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CALMODULIN PROTECTS ANDROGEN RECEPTOR FROM CALPAIN-MEDIATED BREAKDOWN IN PROSTATE CANCER CELLS

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Abstract

Although inactivation of the androgen receptor (AR) by androgen-ablation or anti-androgen treatment has been frontline therapy for disseminated prostate cancer for over 60 years, it is not curative because castration-resistant prostate cancer cells retain AR activity. Therefore, curative strategy should include targeted elimination of AR protein. Since AR binds to calmodulin (CaM), and since CaM-binding proteins are targets of calpain-mediated proteolysis, we studied the role of CaM and calpain in AR breakdown in prostate cancer cells. Whereas the treatment of prostate cancer cells individually with anti-CaM drug or calcimycin, which increases intracellular Ca^{++} and activates calpain, led to minimal AR breakdown, combined treatment led to a precipitous decrease in AR protein levels. This decrease in AR protein occurred without noticeable changes in AR mRNA levels, suggesting an increase in AR protein turnover rather than inhibition of AR mRNA expression. Thus, CaM inactivation seems to sensitize AR to calpain-mediated breakdown in prostate cancer cells. Consistent with this possibility, purified recombinant human AR (rhAR) underwent proteolysis in the presence of purified calpain, and the addition of purified CaM to the incubation blocked rhAR proteolysis. Together, these observations demonstrate that AR is a calpain target and AR-bound CaM plays an important role in protecting AR from calpain-mediated breakdown in prostate cancer cells. These observations raise an intriguing possibility that anti-CaM drugs in combination with calpain-activating agents may offer a curative strategy for the treatment of prostate cancer, which relies on AR for growth and survival.

Keywords

Androgen receptor; calmodulin; calpain; hormone-refractory prostate cancer

Prostate cancer is the most frequently diagnosed non-skin cancer and second leading cause of cancer deaths in American men (Jemal et al., 2009). Androgen, by activating the androgen receptor (AR), plays a pivotal role in prostate cancer cell proliferation and viability (Magi-Galluzzi et al., 1998). Hence, androgen ablation has been frontline therapy for the treatment of advanced prostate cancer. However, androgen-deprivation therapy (ADT) is only palliative and most patients receiving ADT eventually succumb to castration-resistant prostate cancer (CRPC) that is also resistant to chemotherapy (Feldman and Feldman, 2001). Furthermore, whether normal or mutated, AR continues to play an important role in progression of CRPC (Feldman and Feldman, 2001; van der Kwast et al., 1991) and is required for proliferation and survival of both androgen-sensitive as well as androgen-independent AR-positive prostate cancer cells (Haag et al., 2005; Snoek et al.,

2009; Yuan et al., 2006; Zegarra-Moro et al., 2002). Therefore, developing a curative strategy for the treatment of hormone-refractory disease requires identifying effective means to eliminate AR protein in prostate cancer cells (Reddy et al., 2006).

Overexpression of AR is reported to be involved in the progression to CRPC (Chen et al., 2004). Besides AR gene amplification (Ford et al., 2003; Visakorpi et al., 1995), an increased transcription or stabilization of AR mRNA [reviewed in reference (Burnstein, 2005)] may contribute to increased AR protein levels. However, the steady-state level of a protein is determined not only by the rate of its synthesis but also by the rate of its degradation. A number of proteases including serine proteases (de Boer et al., 1987; Gregory et al., 2001; Wilson and French, 1979), thiol proteases caspase (Kobayashi et al., 1998; LaFevre-Bernt and Ellerby, 2003) and calpain (Libertini et al., 2007; Pelley et al., 2006; Yang et al., 2008), and threonine protease the ubiquitin-proteasome system (Lin et al., 2002b; Sheflin et al., 2000) have all been reported to either cleave AR to generate proteolytic fragments of specific functional domains or degrade AR altogether. Interestingly, some of the proteases, such as ubiquitin-proteasome system and calpain, are reported to not only degrade AR but also regulate AR activity. For example, ubiquitin-proteasome system implicated in proteolytic breakdown of AR (Lin et al., 2002b; Litvinov et al., 2006; Sheflin et al., 2000) is reported to regulate nuclear translocation of AR and its interaction with co-regulators (Lin et al., 2002a) and involve in the assembly of AR transcription complexes (Kang et al., 2002). Similarly, calpain implicated in breakdown of AR (Pelley et al., 2006; Yang et al., 2008) is reported to generate a low molecular weight form of AR that is constitutively active (Chen et al., 2010; Libertini et al., 2007). Thus, specific proteases involved in AR turnover and regulatory events contributing to diverse roles of proteases in AR degradation and activation in prostate cancer cells remains obscure.

