Xenoestrogen Action in Prostate Cancer: Pleiotropic Effects Dependent on Androgen Receptor Status

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Abstract

Androgen is critical for prostate development, growth, and survival. Therapies for advanced prostate cancer aim to block androgen receptor (AR) action. However, recurrent tumors ultimately arise, which harbor restored AR activity. One mechanism of such reactivation occurs through AR mutations, rendering the receptor responsive to noncanonical ligands. We have shown previously that a known xenoestrogen, bisphenol A (BPA), activates a tumor-derived AR mutant (*T877A*), leading to androgen-independent prostate cancer cell proliferation. Here, we show that BPA cooperates with androgen to activate AR-T877A as shown by both reporter assays and increased levels of prostate-specific antigen expression. Further investigations using both yeast and mammalian model systems revealed that multiple AR alleles are responsive to BPA, thus expanding the potential influence of xenoestrogens on prostate cancer. Moreover, *in vitro* radioligand binding assay revealed that BPA alters 5α-dihydrotestosterone binding to AR-T877A likely through noncompetitive inhibition. We also show that higher concentrations of BPA block proliferation of AR-positive, androgen-dependent prostate adenocarcinoma cells (LNCaP and LAPC-4), with a more modest inhibitory effect on androgen-independent cells (22Rv-1). By contrast, AR-negative prostate cancer cells failed to show growth inhibition after exposure to high BPA dose. Together, these data show that BPA can serve as a potential "hormone sensitizer" of the mutant ARs present in advanced prostate adenocarcinomas, thereby possibly contributing toward therapeutic relapse in advanced prostate cancer patients and supporting the notion that nonsteroidal environmental compounds can alter the function of nuclear receptor complexes. (Cancer Res 2005; 65(1): 54-65)

Introduction

Prostate cancer is a major health challenge in most Western countries, with an estimated 230,110 new cases and ~30,000 cancer-related deaths (~6% of all cancer deaths) predicted in 2004 in the United States alone (1). Despite its high morbidity, the molecular etiology of advanced prostate cancer is still poorly understood. Although organ-confined tumors are potentially curable by surgical intervention (radical prostatectomy) and/or radiation therapy, treatment options are limited for the disseminated disease. At early stages, prostate tumors are dependent on androgen for growth. Therefore, androgen ablation therapy is the main line of treatment for such patients. Unfortunately, therapeutic efficacy is transient, as most patients relapse and develop therapy-resistant, metastatic, androgen-independent cancer (reviewed in refs. 2, 3). Although the events that regulate the molecular switch from an androgen-dependent to an androgen-independent state are incompletely understood, mounting evidence suggests that the growth of most recurrent prostate cancers is driven by an inappropriately activated androgen receptor (AR; refs. 4–6).

The AR is a ligand-activated transcription factor that plays a pivotal role in regulating function, growth, and differentiation of the prostate gland. In the absence of ligand, AR is held inactive in the cytoplasm by bound heat shock proteins. In the prostate, the critical ligand for the AR is 5α-dihydrotestosterone (DHT), the binding of which to the receptor triggers dissociation of inhibitory heat shock proteins and rapid translocation to the nucleus (reviewed in refs. 5, 7). Once in the nucleus, the AR complex binds to specific androgen response elements on target genes to stimulate transcription. For example, AR stimulates prostate-specific antigen (PSA) expression, which serves as clinical marker for prostate cancer progression (8). Once AR transcription is initiated, it leads to diverse biological outcomes, such as prostate gland secretion, differentiation, and growth (5–7).

At early stages of prostate cancer, AR activity can be effectively ablated by AR antagonists and/or inhibitors of androgen synthesis, whereas in the advanced disease AR becomes reactivated through multiple mechanisms, contributing to relapse of the disease and therapy bypass (reviewed in refs. 5, 6). *AR* gene amplification and a strong nuclear accumulation of the protein have been reported in up to 30% of recurrent prostate tumors (9, 10). Moreover, it has been recently shown that increased AR expression correlates with resistance to anti-androgen therapy (11). A second mechanism that contributes to ligand-independent AR reactivation is the stimulation of signal transduction pathways in response to cytokines and growth factors (12, 13). Thirdly, altered expression and function of AR coactivators have been detected in recurrent tumors (14–16). Lastly, it is known that up to 40% of androgen-independent prostate cancers harbor specific mutant ARs that are capable of evading normal growth regulation by androgens (17–20). Most such mutations map to the ligand binding domain of the AR, resulting in the broadening of ligand specificity, thus allowing activation of the receptor by noncanonical ligands such as nonandrogen steroids and anti-androgens (reviewed in ref. 6). As a result, AR has become a major target in the search for an effective treatment of an advanced prostate cancer treatment.

We showed previously that a common tumor-derived AR, AR-T877A allele, can be inappropriately activated by a nonsteroidal environmental estrogen, bisphenol A (BPA; ref. 21). BPA is

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produced at over 800 million kg/year in the United States and is used in the production of epoxy resins and polycarbonate plastics. These plastics are used in many food and drink packaging applications, whereas the resins are commonly used as interior coatings of food cans, milk containers, baby bottles, food storage vessels, and water supply pipes; BPA is also widely used in dentistry. Human environmental exposure to this compound can be significant, as BPA is detectable in water streams at relatively high concentrations, up to 12 μg/L (~50 nmol/L; ref. 22). Moreover, human exposure can arise from direct contact of food with BPA-containing plastics (23), and microgram quantities of BPA are present in certain canned foods (24–26). Recently, it was shown that BPA is detectable not only in adult serum (up to 6.5 nmol/L; ref. 27) but also in the amniotic fluid and fetal tissues (28, 29), indicating that exposure to this pervasive environmental compound may be persistent and significant.

