incubated at 37°C. Thirty minutes later, A23187 was added and cells were incubated for 24 hours. The cells were harvested, as above under UV treatment, by pipetting the culture medium up and down to detach the cells followed by washing and sonication to prepare the extracts.

**Western blot analysis.** Samples were dissolved in PAGE loading buffer (Bio-Rad, Richmond, CA) and subjected to denaturing 10% or 12% SDS/PAGE and then transferred to nitrocellulose membranes. Individual membranes were probed with rabbit polyclonal antibody (pAb) against the 20 NH2-terminal residues (N20) or murine monoclonal antibody (mAb) against residues 301 to 317 (mAb 441) of human AR (Santa Cruz Biotechnology, Santa Cruz, CA). Other primary antibodies used were mAb against residues 301 to 317 (mAb 441) of human AR (Santa Cruz Biotechnology, Santa Cruz, CA). Other primary antibodies used were mAb against the following: CaM (Upstate Cell Signaling Solutions, Lake Placid, NY); caspase-3 (Santa Cruz Biotechnology); the COOH-terminal domain of poly(ADP-ribose) polymerase (PARP), which reacts with both the intact 112-kDa and the clipped 85-kDa forms of PARP (Santa Cruz Biotechnology); μ-calpain large subunit and calpastatin (Chemicon International, Temecula, CA); and rabbit mAb to cleaved caspase-3 (Cell Signaling Technology, Beverly, MA). Immunoreactive bands were developed by using horseradish peroxidase–conjugated secondary antibodies and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and visualized by using X-ray film.

**CaM-agarose affinity chromatography.** The CaM affinity chromatography procedure was essentially as described by Cifuentes et al. (28). Briefly, nuclear (~2.5 mg protein) and cytoplasmic (~7.5 mg protein) lysates of LNCaP cells prepared as described above were applied to 2 mL CaM-agarose affinity columns (Sigma Chemical) poured in 25-cm Eppendorf columns (Bio-Rad). Nonadherent proteins were washed from the column with buffer B [20 mmol/L Tris (pH 7.4), 4 mmol/L MgCl₂, 2 mmol/L CaCl₂, 10 mmol/L KCl, 1 mmol/L PMSF] until A₂₈₀ returned to the baseline. This nonadherent fraction was called the drop thru. Proteins retained on the column were then eluted with modified buffer B [20 mmol/L Tris (pH 7.4), 4 mmol/L MgCl₂, 10 mmol/L EGTA, 10 mmol/L KCl, 1 mmol/L PMSF] in which the Ca²⁺ chelator EGTA was substituted for Ca²⁺. All chromatography procedures, including cell lysis application, washing, and elution, were carried out without application of pressure to the gel bed other than the hydrostatic pressure of 15-cm (maximum) column of liquid above the gel. Protein in the drop thru and eluate fractions was concentrated by precipitation in ice-cold 5% trichloroacetic acid and dissolved in PAGE loading buffer.

**Immunoprecipitation.** AR immunoprecipitation to analyze AR-interacting proteins was done by modification of the method of Cifuentes et al. (28). Cell extracts were diluted 10-fold in 250 mmol/L NaCl and 50 mmol/L Tris (pH 7.4; dilution buffer) and incubated overnight with 4 μg/mL of N20 or 441 anti-AR antibody at 4°C. The immune complexes were then adsorbed to protein A/G agarose immunoadsorbent (Pierce) by incubation for 1 hour at ambient temperature with gentle agitation. The adsorbed complexes were washed twice in dilution buffer by centrifugation at 4°C and eluted with PAGE loading buffer. Mock immunoprecipitates were obtained by diluting and incubating the extracts without AR antibody. The mixture was then incubated with immunoadsorbent and eluted as above.