We previously reported that calmodulin (CaM), a major Ca^{++} -receptor protein, binds to AR and anti-CaM drugs inhibit AR transcriptional activity in prostate cancer cells (Cifuentes et al., 2004). Several CaM-binding proteins are known to contain PEST sequences (Barnes and Gomes, 1995) and PESTFind, a web-based algorithm, identified a strong PEST sequence located within the hydrophilic hinge region of AR (Fig. S1). PEST motifs are believed to be putative intramolecular signals for proteolytic degradation (Dice, 1987). Calpain is one of the proteases that interact with PEST sequences (Molinari et al., 1995; Shumway et al., 1999) and hydrolyze PEST containing proteins (Carafoli and Molinari, 1998). Calpain is a cytosolic Ca^{++} -activated neutral protease that is known to play a crucial role in various physiological and pathological processes (Goll et al., 2003; Schollmeyer, 1988) and, interestingly, virtually all CaM-binding proteins are known to be calpain targets for proteolysis (Chen and Mallampalli, 2007; Wang et al., 1989). Thus, the PEST sequence (Fig. S1), along with putative CaM-binding motifs in AR (Cifuentes et al., 2004; Pelley et al., 2006), make AR a potential target for calpain-mediated proteolysis. Consistent with this possibility, we observed that Ca^{++} or calcimycin, which is known to activate calpain (Gil-Parrado et al., 2002; Khorchid and Ikura, 2002), cause AR breakdown (Pelley et al., 2006). We have also shown that AR breakdown, in prostate cancer cells treated with a variety of chemotherapeutic agents, including 26S proteasome inhibitors, is attenuated by calpain-inhibitors (Yang et al., 2008). Although these observations implicate calpain in proteolysis of AR, the role of CaM in AR turnover in prostate cancer cells has not been studied.

It has been suggested that CaM binding to its partners may induce conformational changes that protect cleavage sites from calpain attack (Wang et al., 1989), or decrease the rate of proteolysis by calpain (Seubert et al., 1987). Based on these observations, we tested the possibility that AR-bound CaM may play an important role in regulation of AR stability. Our studies demonstrate for first time that the inhibition or knockdown of CaM sensitizes AR to calcimycin-stimulated proteolysis in androgen-sensitive and androgen-independent

prostate cancer cells, and using purified proteins *in-silico* we show that AR is a proteolytic target of calpain, and CaM indeed protects AR from calpain-mediated breakdown.

EXPERIMENTAL PROCEDURES

Cell Culture

LNCaP cells, C4-2B cells and CWR22Rv1 cells were grown in RPMI-1640 medium, and PC-3 cells were grown in DMEM medium. Both media were supplemented with 10% fetal calf serum, 2.5 mM glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin. Cell cultures were maintained in a humidified incubator with 5% CO₂ and 95% air at 37° C. When indicated, exponentially growing cells were treated with calcimycin (Sigma) in the presence or absence of anti-CaM drugs N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), N-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride (W-5), or 10-[3-(4-methylpiperazine-1-yl) propyl]-2-(trifluoromethyl)10H-phenothiazine (trifluoperazine, TFP) for 24 hours. W-7 and W-5 were purchased from Alexis Biochemicals and TFP was from MP Biochemicals.

Cell extracts and Western blot analysis

Cells scraped with a rubber policeman into media were washed once with, and suspended in, Buffer A (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 50 mM NaF, and 0.1 mM Na₃VO₄) supplemented with protease inhibitor mixture (P-8340, Sigma) at $5-8 \times 10^6$ cells/ml. Cells were then subjected twice to 30 pulses of sonication with a Branson Sonifier 250 set at output control of 2 and a duty cycle of 20, with intermittent cooling on ice. The sonicated cell extract was cleared by centrifugation in an Eppendorf centrifuge at 12,500 rpm for 1 min. Protein concentration in cell extracts was assessed with the MicroBCA protein assay kit (Pierce). Equal amounts of protein in cell extracts or purified proteins in Laemmli Sample Buffer were subjected to western blot analysis using rabbit polyclonal antibody (pAb) (N20) against human AR (Santa Cruz Biotechnology), mAb against PARP (Santa Cruz Biotechnology), which reacts with both the intact 112 kDa as well as the cleaved 85 kDa forms of PARP, goat pAb against β -actin (Santa Cruz Biotechnology), and mouse monoclonal antibodies against calpain (Calbiochem). Immunoreactive bands were developed by using horseradish peroxidase-conjugated secondary antibodies and SuperSignal West Pico chemiluminescent substrate (Pierce) and visualized by using X-ray film.