It is well established from extensive *in vitro* and *in vivo* studies that BPA possesses estrogenic activity (reviewed in ref. 30). In addition, BPA directly affects prostate development in rodents (31); recently, it was shown that short-term, low-dose environmental exposure to this compound results in meiotic aneuploidy in mice (32). Although many studies have shown that BPA is an estrogen mimic, capable of binding and stimulating both estrogen receptors (ER) α and β, we reported previously that BPA is capable of inducing activity of the tumor-derived AR-T877A mutant, resulting in proliferation of androgen-dependent prostatic adenocarcinoma cells (LNCaP; ref. 21). Thus, our original findings implicated BPA exposure as another potential mechanism that could facilitate the transition of prostate tumors to therapeutic resistance.

In this study, we examined the effect of BPA on multiple tumor-derived AR mutants and analyzed the outcome of BPA exposure in the context of both early-stage and late-stage cancer models. We show that BPA can enhance transcriptional efficacy of androgen-activated AR-T877A and AR-H874Y in prostate cancer cells at low, environmentally relevant doses. Moreover, BPA activates multiple tumor-derived mutant AR alleles and moderately induces wild-type AR activity in prostate cancer cells. Lastly, we show that exposure to high BPA doses uncouples androgen-mediated transcription from AR-mediated mitogenesis in prostate carcinoma cells. These findings suggest that AR activity and subsequent prostate cancer progression to androgen independence can be influenced by patient exposure to estrogenic environmental compounds.

Materials and Methods

Reagents. DHT, 17β-estradiol, cholesterol, and BPA (4,4′-isopropylidenediphenol) were purchased from Sigma Chemical Co. (St. Louis, MO). Casodex (bicalutamide) was a generous gift from AstraZeneca Pharmaceuticals (London, United Kingdom). These compounds were dissolved in 100% ethanol at 10^{-2} mol/L concentration and stored at −20°C.

Cell Culture and Treatmentxs. LNCaP cells were obtained from American Type Culture Collection (Rockville, MD). 22Rv-1 cell line was the gift of Dr. J. Jacobberger (Case Western Reserve University, Cleveland, OH) and LAPC-4 cells were a gift of Dr. C. Sawyers (University of California at Los Angeles, Los Angeles, CA). LNCaP cells were maintained in IMEM (Cellgro, Mediatech, Herndon, VA) containing 5% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD) and LAPC-4 cells were cultured in Iscove's modified Dulbecco's medium (Cambrex, Walkersville, MD) containing 15% heat-inactivated fetal bovine serum. Both cell types were supplemented with 100 units/mL penicillin-streptomycin and 2 mmol/L L-glutamine (Mediatech, Herndon, VA). Cells were grown at 37°C in a 5% CO_2 humidified incubator. 22Rv-1 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, and 100 units/mL penicillin-streptomycin (Mediatech). For growth in steroid-free conditions, cells were seeded in phenol red–free IMEM (LNCaP) or DMEM (LAPC-4 and 22Rv-1) containing charcoal/dextran-treated fetal bovine serum (CDT serum, 5% for LNCaP cells and 10% for LAPC-4 and 22Rv-1 cells).

Plasmids. Plasmid encoding the LNCaP tumor-derived AR (*T877A*) was a gift of Dr. D. Feldman (Stanford University, Stanford, CA) and has been described previously (33). Plasmid encoding β-galactosidase was a gift of Dr. J.Y.J. Wang (University of California at San Diego, San Diego, CA). Generation of the probasin reporter plasmid, ARR2-LUC, was described in ref. 34. pcDNA3.1 vector was purchased from Invitrogen, Inc. (Carlsbad, CA).

Transfection and Reporter Assays. LNCaP cells were seeded 24 hours prior to transfections in IMEM supplemented with 5% CDT serum on poly-L-lysine-coated six-well dishes at a density of 3.5×10^5 cells per well. Cells were transfected using Lipofectin transfection reagent (Invitrogen), in accordance with the manufacturer's protocol, with ARR2-LUC reporter (1 μg), CMV-β-galactosidase (0.5 μg), and pcDNA3.1 (1.5 μg). 22Rv-1 cells were seeded in six-well dishes in DMEM supplemented with 10% CDT serum at a density of 2×10^5 cells per well. LAPC-4 cells were seeded in the same medium as 22Rv-1 cells on poly-L-lysine-coated six-well dishes at a density of 3.5×10^{-5} cells per well. On the following day, cells were transfected using FuGene6 transfection agent (Roche Molecular Biochemicals, Indianapolis, IN) as described in the manufacturer's protocol with ARR2-LUC (1 μg), CMV-β-galactosidase (0.5 μg), and pcDNA3.1 (1.5 μg). Post-transfection, the medium were replaced and supplemented with corresponding treatments for 48 hours. For experiments involving Casodex, cells (LNCaP, LAPC-4, and 22Rv-1) were pretreated with 10^{-6} mol/L Casodex for 4 hours prior to stimulation, as indicated, for 48 hours. Cells were then harvested and processed for luciferase activity according to the manufacturer's protocol (Promega, Madison, WI). β-galactosidase activity was used as an internal control for transfection efficiency and measured using Galacto-Star system (Tropix, Bedford, TX). Averages and SD from at least six independent samples are shown.