**Results**

**CaM-binding site in the NH₂-terminal region of AR.** In LNCaP cell extracts prepared from cells harvested by trypsinization, there was a noticeable breakdown of AR to fragments with approximate molecular weights of ~50 and 31/34 kDa. Like intact AR, each of these AR fragments was detected on Western blots using affinity-purified pAbs raised against the most NH₂-terminal 20 amino acids of human AR (pAb N20, Santa Cruz Biotechnology; Fig. 1). Therefore, the AR fragments detected by pAb N20 are referred to as AR ATD fragments. Following up on our observation that AR in LNCaP cell extracts binds to CaM (28), we attempted to map the CaM-binding region on AR by testing whether the ~50-kDa and 31/34-kDa AR ATD fragments retain CaM-binding ability. CaM-agarose affinity chromatography of lysate from trypsinized LNCaP cells showed strong binding of both the ~50-kDa and 31/34-kDa AR ATD fragments to CaM. Although Ponceau S stainable protein could be detected equally well in both the “drop thru” and EGTA eluate fractions of a CaM-agarose affinity column (data not shown), the pAb N20 immunoreactive bands of AR and AR ATD fragments could only be detected in the EGTA eluate (Fig. 1, lane 2). In addition, we
observed that, whereas the pAb N20 detected all ATD fragments of AR, mouse mAb (32) raised against amino acids 301 to 317 of the AR (mAb 441, Santa Cruz Biotechnology) detected intact AR and the ∼50-kDa ATD fragment but not the 31/34-kDa ATD fragment (data not shown). These observations together suggest that the CaM-binding site lies within the 31/34-kDa NH₂-terminal region of AR and that the 31/34-kDa fragment lacks the 301 to 317 epitope.

AR cleavage is accentuated in LNCaP cell extracts subjected to CaM affinity column. Quantitative analysis of the relative distribution of pAb N20 immunoreactive bands of intact and ATD fragments of AR in cell lysates versus those enriched in the EGTA eluate of a CaM affinity column suggested that AR in LNCaP cell lysates may have undergone additional proteolytic breakdown during the course of affinity chromatography despite the presence of PMSF in the buffer. Densitometry of pAb N20 immunoreactive bands in cell lysate showed an approximate distribution of intact AR and the ∼50-kDa and 31/34-kDa ATD fragments to be 38%, 36%, and 26%, respectively (Table 1). By comparison, the same cell lysates, when subjected to CaM affinity chromatography, yielded EGTA eluate that contained pAb N20 immunoreactive bands of intact AR and the ∼50-kDa and 31/34-kDa ATD fragments with a density distribution of 9%, 43%, and 43%, respectively (Table 1). Thus, there was a significant decrease of intact AR and an increase of 50-kDa and 31/34-kDa ATD fragments in the EGTA eluate fraction compared with that in the cell lysate before CaM affinity chromatography.

Ca²⁺ stimulates AR breakdown in LNCaP cell extracts. The cleavage of AR on chromatography on CaM affinity columns observed above occurred in the presence of 1 mmol/L PMSF, suggesting that AR proteolysis was caused by an enzyme that was not a serine esterase. Given that the loading buffer for applying extracts to the CaM affinity column contained 2 mmol/L Ca²⁺, we sought to determine if Ca²⁺ was activating a proteolytic factor in the cell extracts. Therefore, LNCaP nuclear extracts (prepared from cells harvested by scraping rather than trypsinization) were incubated for varying times in the presence of increasing concentrations of Ca²⁺ before being separated by PAGE and analyzed by Western blots using antibodies to AR, µ-calpain, or caspase-3 as described in Materials and Methods. Data are from one of four chromatographic runs.

Ca²⁺-stimulated breakdown of AR in LNCaP cell extracts is attenuated by N-acetyl-leu-leu-norleu. One of the best-characterized Ca²⁺-activated proteases is calpain. Therefore, we tested whether the breakdown of AR into ATD fragments from AR is Ca²⁺ dependent.

![Figure 3](https://example.com/figure3.png) Increasing intracellular Ca²⁺ by ionophore increases AR breakdown, and chelation of intracellular Ca²⁺ decreases AR breakdown. LNCaP cells were incubated for 24 hours with the ionophore calcimycin (A23187) in the presence or absence of the intracellular Ca²⁺ chelator AM-BAPTA. Cells were then harvested by scraping into medium, and experimental and control (whole cell) lysates were prepared at a concentration of 2 × 10⁷ cells/mL. PAGE was done, and the blots were analyzed with antibodies to AR (pAb N20), calpastatin, CaM, or actin as described in Materials and Methods. The experiment was repeated twice, and representative data are shown.