Measurement of intracellular free calcium

Exponentially growing LNCaP cells were harvested in 10 mM HEPES-buffered 0.9% saline, pH 7.4, containing 0.05% EDTA, washed once in Buffer B [5.4 mM KCl, 137 mM NaCl, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 0.34 mM Na₂HPO₄, 1mM MgCl₂, 2 mM CaCl₂, 5 mM HEPES (pH 7.4), 11 mM D-glucose, and 0.1% bovine serum albumin (BSA)] then loaded with 3 µM FURA-2AM (Molecular Probes) as described by Savino et al (Savino et al., 2006). After 30 min at 37°C, cells were washed twice in Buffer A. FURA-2AM fluorescence was recorded continuously at 25°C in a spectrofluorometer (Photon Technology International, Inc., Birmingham, NJ) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm. After establishing the baseline, calcimycin, W-7, or trifluoperazine was added, and the increase in Ca⁺⁺ levels (as indicated by the increase in 340/380 nm fluorescence ratio) in 1×10^6 cells was monitored (Savino et al., 2006).

CaM siRNA transfection

Exponentially growing LNCaP cells were transfected with 150 nM scramble (control) or CaM-siRNAs (Santa Cruz Biotechnology) using lipofectamine (Invitrogen) following manufacturer's instructions.

RT-PCR Analysis

Total RNA was prepared as described (Bai et al., 2005). RNA was reverse transcribed using random hexamers or oligo (dT) primer and Transcriptor Reverse Transcriptase (Roche Applied Science) according to the manufacturer's instructions. PCR of cDNA was carried out using Platinum PCR SuperMix (Invitrogen). PCR primers for AR were 5'-tcagttcacttttgacctgctaa (forward) and 5'-gtggaaatagatgggcttga (reverse), and for GAPDH were 5'-gagatccctccaaatcaagt (forward) and 5'-ccttccacgatacacaagtgt (reverse). Cycle parameters were 94°C for 2 min, 94°C for 30 sec, 55°C for 30 sec and 68°C for 1 min. AR was amplified for 30 cycles and GAPDH for 25 cycles. PCR products were run on 2% agarose gels and quantitation was carried out by digital analysis of band intensity in the gel with an Eagle Eye II Still Video system, using the EagleSight software (version 3.2; Stratagene, La Jolla, CA).

In-vitro AR breakdown assays

Purified recombinant human androgen receptor (rhAR) was used as substrate for *in vitro* AR degradation assays. rhAR was purified from His-tagged wild type hAR expressing baculovirus (BV_AR10, Magene Life Sciences Pvt. Ltd., Hyderabad, India) infected High Five insect cells using a Ni-NTA column (Invitrogen). For studies with PC-3 cell extracts, 1–2 ng purified rhAR was incubated with PC-3 cell extract in buffer A (100 µg protein) in the presence of various concentrations of CaCl₂ at room temperature for 1 hour. The reactions were terminated by adding 5X Laemmli Sample Buffer and boiling for 5 min. Residual AR in each sample was assessed by Western blot analysis.

In studies with purified proteins, 1 ng rhAR was incubated with 0.9 units calpain (Calbiochem) in the presence or absence of 1 ng CaM in 25 µl of calpain buffer (20 mM Tris, pH 7.5, 2 mM dithiothreitol, 1% Tween-20 and 0.015% Triton X-100) supplemented with 1 mg/ml bovine serum albumin (BSA). Incubation was terminated by adding 10 µl Laemmli Sample Buffer, and rhAR, calpain, and CaM were assessed by western blot analysis.

RESULTS

Effect of anti-CaM drugs and calcium on AR stability in prostate cancer cells

We reported previously that CaM binds to AR and that treating LNCaP cells with the anti-CaM drug W-7 inhibits proliferation and suppresses the expression of the AR-target gene prostate specific antigen (PSA) (Cifuentes et al., 2004). We investigated whether these inhibitory effects were due to a decrease in AR. We observed that both W-7 and another CaM inhibitor trifluoperazine (TFP) caused a dose-dependent decrease in AR protein levels (Fig. 1A). It is reported that W-7 is a highly specific inhibitor of CaM (Hidaka et al., 1981); nevertheless, to further confirm a CaM role in AR stability, we also tested the effect of a less active analog of W-7, W5 on AR protein levels in LNCaP cells. As shown in Fig. 1A, whereas both TFP and W-7 caused a dose-dependent decrease in AR levels, W-5 had no noticeable effect on AR. These data demonstrate an important role of CaM in maintaining AR protein levels in LNCaP cells.