Immunoblotting. LNCaP cells (~8×10^5) in 10-cm dish were seeded in 5% CDT serum–containing IMEM supplemented with either vehicle (0.1% ethanol) or indicated drugs for 72 hours. Treatments were replenished every 48 hours. Post-treatment, cells were pelleted and whole cell lysates were prepared. Briefly, lysates were subjected to brief sonication and clarified by centrifugation. Equal protein concentrations (15 μg) were loaded and subjected to SDS-PAGE. Proteins were transferred to Immobilon membrane (Millipore Corp., Bedford, MA) and immunoblotted for AR, cyclin A, cyclin D1, $p27^{kip1}$, $p21^{cip1}$, CDK4, and β-tubulin (all antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA). Goat antirabbit (for detecting AR, cyclin A, cyclin D1, $p27^{kip1}$, $p21^{cip1}$, and CDK4) or goat anti-mouse (for detection of β-tubulin) horseradish peroxidase–conjugated secondary antibodies (Pierce, Inc., Rockford, IL) were used to visualize the antibody-antigen complex via enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Radioligand Binding Assay. Tumor-derived AR-T877A was generated using TnT-coupled *in vitro* transcription/translation rabbit reticulocyte system (Promega) in accordance with the manufacturer's protocol; 10 μL were used per binding reaction. Binding reactions were done in the total volume of 100 μL in binding buffer (10 mmol/L Tris-HCl, 1 mg/mL bovine serum albumin, 2 mmol/L DTT) containing indicated concentrations of $[^3H]$DHT (5α-dihydro[1α,2α(*n*)-^{3}H]testosterone, 51.0 Ci/mmol, 1.0 mCi/mL, Amersham Pharmacia Biotech, Piscataway, NJ). Competition analyses were performed in the presence of corresponding concentrations of $[^3H]$DHT and unlabeled competitors (either BPA, DHT, or cholesterol) at an excess of 1- to 10,000-fold as described (35). Binding or competition reactions were done overnight at 4°C. Following incubation, bound and free ligands were separated by hydroxyapatite (type 1, buffered aqueous suspension, Sigma Chemical) in which 200 μL of 50% hydroxyapatite slurry were added into each tube and samples were incubated on ice for 15 minutes, vortexing every 5 minutes. Hydroxyapatite pellets were washed thrice in 1 mL wash buffer (40 mmol/L Tris-HCl, 100 mmol/L KCl, 1 mmol/L EDTA, and 1 mmol/L EGTA). The pellets were resuspended in 200 μL of 95% ethanol and transferred to scintillation vials, at which point 5 mL of scintillation

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fluid were added (Budget-Solve, Fisher Scientific, Hampton, NH). The receptor-bound [^{3}H]DHT in each vial was measured in a Beckman LS-6500 scintillation counter. Specific binding was calculated by subtracting nonspecific binding obtained in the presence of 500-fold excess unlabeled DHT. Radioligand binding and dose-response competition data were graphed and analyzed using graphical and statistical program Prism (GraphPad Software, San Diego, CA).

Yeast Assay of AR Function. Yeast strains with the genotype *MAT***a**ade2-1 leu2-3, 112trp1-1his3-11, 15can1-100ura3-1 URA3 *3xARE::pCYC1::ADE2* expressing distinct AR alleles were maintained and grown as described previously (36). Briefly, single yeast colonies were grown for 3 days at 30°C in 3 mL minimal selective medium (Difco yeast nitrogen base without amino acids, Becton Dickinson, Franklin Lakes, NJ) containing 0.5% adenine, 1% histidine, and 1% leucine and supplemented with arginine, valine, phenylalanine, aspartic acid, isoleucine, serine, methionine, threonine, and glutamic acid. Yeast culture (~3 μL) was inoculated onto selection plates containing indicated concentrations of one of the ligands studied (either 10^{-8} mol/L DHT, 10^{-8} mol/L 17β-estradiol, or 10^{-3} mol/L BPA) and grown at 35°C for 3 days. Based on the color of the colony, the transactivational activity of AR was measured. White colonies indicate complete activity (+++), white-pink colonies indicate moderate activity (++), pink indicates partial AR activity (+), and red indicates lack of activity (−).

Quantitative Reverse Transcription-PCR. Total RNA was extracted from LNCaP cells treated with indicated drugs for 48 hours using Trizol reagent (Invitrogen, Inc., Carlsbad, CA). RNA (5 μg) was reverse transcribed using SuperScript II reverse transcriptase and random hexamers (Invitrogen) according to the manufacturer's protocol. PSA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used were described previously (21). PCR was done in a 50-μL volume containing 2 μL of corresponding cDNA, 50 ng of each primer set, 0.25 units Taq DNA polymerase (Promega), and 5 μCi [α-^{32}P]dCTP (Perkin-Elmer Life Sciences). PCR variables were 94°C for 2 minutes, 23 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds with a final extension at 72°C for 10 minutes. PCR products were separated on a 6% polyacrylamide gel and visualized and quantified a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). PSA expression was normalized to GAPDH and set to 1 in vehicle (0.1% ethanol)–treated cells. Averages and SDs are shown from multiple experiments with each condition tested at least in duplicate.

Bromodeoxyuridine Incorporation Assay. LNCaP and LAPC-4 cells were seeded in six-well dishes on poly-L-lysine-coated coverslips at a density of 3.5 × 10^5 cells per well in phenol red–free corresponding medium supplemented with either vehicle (0.1% ethanol), 10^{-9} DHT (LAPC-4 cells), 10^{-10} mol/L DHT (LNCaP cells), 10^{-5} mol/L BPA, and DHT with 10^{-5} mol/L BPA for 72 hours. PC-3 and DU-145 cells were seeded on glass coverslips in six-well dishes at a density of 2 × 10^5 cells per well and corresponding drugs were added for 72 hours. Following treatments, all cell types were labeled with Cell Proliferation labeling reagent (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Labeling continued for 16 hours and cells were then processed to detect bromodeoxyuridine (BrdUrd) via indirect immunofluorescence as described previously (37). Experiments were done at least twice in duplicate and ~300 cells per experiment were counted. Averages and SDs are shown.