![Figure 4](https://example.com/figure4.png) Calcium-dependent binding of AR and calpain, but not caspase-3, to CaM-agarose columns. LNCaP cells were harvested by trypsinization, extracts were subjected to CaM-agarose affinity chromatography, and the unbound flow-through protein fraction (Drop-Thru) and the protein fraction eluted with EGTA (EGTA Eluate) were subjected to Western blot analysis using antibodies to AR, µ-calpain, or caspase-3 as described in Materials and Methods. Data are from one of four chromatographic runs.
N-acetyl-leu-leu-norleu (calpain inhibitor I or ALLN). Addition of 20 μmol/L ALLN to the incubation system described above in Fig. 2A resulted in protection of AR from Ca2+-activated proteolytic cleavage into ATD fragments (Fig. 2B). A similar inhibitory effect of ALLN on Ca2+-stimulated AR breakdown was observed in cytosolic extracts incubated at 4°C and with both nuclear and cytosolic extracts incubated at 23°C (data not shown). Thus, Ca2+-stimulated AR cleavage and ALLN attenuation of Ca2+-stimulated cleavage suggest the involvement of calpain in AR cleavage.

Ca2+ ionophore stimulates and cell-permeable Ca2+ chelator suppresses breakdown of endogenous AR in LNCaP cells. Given that the breakdown of AR in vitro is accelerated by increasing the levels of Ca2+, we investigated the effect of artificially altering intracellular levels of Ca2+. We cultured cells in the presence of the cell-permeable Ca2+ chelator acetomethyl ester form of BAPTA (AM-BAPTA) and/or in the presence of A23187, an ionophore that raises intracellular Ca2+ levels. Cells in this study were harvested by scraping rather than trypsinization. Treatment of LNCaP cells with AM-BAPTA had little effect, per se, on the level of intact AR but attenuated the formation of the ~50-kDa and 31/34-kDa fragments of AR (Fig. 3, compare lanes 1 and 2). This is consistent with a potential role of Ca2+-dependent AR cleavage. On the other hand, treatment of LNCaP cells with the A23187 calcimycin ionophore had a striking effect. Cell rounding and detachment were observed, consistent with induction of apoptosis, although, within the 24-hour course of the experiment, cellular integrity was unaffected as evidenced by the equal amount of actin in equal volumes of extract (Fig. 3). Relative to actin, A23187 calcimycin caused a substantial decrease in intact AR and AR fragments (Fig. 3, lane 3). This A23187-induced decrease in AR was also associated with a decrease in calpastatin, a natural inhibitor of calpain, and calpastatin breakdown products (Fig. 3). Pretreatment of LNCaP cells with AM-BAPTA partially ameliorated the effect of calcimycin ionophore (Fig. 3, lane 4).

Calpain, but not caspase-3, binds to CaM affinity columns along with AR. Cleavage of AR by calpain has not been previously reported, whereas there are numerous reports of caspase-3-mediated AR breakdown (19–22). Furthermore, ALLN is not specific for calpain. Therefore, we sought further evidence for the involvement of calpain in AR degradation. LNCaP cell extracts were subjected to CaM-agarose affinity chromatography under the conditions described in Fig. 1, a procedure found to accelerate AR cleavage. The drop thru and EGTA eluate fractions were then analyzed by Western blots probed with antibodies to caspase-3, μ-calpain, and AR (Fig. 4). Caspase-3 failed to bind to CaM and was present only in the drop thru fraction (Fig. 4, lane 1). Furthermore, the molecular weight of the material immunoreactive with the antibody to caspase-3 was consistent with the presence of caspase-3 in its inactive form. No material was observed in these extracts that reacted with antibody to cleaved caspase-3 (data not shown), which is the active form of the enzyme. All of the AR bound to the CaM column and was eluted with EGTA (Fig. 4, lane 2). Calpain bound to the CaM-agarose column and was present in the EGTA eluate (Fig. 4, lane 2). Thus, the Ca2+-dependent binding of AR and calpain to CaM affinity columns is consistent with a potential role of calpain in AR cleavage.