We reported previously that treatment of LNCaP cells with 20 µM calcimycin, a Ca⁺⁺ ionophore that activates calpain by increasing Ca⁺⁺ (Gil-Parrado et al., 2002; Khorchid and

Ikura, 2002), led to calpain-mediated AR degradation (Pelley et al., 2006). Fig. 1B shows a dose-dependent effect of calcimycin on AR breakdown in LNCaP cells. Since CaM inhibitor and calcimycin both decreased AR levels, we wondered whether the effect of CaM inhibitors on AR degradation was due to an increase in intracellular Ca^{++} , which activated calpain and led to calpain-mediated AR breakdown. Therefore, we tested the effect of W-7, TFP, and calcimycin on intracellular Ca^{++} levels by using a FURA-2 fluorescence assay. As shown in Fig. 1C, 0.6 μM calcimycin or 30 μM TFP caused a significant increase in intracellular Ca^{++} . Interestingly, TFP led to an increase in Ca^{++} ; this suggests that the effect of TFP on AR protein may be due to a dual effect of TFP on CaM inhibition and increase in Ca^{++} . By contrast, W-7, even at 60 μM concentration, had no effect on intracellular Ca^{++} levels (Fig. 1C), indicating that the effect of W-7 on AR protein was not due to increase in Ca^{++} . We inferred from these observations that the effect of W-7 on AR breakdown involved basal activity of calpain, and we hypothesized that further activation of calpain (e.g., by calcimycin) would enhance AR breakdown.

CaM inhibitor sensitizes AR to calcimycin-stimulated breakdown in prostate cancer cells

To test the hypothesis that CaM inhibition sensitizes AR to calpain, we compared the effect of W-7 on AR breakdown in cells treated with and without calcimycin. We chose concentrations of CaM inhibitor and calcimycin that each alone had only a modest effect on AR levels. Accordingly, as shown in Fig. 2, 60 μM W-7 or 0.4 μM calcimycin alone had a modest effect on the AR protein level. However, combined treatment with 60 μM W-7 and either 0.2 μM or 0.4 μM calcimycin caused a dramatic decrease in AR protein in androgen-sensitive LNCaP cells (Fig. 2A) as well as in androgen-independent C4-2B cells (Fig. 2B). A similarly dramatic decrease in AR protein was also observed when 15 or 30 μM TFP was combined with 0.2 – 0.4 μM calcimycin in LNCaP and C4-2B cells (Fig. S2, Supplemental Information). Interestingly, the decrease in AR protein levels in these cells was associated with induction of PARP cleavage (PARP activation) (Figs. 2A and 2B, and Figs. S2A and S2B) and changes in cellular morphology reminiscent of apoptotic cells (Fig. S3, Supplemental Information).

It has been reported that an ~80 kDa isoform of AR in CWR22Rv1 and VCaP cells and in human prostate tumor tissues represents AR splice variants generated by splicing of novel cryptic exons in intron 2 or intron 3 of AR, and that these splice variants may be responsible for hormone-refractory growth of prostate cancer cells, since they encode an AR that is missing the ligand-binding domain (LBD) and is constitutively active (Dehm et al., 2008; Hu et al., 2009). We investigated whether anti-CaM drugs sensitize the ~80 kDa AR isoform in CWR22Rv1 cells to calcimycin-stimulated breakdown. As shown in Fig. 2C, both the ~80 kDa isoform and 114 kDa full-length AR in CWR22Rv1 cells were equally affected by CaM inhibitors, W-7 or TFP, and Ca^{++} -ionophore calcimycin. Thus, CaM inhibitors sensitized both full-length AR and variant AR (~80 kDa) to calcimycin-stimulated breakdown in prostate cancer cells. The AR breakdown in CWR22Rv1 cells, as in LNCaP and C4-2B cells, was associated with PARP cleavage (Fig. 2C).

In order to quantitate the impact of CaM inhibition on AR stability, we estimated the half-life of AR in LNCaP cells treated with CaM inhibitor in the absence or presence of calcimycin. As shown in Fig. 3A, 0.4 μM calcimycin or 60 μM W-7 caused a time-dependent decrease in AR protein levels. The half-life of AR in the presence of W-7 alone or calcimycin alone was about 20 hours (Fig. 3B). However, in cells treated with W-7 plus calcimycin there was a precipitous decrease in AR levels (Fig. 3A) and the half-life of AR was about 8 hours (Fig. 3B). A similar observation was made in cells treated with TFP and calcimycin (Fig. S4, Supplemental Information). Thus, CaM inhibition resulted in a 2- to 3-fold decrease in AR stability.