Statistical Assessment. Quantitative results are expressed as means ± SD. Statistical analyses were performed using two-tailed Student's *t* test and GraphPad software. The criterion for statistical significance was $P < 0.05$.

Results

Bisphenol A Potentiates Ligand-Dependent AR Transactivation in Androgen-Dependent Prostate Cancer Cells. Previously, we have shown that nanomolar concentrations of an endocrine-disrupting compound, BPA, provide inappropriate mitogenic stimuli to prostatic adenocarcinoma cells (LNCaP) in the absence of androgens. Moreover, we have shown that BPA activates the AR-T877A tumor-derived mutant using both endogenous PSA expression and reporter assays (PSA promoter) as readout (21). To further characterize the mechanism and pharmacology of BPA action in a physiologically relevant environment, we examined transcriptional activity of AR in the presence of increasing concentrations of BPA with or without physiologic doses of DHT (10^{-10} to 10^{-9} mol/L). For these experiments, androgen-dependent LNCaP cells that express the tumor-derived mutant AR-T877A were cultured in medium devoid of steroids. The cells were cotransfected in the absence of steroids with plasmids encoding β-galactosidase and an AR-responsive probasin reporter plasmid (ARR2-LUC; described in ref. 34). After transfection, cells were stimulated with either vehicle alone (0.1% ethanol), 10^{-9} mol/L DHT, or increasing concentrations of BPA for 48 hours. As expected, 10^{-9} mol/L DHT served as a potent agonist of the endogenous AR in LNCaP cells (Fig. 1*A*), stimulating luciferase activity ~90-fold over basal levels. Treatment of LNCaP cells with 10^{-8} mol/L BPA also resulted in stimulation of AR-T877A activity (~15 fold), consistent with our previous findings (21). Interestingly, transcriptional activity of AR did not increase significantly with increasing concentrations of BPA (20.2 fold with 10^{-6} mol/L and 21.9 fold with 10^{-5} mol/L BPA). Thus, BPA activated AR with a maximal induction of ~20-fold over basal levels. In combination with our previous findings, these data also verify that BPA induces AR-T877A activity on multiple AR target genes (both PSA and probasin).

Previously, we examined the effects of BPA exposure on prostate cancer cells at castrate levels of androgens (such as in patients undergoing hormone therapy; ref. 21); however, because exposure to BPA would also be compounded with normal androgen levels (in untreated patients), we extended our studies on BPA exposure in the presence of physiologic concentrations of androgens (10^{-10} to 10^{-9} mol/L DHT). LNCaP cells were propagated in medium supplemented with CDT serum that contains growth factors but is devoid of steroids and supplemented with corresponding treatments for 48 hours post-transfection (Fig. 1*B*). Surprisingly, BPA significantly enhanced transcriptional efficacy of androgen-activated AR-T877A, as shown in Fig. 1*B*. Maximal potentiation of AR transactivation was achieved at a low (10^{-8} mol/L) dose of BPA: 90-fold (10^{-9} mol/L DHT alone) versus ~300-fold induction over basal levels. Higher (10^{-6} and 10^{-5} mol/L) doses of BPA also potentiated DHT-mediated AR transactivation: 200- and 131-fold increase over basal activity, respectively. These data indicate that BPA can act cooperatively with endogenous levels of androgen in mediating AR transactivation. Next, we examined whether BPA actions on the probasin reporter in LNCaP cells were dependent on the AR-T877A activity. For these experiments, we used a nonsteroidal pure AR antagonist, Casodex, which acts as a competitive inhibitor of ligand (38). As shown in Fig. 1*C*, Casodex effectively inhibited both DHT-induced AR activity (>80% reduction) and ability of BPA to transactivate AR (with ~60% reduction) in LNCaP cells. In addition, Casodex treatment blocked BPA-induced enhancement of DHT-mediated AR transactivation (>90% reduction in AR-T877A activity), confirming that BPA actions on the probasin reporter in LNCaP cells are dependent on the AR.

To examine the biological outcome of an increased AR activity, we monitored the expression of PSA gene, which is an endogenous AR target in LNCaP cells. For these studies, LNCaP cells were cultured as in Fig. 1 and treated with 10^{-10} mol/L DHT in the presence of increasing concentrations of BPA for 48 hours (Fig. 2). Cells were treated as indicated for 48 hours; then, RNA was subjected to reverse