Calpain, calpastatin, and CaM, but not caspase-3, are in AR immunoprecipitates. The previous experiments suggested that calpain, but not caspase-3, was responsible for calcium-activated cleavage of AR to ATD fragments. Therefore, we sought evidence using immunoprecipitation that calpain is associated with AR in whole-cell extracts. Caspase-3, although present in crude lysates, did not coprecipitate when AR was immunoprecipitated with either mAb 441 or pAb N20 (Fig. 5), consistent with our conclusion that caspase-3 was not responsible for AR breakdown in our experiments. CaM, on the other hand, coprecipitated with AR in immunoprecipitates prepared with both antibodies (anti-AR mAb 441 and pAb N20), confirming our earlier report of AR binding to CaM (28). Calpain and calpastatin also coprecipitated with AR (Fig. 5). Coprecipitation of calpastatin with AR was more apparent with the mAb 441 than with pAb N20. We infer from this that the binding of calpastatin to AR may be disrupted when pAb N20 binds to the most NH2-terminal residues of AR. Thus, it seems that, in crude lysates at a concentration of Ca2+ that is physiologic for the intracellular milieu, AR, CaM, calpain, and calpastatin, and Ca2+ form a complex.

Changes in calpain and calpastatin levels are associated with progression of synchronized LNCaP cells from G0-G1 to S phase. Given that AR, CaM, calpain, and calpastatin seem to form a complex in the living cell and given that intracellular Ca2+ fluctuates significantly during the cell cycle (33), we examined the possibility that variation of AR levels throughout the cell cycle might be associated with concomitant changes in the levels of calpain and calpastatin. For this, we used LNCaP cells synchronized by isoleucine deprivation that progress synchronously from G0-G1 to S phase on release from isoleucine deprivation.
block (29). In these cells, AR levels fluctuated during the cell cycle from a low at G₀-G₁ (0–4 hours after release from isoleucine block) to a maximum just as S phase begins (12–16 hours after release from isoleucine block; Fig. 6). These changes were more pronounced in nuclear extracts (Fig. 6B, top) than in cytosolic extracts (Fig. 6A, top). In both compartments, AR ATD fragments generally tended to increase as the cell cycle progressed.

The levels of calpain and calpastatin also varied during the cell cycle. Cytosolic calpain levels were highest early in the cell cycle, decreased in late G₁, and increased as S phase approached (Fig. 6A). Cytosolic calpastatin levels roughly mirrored calpain levels (Fig. 6A). The pattern of increasing cleavage of AR to ATD fragments seems to correlate to the increasing levels of calpain. Nuclear calpain was lowest during early G₁ (Fig. 6B) and increased as the cell cycle progressed, correlating with the progressive increase in AR ATD fragments. Calpastatin levels, on the other hand, remained relatively steady throughout the cell cycle, although the level of the two highest molecular weight forms fluctuated.

In summary, there seems to be a correlation, more pronounced in the nucleus, between increases in calpain expression during progression through the cell cycle and the appearance of AR ATD fragments. This suggests that calpain might be responsible for AR cleavage as cells enter S phase. Although calpastatin is present in both nuclear and cytosolic extracts, greater variation is seen in cytosolic levels of calpastatin, suggesting different effects in the two compartments.