CaM knockdown sensitizes AR to calcimycin-stimulated breakdown

To confirm that the sensitizing effect of W-7 or TFP on calcimycin-stimulated breakdown of AR was specifically due to CaM inactivation, we used CaM-siRNA to decrease CaM levels. As shown in Fig. 4A, CaM mRNA as well as protein levels decreased significantly in CaM-siRNA treated LNCaP cells as compared to controls, and CaM knockdown sensitized AR protein to calcimycin-stimulated breakdown, with no change in AR mRNA. These observations further support the conclusion that the effect of CaM inhibitors on AR breakdown in calcimycin treated cells was specifically due to CaM inactivation.

Down regulation of AR in CaM-inhibitor and calcimycin treated cells is a post-translational event

We investigated whether the effect of CaM inhibitor and calcimycin on AR protein levels was due to suppression of AR mRNA expression. As shown in Fig. 4B, neither 60 μ M W-7 nor 0.4 μ M calcimycin inhibited AR mRNA levels in LNCaP or C4-2B cells. These observations demonstrate that the down regulation of AR by CaM inhibitor and calcimycin is a post-translational event caused by proteolytic breakdown.

CaM inhibitors sensitize AR to Ca^{++} -stimulated breakdown in cell-free extracts

We considered the possibility that CaM inhibition and/or an ionophore-mediated increase in intracellular Ca^{++} in LNCaP, C4-2B, and CWR22Rv1 cells might interfere with signaling pathways that control AR stability and thereby indirectly down regulate AR protein levels. To evaluate such a possibility, we used a cell-free system in which we incubated purified recombinant human wild type AR (rhAR) with AR-negative PC-3 cell extracts in the presence of Ca^{++} and/or CaM inhibitors. As shown in Fig. 5A, rhAR added to PC-3 cell extracts was readily degraded in the presence of 2 mM CaCl_2 , but not in the presence of 5 mM EGTA. In order to investigate the combined effect of Ca^{++} and CaM-inhibitor, we used a lower concentration of Ca^{++} (0.5 mM) to minimize the effect of Ca^{++} on rhAR degradation (Fig. 5B). We observed that W-7 or TFP alone had very little effect on rhAR (Fig. 5B). However, there was a dramatic decrease in rhAR when CaM inhibitors were combined with 0.5 mM CaCl_2 . The less active analogue of W-7, W-5, did not have this effect (Fig. 5B). These observations indicate that AR degradation caused by CaM inhibitors and calcimycin does not involve intracellular signaling.

Calcimycin-stimulated breakdown of AR is due to calpain activation

Based on our earlier studies with specific protease inhibitors, calcimycin- and Ca^{++} -stimulated breakdown of AR in intact cells and cell-free extracts appears to be due to calpain, not caspase-3 or 26S proteasome (Pelley et al., 2006; Yang et al., 2008). Since significant breakdown of AR in LNCaP cell extracts occurred only when the Ca^{++} concentration was above 0.5 mM (Pelley et al., 2006), and since this concentration of Ca^{++} is required for the activation of m-calpain (calpain-2) (Goll et al., 2003), we reasoned that activation of calpain-2, rather than μ -calpain (calpain-1), caused AR breakdown in prostate cancer cells. To test this, we treated purified rhAR with purified calpain-2 in the presence of increasing concentrations of CaCl_2 . As shown in Fig. 6A (top panel), we observed a significant decrease in the rhAR level at Ca^{++} concentrations that activate calpain-2 (Goll et al., 2003). Calpain-2 activation at 0.32 to 0.62 mM Ca^{++} was evident from the autolytic cleavage of calpain to the 18 kDa fragment (Crawford et al., 1987; Saido et al., 1994) (Fig. 7A, bottom panel). Thus, it is likely that calpain-2 is the protease that is activated by the calcimycin-mediated increase in intracellular Ca^{++} levels (Fig. 1C) and responsible for AR breakdown in prostate cancer cells.

CaM protects AR from calpain-mediated breakdown

The sensitizing effect of CaM inhibitor on calcimycin-stimulated AR degradation in intact cells, and of CaM inhibitor on Ca^{++} -stimulated AR breakdown in cell-free extracts, raises the intriguing possibility that CaM, by binding to AR (Cifuentes et al., 2004; Pelley et al., 2006), may protect AR from calpain-mediated breakdown and that disruption of the CaM-AR interaction renders AR susceptible to proteolytic breakdown. We tested this possibility by treating purified rhAR with activated calpain in the presence and absence of CaM in a test tube. As shown in Fig. 6B, calpain-mediated breakdown of AR was attenuated in the presence of CaM. This effect was not due to inhibition of calpain activity since calpain was equally active, as indicated by the autolytic cleavage of calpain to the 18 kDa fragment, both in the presence and absence of CaM (Fig. 6B, bottom panel). Thus, studies with purified proteins demonstrate that CaM can protect AR from calpain-mediated proteolytic breakdown.