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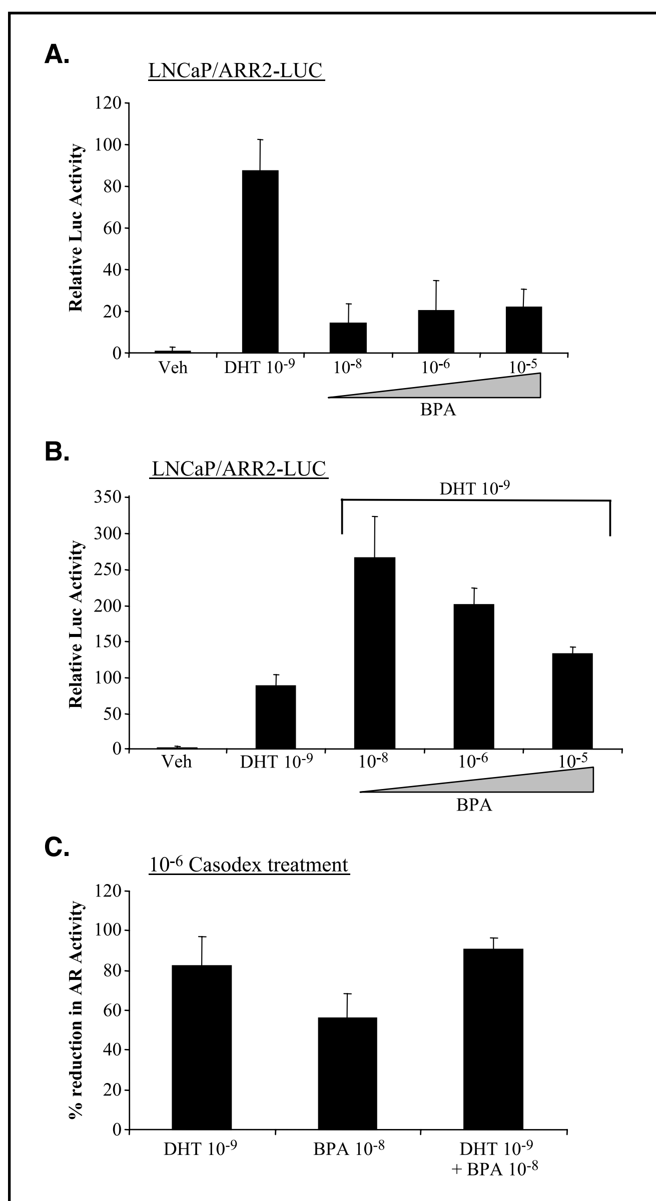

Figure 1. BPA activates endogenous AR-T877A and potentiates androgen-mediated AR transactivation. LNCaP cells were transfected in the absence of steroids with the probasin reporter (ARR2-LUC, 1.0 μg), CMV-β-galactosidase expression plasmid (0.5 μg), and an empty vector (pcDNA3.1, 1.5 μg). After transfection, cells were stimulated as indicated with increasing concentrations of BPA either in the absence *A,* or in the presence *B,* of 10^{-9} mol/L DHT for 48 hours. *Columns,* average relative luciferase activities from at least three independent experiments; *bars,* SD. *C,* cells were pretreated with 10^{-6} mol/L Casodex for 4 hours prior to the addition of corresponding treatments. Cells were stimulated for 48 hours and luciferase activity was measured. *Columns,* average percentage reduction in AR activity in the presence of Casodex compared with corresponding AR activity in the absence of the drug; *bars,* SD.

transcription and subsequent PCR analyses in the presence of [α-^{32}P]dCTP to quantify expression of both PSA and GAPDH. This method has been shown to allow accurate quantitative detection of changes in template levels over a large linear range (39). Representative data are shown in Fig. 2*A*. GAPDH mRNA levels remained constant throughout treatments and served as an internal control. Consistent with previous reports, little PSA was detected in the vehicle alone–treated cells (*lanes 1* and *5*), whereas both 10^{-10} mol/L DHT and 10^{-9} mol/L BPA induced PSA mRNA levels (*lanes 2* and *3*, respectively). Cell exposure to both DHT and BPA (either low, 10^{-9} mol/L, or a high, 10^{-5} mol/L, dose) resulted in a significant increase of PSA mRNA expression (compare *lanes 2* and *4* and *lanes 6* and *7*). Additionally, Casodex cotreatment of LNCaP cells stimulated with both DHT and 10^{-5} mol/L BPA resulted in decreased PSA levels (compare *lanes 7* and *9*). Similar results were observed with cells treated with DHT in the presence of BPA at different concentrations (10^{-9} and 10^{-6} mol/L; data not shown). LNCaP cells treated with DHT in the presence of Casodex served as a control (compare *lanes 6* and *8*). Data from multiple experiments were quantified and are shown in Fig. 2*B*. As shown, we observed at least 3-fold enhancement (when compared with DHT alone) of the endogenous PSA expression on exposure to both DHT (10^{-10} mol/L) and a range of BPA concentrations (10^{-9} and 10^{-5} mol/L). In addition, the use of a pure anti-androgen (10^{-6} mol/L Casodex) in cells treated with either DHT or DHT plus 10^{-5} mol/L BPA brought PSA mRNA expression almost to basal levels, confirming that cooperative action of BPA in LNCaP cells are AR dependent. To verify that BPA actions at different concentrations were dependent on the AR in LNCaP cells, cells were treated with either 10^{-9} or 10^{-5} mol/L BPA in the presence or absence of 10^{-6} mol/L Casodex, and reverse transcription-PCR analyses of PSA mRNA were done as in Fig. 2*A* and *B*. As shown in Fig. 2*C*, Casodex blocked both DHT-induced and BPA (10^{-9} mol/L)-induced PSA mRNA expression by ~80%, confirming our earlier findings (21). Similarly, Casodex treatment resulted in >80% reduction in PSA expression in LNCaP cells stimulated with 10^{-5} mol/L BPA. Together, these data show that BPA potentiates androgen-induced transcription of an endogenous AR target (PSA) in prostate carcinoma cells, and BPA action in this system is AR dependent.