UV light activates caspase-3, triggers apoptosis in LNCaP cells, and causes decreased AR levels but does not generate ATD fragments. Caspase-3 has been implicated in the proteolytic cleavage of AR, but the experiments described above failed to reveal activated caspase-3 in exponentially growing or synchronized LNCaP cells. Therefore, we exposed LNCaP cells to doses of UV radiation known to trigger programmed cell death in cultured cancer cells and examined the effect on activation of caspase-3 and AR. Twelve hours after UVB exposure, LNCaP cells showed a mild degree of rounding up but little detachment from their substratum; by 24 hours after exposure, the apoptotic pathway was activated as evidenced by rounding and detachment of ~20% of the cells at the higher UV dose (data not shown). There was significant, UV dose-dependent cleavage of PARP (Fig. 7A), a classic marker of programmed cell death. Although UV treatment did not affect the level of intact caspase-3 (Fig. 7B, top), it led to the appearance of cleaved caspase-3 fragments. This confirms that caspase-3 is present only in the inactive form in untreated, exponentially growing, or synchronized LNCaP cells but that it can be generated under specific conditions.

Although there was no change in the actin level, the level of AR was dramatically reduced by 24 hours after UVB irradiation at a dose of 600 J/m² (Fig. 7A). However, this decrease in AR was not accompanied by an increase in ATD fragments of AR. Rather, compared with unexposed controls or low-dose UV at 24 hours, the 31/34-kDa ATD fragments were less prominent in cells undergoing programmed cell death.

Discussion

Our studies are the first to show that Ca²⁺ stimulates AR breakdown in vitro and in vivo and that CaM bound to AR seems to play a role in calpain-mediated cleavage of AR in prostate cancer cells. When AR binds to CaM in the presence of 2 mmol/L Ca²⁺, AR breakdown is accelerated despite the presence of PMSF (Fig. 1). AR, calpain, and its inhibitor calpastatin all bind to CaM in a Ca²⁺-dependent manner and...
are eluted with the calcium chelator EGTA (Fig. 4). Similarly, AR immunoprecipitates contain CaM, calpain, and calpastatin (Fig. 5). In vitro, Ca\(^{2+}\) activates AR breakdown in both nuclear and cytosolic extracts, and the inhibitor of thiol proteases, ALLN, prevents Ca\(^{2+}\)-stimulated AR breakdown (Fig. 2). Based on AR breakdown fragment sizes and AR antibody epitope locations, we propose a model of AR cleavage by calpain (see Fig. 8A). We suggest that calpain cleaves AR at protease-sensitive sites between the LBD and DBD (yielding an ∼75-kDa ATD fragment), between the ATD and DBD (yielding an ∼50-kDa ATD fragment), and approximately in the middle of the ATD (yielding a 31/34-kDa NH\(_2\)-terminal fragment; Fig. 8A).

Proteins that bind to Ca\(^{2+}\) and/or are activated by Ca\(^{2+}\)-binding proteins, such as CaM, act as decoders of the Ca\(^{2+}\) signal. In vitro, Ca\(^{2+}\) is known to change calpain conformation, effecting its autocatalytic cleavage and activation (34). Calpain heavy and light chains both have CaM homology domains (35, 36). Calpastatin binds to these CaM homology domains and inactivates calpain (37). Interestingly, virtually all proteins that bind to CaM are also substrates for calpain (38). Calpain, calpastatin, and CaM exist in a complex with AR (Fig. 5), and Ca\(^{2+}\) stimulates AR degradation (Fig. 3) in LNCaP cells. As depicted in Fig. 8B, these observations raise the intriguing possibility that CaM, bound to AR, recruits calpain and calpastatin to cleavage sites on AR. At low Ca\(^{2+}\), CaM-bound calpain is enzymatically inactive. However, at high Ca\(^{2+}\), the conformation of the calpain-CaM-calpastatin complex is altered in such a way that calpain is released from the inhibitory effect of calpastatin, thereby allowing calpain to cleave AR (Fig. 8B). This model is also consistent with our earlier observation that the anti-CaM drug N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, which can alter CaM conformation, induces AR degradation in LNCaP cells (28).