DISCUSSION

These studies demonstrate for the first time that CaM protects AR from proteolytic breakdown in prostate cancer cells and provide a direct evidence for calpain involvement in AR turnover. CaM inactivation together with calpain activation caused a precipitous decrease in AR protein levels leading to PARP cleavage, a marker of apoptosis, in both androgen-sensitive (LNCaP) and androgen-independent (C4-2B and CWR22Rv1) prostate cancer cells. Thus, anti-CaM drugs in combination with calpain-activating agents may offer a curative strategy for the treatment of prostate cancer, which relies on AR for growth and progression. Importantly, AR-bound CaM thus represents a novel target to get rid of AR without relying on ligand binding domain (LBD), which may or may not present in the AR of hormone-refractory prostate cancer cells (Dehm et al., 2008; Guo et al., 2009; Hu et al., 2009), to suppress AR activity.

W-7 and TFP have been used extensively to study the role of CaM (Hidaka et al., 1981; Osborn and Weber, 1980). These drugs bind to CaM and inhibit binding of CaM to its binding partner (Craven et al., 1996; Hidaka et al., 1981; Osawa et al., 1998). Compared to W-7, W-5 has a 6- to 7- fold lower affinity for binding to CaM and, therefore, is a very poor inhibitor of CaM (Hidaka et al., 1981; Ito and Hidaka, 1983). Accordingly, W-7 or TFP caused a dose-dependent decrease in AR protein levels in LNCaP cells, but W-5 had very little effect (Fig. 1), indicating that AR breakdown in the presence of anti-CaM drugs was specifically due to CaM inactivation. This was further corroborated by our observation that siRNA-induced knockdown of CaM sensitized AR to proteolytic breakdown (Fig. 4A). In comparison to W-7, TFP caused a significant increase in intracellular Ca^{++} (Fig. 1C). Thus, TFP not only inhibits CaM (Osborn and Weber, 1980), but also increases intracellular Ca^{++} , which leads to the activation of calpain (Khorchid and Ikura, 2002). This dual effect may account for the higher efficacy of TFP (Fig. S2), as compared to W-7 (Fig. 2), in causing AR breakdown in androgen sensitive (LNCaP) and androgen-independent (C4-2B and CWR22Rv1) cells.

Calcium ionophores such as calcimycin (A23187) and ionomycin are known to activate ubiquitous calpains by increasing intracellular Ca^{++} levels (Gil-Parrado et al., 2002; Molinari et al., 1995). Calcimycin stimulated intracellular Ca^{++} (Fig. 1C) and decreased AR protein levels (Fig. 1B). The concentration of calcimycin required for AR turnover in these studies was $<0.5 \mu\text{M}$ (Fig. 1B). At these concentrations there was no noticeable effect on AR mRNA levels (Fig. 4B). Consistent with these observations, Gong et al (Gong et al., 1995) also found no significant change in AR mRNA levels in LNCaP cells treated with $<0.5 \mu\text{M}$ A23187 (calcimycin). However, at higher concentrations ($1 \mu\text{M}$) calcimycin seems to decrease both AR mRNA and protein levels (Gong et al., 1995). The fact that AR protein

levels were decreased in cells treated with low concentrations of calcimycin that had no effect on AR mRNA suggests that the decrease in AR protein levels at these concentrations was a post-translational event involving proteolytic breakdown rather than the inhibition of AR mRNA expression.