It has been reported that both *in vitro* and *in vivo* exposure to certain phytoestrogens and environmental estrogens results in modulation of AR gene and/or protein expression levels (40–43). Therefore, we examined endogenous AR protein levels in LNCaP cells treated with increasing concentrations of BPA. For these experiments, cells were propagated in medium devoid of steroids and supplemented with either vehicle (ethanol) or increasing concentrations of either DHT or BPA for 72 hours. Cells were then harvested and processed for protein analyses as described in Materials and Methods. Detection of CDK4 served as a loading control. As shown in Fig. 2*D*, immunoblotting analyses showed that treatment of LNCaP cells with either vehicle (*lane 1*) or DHT at 10^{-9} or 10^{-5} mol/L concentrations did not affect AR expression (*lanes 2* and *3*, respectively). Similarly, treatment with either low (10^{-9} mol/L, *lane 4*) or high (10^{-5} mol/L, *lane 5*) doses of BPA did not affect AR protein levels in LNCaP cells, indicating that enhancement of androgen-mediated AR transactivational activity in the presence of BPA was not due to the changes in AR protein levels. Thus, our data indicate that BPA can enhance DHT-mediated activation of AR-T877A without affecting AR protein levels.

Bisphenol A Is a Noncompetitive Inhibitor of DHT Binding to AR-T877A. To probe the ability of BPA to modulate DHT binding to the AR-T877A, cell-free radioligand binding/competition assays were done. For these experiments, AR-T877A was *in vitro* transcribed/translated and incubated with [^{3}H]DHT in the presence or absence of indicated concentrations of cold competitors. Initially, [^{3}H]DHT saturation binding experiments were done to determine the DHT dissociation constant (K_d) for

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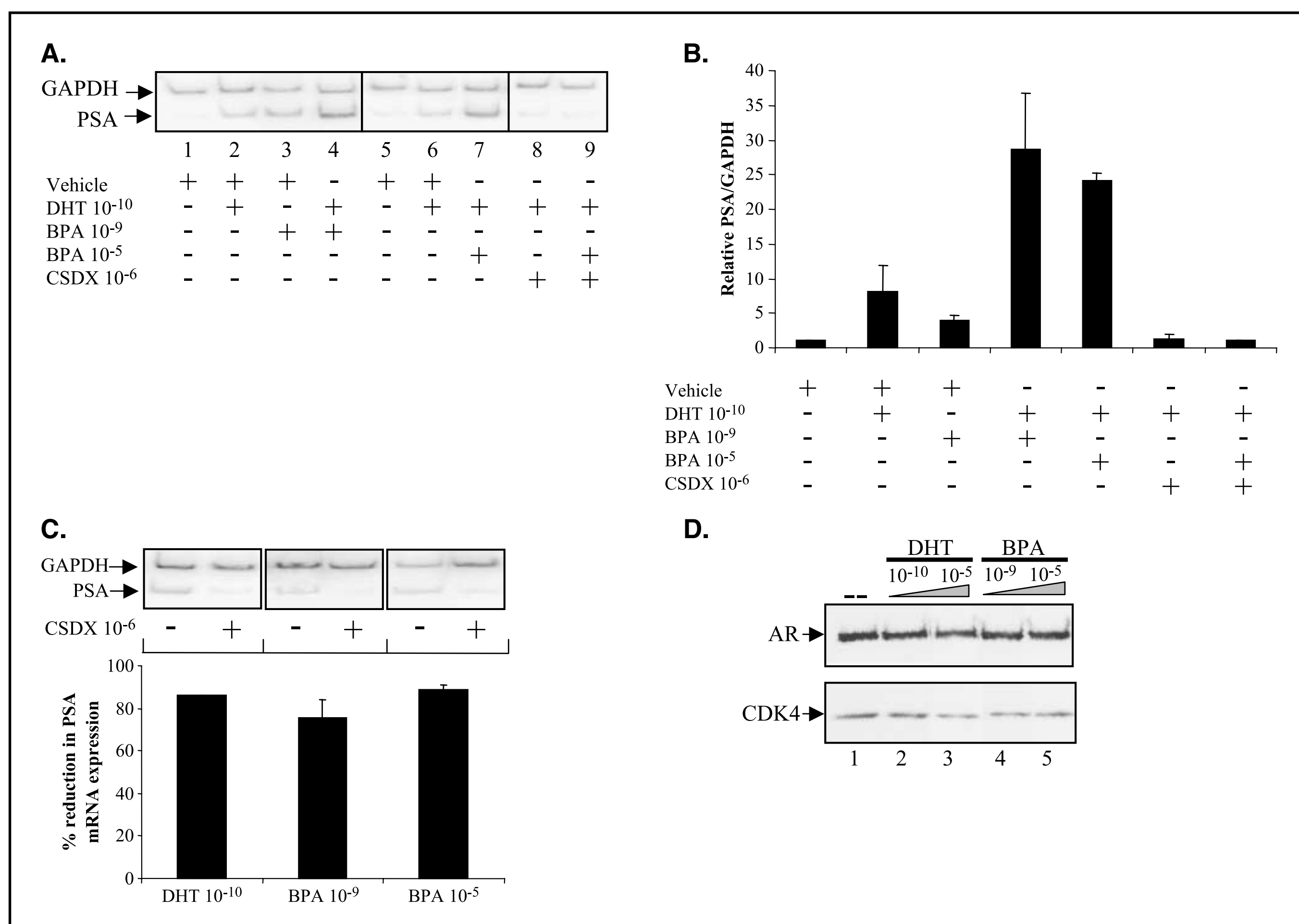