Ca\(^{2+}\), as a second messenger, enables cells to walk a tightrope between life and death. In exponentially growing rat prostate cancer cells, increasing intracellular Ca\(^{2+}\) shunts cells into G\(_0\)-G\(_1\) phase and induces the growth arrest gene gadd153 (39). Thapsigargin (39–41), β-lapachone (42), and ionomycin (40, 43) all induce striking increases in intracellular Ca\(^{2+}\) in prostate cancer cells and trigger programmed cell death. This process is accompanied by growth arrest (39) and down-regulation of AR expression (40). Our studies show that, both in cultured LNCaP cells and in cell-free extracts, increasing Ca\(^{2+}\) allowed AR degradation whereas decreasing Ca\(^{2+}\) (by chelation either in vitro with EDTA or intracellularly with AM-BAPTA) attenuated AR breakdown (Figs. 2 and 3). Thus, an increase in intracellular Ca\(^{2+}\) both suppresses the expression and stimulates the breakdown of proteins necessary for proliferation and viability of prostate cancer cells.

That AR is a substrate for calpain is not surprising. Estrogen receptor (44) and progesterone receptor (45) are cleaved by what we now know to be calpain. In LNCaP cells, cleavage of β-catenin, a key molecule in the Wnt signaling pathway that controls AR expression, is mediated by calpain (46). In this study, we observed that AR levels inversely correlated with calpain levels during the cell cycle in LNCaP cells; when AR levels were increasing, calpain levels were low in early G\(_1\) phase, whereas AR levels were beginning to decline, calpain levels were at a maximum in S phase (Fig. 6).

![Figure 7](https://example.com/figure7.png)

Figure 7. Exponentially growing LNCaP cells were exposed to either 300 or 600 J/m\(^2\) of UV radiation; cells were scraped into medium 12 or 24 hours later, and whole-cell extracts were prepared and subjected to PAGE. A. Western blots were incubated with antibody to PARP to determine if the apoptotic process was initiated (top) and with the pAb N20 to the ATD of AR (middle) or antibody to actin to assess uniformity of loading (bottom). B, blots were incubated with antibody to intact caspase-3 (top) or antibody to cleaved caspase-3 (bottom). Results are from a single experiment.
Caspase-3 does not seem to be the enzyme that cleaves AR under physiologic conditions. AR with expanded polyglutamine repeats has been implicated in neurodegenerative disease wherein AR is cleaved between the DBD and LBD into a toxic, "truncated" ATD-DBD moiety (18). Caspase-3 is suggested to be the enzyme responsible for the cleavage of this pathologic AR (19–22). Based on these observations of AR in neurodegenerative diseases, caspase-3 is implicated to cleave AR in prostate cancer cells (15, 23). However, we found that, under physiologic conditions, caspase-3 is not present in its activated form in exponentially growing LNCaP cells and caspase-3 is not part of the AR-CaM complex. Furthermore, activation of caspase-3 following irradiation of LNCaP cells with UVB radiation is not associated with cleavage of AR into 76-kDa, 50-kDa, or 31/34-kDa ATD fragments. Thus, caspase-3 could play a role in AR breakdown either directly or indirectly only under stress conditions; under normal growth conditions, it is an unlikely candidate for AR degradation during the cell cycle.

The degradation of AR is complex and multifaceted, and different proteolytic systems seem to be involved in transcription of genes regulated by AR, degradation of damaged AR, progress through the cell cycle, and programmed cell death. Although initial studies with proteasome inhibitors suggested that the proteasomal pathway was a major route for degradation of AR (5), subsequent studies (7, 9) have indicated that the primary effect of proteasome inhibitors in prostate cancer cells is to alter the Akt pathway, thereby altering transcription and translation of AR. On the other hand, the proteasome is critical for AR transactivating activity [i.e., AR binding to the ARE (6, 8)]. This study adds to the complexity by implicating a proteolytic enzyme, calpain, novel to AR degradation but well established for cleaving key regulatory molecules. Certainly, breaking down AR by a Ca2+-dependent, CaM-interacting, calpastatin-regulated mechanism is consistent with our concept of the importance of protein-protein interactions during the cell cycle. The localization of CaM-binding site(s) to the ATD of AR focuses our attention on a region of AR ripe for targeted therapy development (47). We envision a critical regulatory role for a CaM-AR-calpastatin-calpain complex in the proliferation of prostate cancer cells and as targets of opportunity in the development of agents for treating prostate cancer.

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References


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