Calpain has been implicated in the breakdown of AR protein in both cycling (Pelley et al., 2006) and apoptotic (Yang et al., 2008) prostate cancer cells. It has been suggested that calpain generates a truncated ~80 kDa AR in LNCaP cells treated with 10 μ M calcimycin for 20 min (Libertini et al., 2007), and that the ~80 kDa AR generated by calpain is responsible for growth of hormone-refractory prostate cancer cells (Chen et al.,; Libertini et al., 2007; Tepper et al., 2002). Although an 80 kDa truncated AR that lacks the ligand-binding domain (LBD) is constitutively active in the absence of androgen (Jenster et al., 1991; Simental et al., 1991), and could therefore contribute to hormone-refractory prostate cancer cell proliferation, the ~80 kDa AR generated by 10 μ M calcimycin treatment was not demonstrated to represent a LBD-truncated AR and there is no evidence that calcimycin treatment leads to androgen-independent growth of LNCaP cells. By contrast, we observed that, in LNCaP cells treated with <0.5 μ M calcimycin for 24 hours, AR undergoes proteolysis without any noticeable accumulation of intermediary proteolytic products (Fig. 1B). Besides full-length AR (114 kDa), the only other peptide that was detected by anti-AR(N20) antibodies was ~76 kDa, which was present in 0.1 – 0.2 μ M calcimycin treated cells as well as in vehicle treated control cells (Fig. 1B). Thus the generation of ~76 kDa peptide does not depend on calpain activation in LNCaP cells. Moreover, not only did calcimycin-treatment not generate any intermediary proteolytic products of AR, but it also caused proteolytic breakdown of the low molecular weight AR that is present in hormone-refractory CWR22Rv1 prostate cancer cells (Fig. 2C). In addition, purified calpain failed to generate any intermediary proteolytic products from purified full-length rhAR (Fig. 6). Therefore, we conclude that calpain does not generate AR proteolytic product(s) that could contribute to growth of hormone-refractory prostate cancer cells. Actually, cloning and sequencing analysis has revealed that low molecular weight AR isoform(s) are expressed from splice variants that are generated by the splicing of novel cryptic exons in intron 2 or intron 3 of AR mRNA, and that lead to premature translation termination and the generation of truncated AR that lacks the LBD in hormone-refractory prostate cancer cells (Dehm et al., 2008; Hu et al., 2009).

Observations of accumulation of ubiquitinated AR in cells treated with the 26S proteasome inhibitor, MG132, have been inferred to implicate a role of the ubiquitin/proteasomal pathway in AR degradation (Lin et al., 2002b; Sheflin et al., 2000). However, AR protein levels actually decrease in prostate cancer cells treated with 26S proteasome inhibitors (Yang et al., 2008). For instance, the proteasome inhibitor PS-341 not only decreases basal AR protein levels, but also fails to prevent the decrease in AR protein levels elicited by the antiestrogen, fulvestrant, in LNCaP cells (Bhattacharyya et al., 2006). Furthermore, heat shock treatment, which impairs 26S proteasome function, dramatically decreases AR protein levels in LNCaP cells (Pajonk et al., 2005). Proteasome inhibition has been reported to activate calpain (Li et al.). Consistent with this, we observed that proteasome inhibitor-induced suppression of AR protein levels is due to calpain activation in prostate cancer cells (Yang et al., 2008). Interestingly, 26S proteasome has been suggested to play a role in the regulation of AR nuclear translocation and AR interaction with coregulators (Lin et al., 2002a), and in the dynamic assembly of the AR transcription complex (Kang et al., 2002). Therefore, the ubiquitin/proteasome system may regulate AR through mechanisms that may not involve receptor turnover.

In summary, as depicted in Fig. 7, our observations suggest that CaM, by binding to AR, protects AR from proteolysis by calpain in prostate cancer cells. In the presence of CaM

inhibitors W-7 or TFP, AR-bound CaM is displaced and AR becomes susceptible to degradation by calpain. A similar model of a role of CaM in protecting other CaM-binding proteins from calpain-mediated proteolysis has been proposed (Chen and Mallampalli, 2007; Wang et al., 1989). In this scenario, we anticipate that agents that disrupt CaM-AR interaction offer a curative strategy for the treatment of prostate cancer by causing AR degradation. Unlike the currently available therapies, this treatment strategy does not rely on targeting the ligand-binding domain (LBD), which is reported to be lost in the AR of castration-resistant prostate cancers (Dehm et al., 2008; Guo et al., 2009; Hu et al., 2009), to suppress AR activity. Therefore, in the future, identification of CaM-binding sequence in AR should lead to the development of novel therapeutic agents that can selectively disrupt CaM-AR interaction and render AR susceptible to proteolytic breakdown, and suppress the growth of hormone-sensitive as well as hormone-refractory prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Effect of anti-CaM drugs and ionophore calcimycin on AR and intracellular Ca^{++} in LNCaP cells. Cell extracts, prepared from exponentially growing LNCaP cells that had been treated for 24 hours with increasing concentrations of trifluoperazine, W-7 or W-5 (A), and calcimycin or W-7 (B) were subjected to Western blot analysis of AR and β -actin levels. The data presented in A and B are representative of four independent experiments. C) Calcimycin (0.6 μ M), W-7 (60 μ M), or trifluoperazine (30 μ M) was added to LNCaP cell suspension and changes in intracellular calcium were measured using the fluorescent calcium indicator FURA-2AM as described in Materials and Methods. The data are representative of two independent experiments.