Figure 2. BPA potentiates ligand-dependent transactivation of endogenous AR targets in prostate carcinoma cells. *A,* LNCaP cells were treated as indicated for 48 hours and RNA was harvested and used for cDNA synthesis. Amplification of PSA and GAPDH was done in the presence of [α-^{32}P]dCTP. PCR products were separated on nondenaturing 6% PAGE and visualized on a PhosphorImager. *B,* PSA expression was normalized to GAPDH (internal control) and quantified using a PhosphorImager. Relative expression from at least two independent experiments. *Bars,* SD. *C,* LNCaP cells were pretreated with 10^{-6} mol/L Casodex (*CSDX*) for 4 hours before the addition of either 10^{-10} mol/L DHT, 10^{-9} mol/L BPA, or 10^{-5} mol/L BPA for 48 hours. Reverse transcription-PCR was done and PSA mRNA expression was quantified as in *A. Columns,* average percentage reduction in PSA mRNA expression in Casodex-treated samples. *D,* LNCaP cells were cultured in steroid-free medium and treated as indicated for 72 hours. Cells were harvested and subjected to SDS-PAGE followed by immunoblotting for AR and CDK4. Levels of CDK4 expression served as a loading control.

AR-T877A. In our system, DHT bound mutant AR-T877A with a K_d of 3×10^{-9} mol/L (Fig. 3*A*). These data agree with previously published results indicating that the K_d for AR-T877A is in the nanomolar range (44). In support of these findings, incubation of the receptor with 2.5×10^{-9} mol/L cold DHT resulted in ~50% inhibition of [^{3}H]DHT binding to AR-T877A (Fig. 3*B*). Because labeled [^{3}H]BPA is not commercially available, competition analyses were done in the presence of increasing concentrations of cold BPA for displacement of [^{3}H]DHT from the receptor. Cholesterol was used as a negative control. As shown in Fig. 3*B*, even high concentrations (up to 10^{-4} mol/L) of cholesterol did not affect [^{3}H]DHT binding to AR-T877A. In contrast, we found that 10^{-4} mol/L BPA inhibited [^{3}H]DHT binding to AR-T877A almost to basal levels (Fig. 3*B*), but the inhibition did not fit a simple, one-site competitive binding model, as a biphasic pattern of BPA inhibition is evident. To confirm that BPA inhibition is indeed noncompetitive, the same radioligand binding/competition experiments were done with a 10-fold higher concentration (20×10^{-9} mol/L) of the labeled [^{3}H]DHT. As shown in Fig. 3*B*, we observed a very similar, biphasic competition curve without a significant shift toTLBF the right, indicating that BPA inhibits androgen binding to AR-T877A in a noncompetitive manner.

Bisphenol A Mediates Transcriptional Activity of Multiple Tumor-Derived Mutant AR Proteins. We have shown that BPA enhances androgen-induced AR-T877A activity (Figs. 1 and 2) and that BPA noncompetitively alters DHT binding to AR-T877A (Fig. 3). To determine whether BPA activity is limited to this AR mutant, we examined the effect of BPA exposure on other tumor-derived AR mutant. First, a previously characterized yeast assay of AR function was used (36). We examined the effects of BPA on multiple tumor-derived AR mutant proteins in the yA(G)RE yeast strain that contains an integrated *ADE2* sequences as a reporter gene under the control of an *ARE* promoter and expresses distinct AR alleles. When the reporter *ADE2* gene is transactivated (adenine is produced as a result), it restores ADE2 expression, resulting in the formation of white or pink yeast colonies. The intensity of the color development depends on the extent of activation by the AR. Failure to transactivate the *ADE2* leads to the formation of red colonies.

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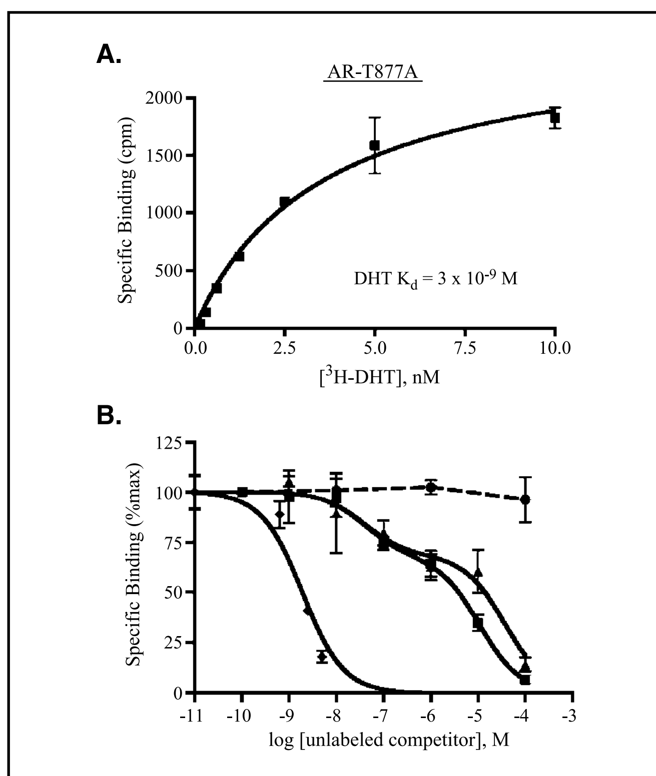

Figure 3. BPA inhibits androgen binding to AR-T877A in a noncompetitive manner. *A,* AR-T877A protein was produced *in vitro* and incubated with increasing concentrations of [^{3}H]DHT as indicated. Reactions were carried out for 16 hours at 4°C. Ligand-bound receptor and unbound ligands were separated using hydroxyapatite beads. Counts per minute were measured using the scintillation counter, and values were adjusted for nonspecific binding determined by adding 500-fold excess unlabeled DHT. Two independent experiments analyzed and graphed using Prism software with the following formula: binding = max × [[^{3}H]ligand / (K_d + [^{3}H]ligand)], where max = maximum possible binding. *B,* AR-T877A protein was produced *in vitro* as in *A* and incubated with 2 × 10^{-9} mol/L [^{3}H]DHT and increasing concentrations of either DHT (♦) or BPA (■). Cholesterol (*CHL*; ●) served as a negative control. Competition analyses in the presence of 20 × 10^{-9} mol/L [^{3}H]DHT and increasing concentrations of BPA (▲) are also shown. Three independent experiments analyzed as in *A.* DHT competition curve was fit to the formula: binding = [^{3}H]ligand × max / ([^{3}H]ligand + K_d [1 + (competitor)/K_i]), where max = maximum possible binding. BPA competition curves did not fit this simple, one-site competitive binding model.