Figure 2.

Combined effect of anti-CaM drugs and calcimycin on AR levels and PARP cleavage in androgen-sensitive and androgen-independent prostate cancer cells. Exponentially growing cells were treated for 24 h with increasing concentrations of W-7 or TFP in the absence or presence of 0.2 or 0.4 μ M calcimycin. Cell extracts prepared from LNCaP (A), C4-2B (B), and CWR22Rv1 (C) cells were subjected to Western blot analysis of AR, PARP, and β -actin or GAPDH. The data are representative of four independent experiments with each cell line.

Figure 3.

Effect of W-7 and calcimycin on AR half-life in LNCaP cells. Exponentially growing LNCaP cells were treated with 0.4 μ M calcimycin (Cal), 60 μ M W-7, or calcimycin plus W-7, cell extracts were prepared at 6-hour intervals, and Western blot analysis of AR and GAPDH was performed (A). The optical density of the AR band was normalized to the optical density of the GAPDH band in the same sample, and plotted as a percentage of that at zero time (B); the mean of two separate gels for each experiment (n=4) is presented (B).

Figure 4.

Figure 4A: Effect of CaM knockdown on calcimycin-stimulated AR breakdown in LNCaP cells. Exponentially growing LNCaP cells were treated with CaM-siRNA (CaM) or scrambled siRNA (cont.) for 72 hours and then with 0.4 μ M calcimycin for an additional 24 hours. Cell extracts and total RNA were subjected to Western blot and RT-PCR analysis, respectively, to determine CaM, AR, and GAPDH levels.

Figure 4B: Effect of W-7 and calcimycin on AR mRNA in LNCaP and C4-2B cells.

Exponentially growing LNCaP and C4-2B cells were treated with 0.4 μ M calcimycin (Cal), 60 μ M W-7, or Calcimycin plus W-7 for 24 hours. Total RNA was subjected to RT-PCR analysis to determine AR and GAPDH mRNA levels. Band intensities of RT-PCR products resolved on agarose gels were determined and AR mRNA levels normalized to GAPDH mRNA levels are expressed relative to control treatment, set as 1.0. PCR reactions without reverse transcriptase (No RT) or without RNA (No RNA) were negative controls.

Figure 5.

Synergistic effect of anti-CaM drugs and Ca^{++} on breakdown of recombinant human AR (rhAR) added to a PC-3 cell extract. Purified rhAR was added to AR-negative PC-3 cell extracts and incubated in the absence (control) or presence of 5 mM EGTA or 2 mM CaCl_2 at room temperature for 1 hour (A). In a separate experiment (B), rhAR added to PC-3 cell extract was treated with 5 mM EGTA (Control) or 0.5 mM CaCl_2 (Ca^{++}), or with 50 μM W-5, W-7 or TFP in the presence or absence of 0.5 mM CaCl_2 for 1 hour at room temperature. At the end of the incubation, sample buffer was added and Western blot analysis was performed to detect AR and β -actin. Boiled Lys, cell extract was boiled before adding rhAR (A).

Figure 6.

Figure 6A: Ca^{++} -dependent calpain activation causes rhAR breakdown *in-vitro*. Purified rhAR and calpain were incubated at room temperature for 30 min in calpain buffer containing increasing concentrations of CaCl_2 . Reactions were terminated by adding sample buffer and equal aliquots of reaction mixture were subjected to western blot analysis using AR and calpain antibodies.

Figure 6B: CaM protects AR from calpain-mediated breakdown *in-vitro*. Purified rhAR was incubated with calpain (Cpn) in the presence (+) or absence (-) of CaM in a reaction mixture containing 0.5 mM CaCl_2 for 30 min at room temperature. Reactions were terminated by adding sample buffer and samples were then subjected to western blot analysis using AR, CaM and calpain antibodies. Bovine serum albumin (BSA) added to the reaction mixture was detected in the background on Western blots developed with calpain antibody.

Figure 7.

Model depicting protective role of CaM in calpain-mediated breakdown of AR. AR-bound CaM is hypothesized to prevent calpain from degrading AR. In the presence of anti-CaM drugs W-7 or TFP, CaM is displaced from AR, thereby allowing calpain to bind to and degrade AR. AR (green ribbon) has a putative PEST sequence (Fig. S1) (hatched area), which makes it a potential target for proteases such as calpain. The dotted region represents putative CaM or calpain-binding site.