We examined the effects of BPA on five tumor-derived mutant AR alleles and the wild-type AR in this system. The assays were done at least in triplicate and representative results are shown in Fig. 4*A*. Yeast colonies grown in the presence of the vehicle alone (ethanol) developed red color, indicating no AR transactivation. All functional mutant AR alleles (T877A, V715M, L701H, and K580R) and the wild-type were activated by 10^{-8} mol/L DHT and 10^{-8} mol/L estradiol, in agreement with previous results (36). As expected, the gain-of-function mutant AR-K580R, showed strongest activation by DHT. The nonfunctional AR-C784Y mutant (isolated from a patient with complete androgen insensitivity syndrome) served as a negative control. Strikingly, all examined functional AR mutants (*T877A*, *V715M*, *L701H*, and *K580R*) were activated by BPA, except the wild-type AR that failed to be transactivated by this estrogenic compound. These results show that an endocrine disruptor BPA is able to act on multiple tumor-derived AR alleles.

To verify these results in mammalian system, we examined BPA effects on endogenous AR transactivation in another prostatic adenocarcinoma cell line, 22Rv-1. These cells were derived from a human prostatic adenocarcinoma xenograft and express the mutant AR-H874Y allele (45). AR-H874Y activity was monitored using the probasin reporter (ARR2-LUC), activity of which was normalized to β-galactosidase levels. Following transfection, cells were stimulated with either vehicle (0.1% ethanol) or the indicated concentrations of BPA for 48 hours. As shown in Fig. 4*B*, 22Rv-1 cells transfected with the ARR2-LUC reporter gene and stimulated with BPA showed up to 10-fold induction (10^{-6} and 10^{-5} mol/L BPA) in AR-H874Y activity when compared with the vehicle. Interestingly, BPA also significantly enhanced DHT-mediated AR-H874Y transactivation at 10^{-6} mol/L concentration ($P < 0.05$), as shown in Fig. 4*C*. To verify that BPA action in 22Rv-1 cells is dependent on AR-H874Y activity, experiments were repeated in the presence of Casodex (Fig. 4*D*). As expected, Casodex treatment of 22Rv-1 cells stimulated with DHT alone resulted in ~60% reduction in AR-H874Y activity. Confirming that BPA action is dependent on AR-H874Y activity in 22Rv-1 cells, Casodex treatment reduced BPA-induced AR activity >60%, as shown in Fig. 4*D*. Moreover, we showed that Casodex inhibits the ability of BPA to enhance DHT-mediated transactivation (Fig. 4*D*). In addition, CV-1 cells (that express no endogenous AR or other nuclear receptors) transfected with plasmids encoding two additional mutant AR proteins (AR-T877S and AR-V715M) showed a modest increase (up to 3-fold) in AR activity when stimulated with 10^{-9} mol/L BPA (data not shown, but summarized in Fig. 4*E*). Together, these findings show that BPA exhibits partial agonist activity on multiple tumor-derived AR alleles and its actions are AR dependent.

Next, we expanded our studies to examine BPA effects on the wild-type AR. For these experiments, LAPC-4 cells were used. These cells were derived from an androgen-dependent prostate cancer xenograft and harbor wild-type AR (13). LAPC-4 cells were propagated in phenol red–free medium devoid of steroids and transfected with an AR-responsive probasin reporter gene (ARR2-LUC) as described in Figs. 1 and 4. As shown in Fig. 5*A*, LAPC-4 cells stimulated with 10^{-9} mol/L DHT showed ~130-fold increase in luciferase activity over basal levels (0.1% ethanol). Treatment with either 10^{-8} or 10^{-5} mol/L BPA resulted in only moderate (~2.5-fold) activation of the wild-type AR, indicating that BPA has only marginal effects on this receptor. Because we observed a significant enhancement of androgen-activated tumor-derived AR (AR-T877A and AR-H874Y) activity by BPA (Figs. 1 and 4), we also examined BPA effects on DHT-mediated activation of the wild-type AR. As shown in Fig. 5*A*, LAPC-4 cells treated with both DHT and 10^{-8} mol/L BPA failed to exhibit enhanced AR activity, demonstrating ~100-fold induction over basal levels compared with activity induced by DHT alone (~130-fold). Further analyses of BPA action on the wild-type AR in LAPC-4 cells by using Casodex revealed that the moderate BPA-induced activity was indeed mediated by the receptor (Fig. 5*B*). Casodex treatment reduced AR activation by >70%, similar to the reduction seen in DHT-mediated AR transactivation in this system. Although we did not observe an enhancement of DHT-mediated, wild-type AR transactivation on BPA treatment, Casodex again blocked AR activity by >70% in cells treated with both DHT and BPA, as shown in Fig. 5*B*. Together, these data indicate that although BPA alone can marginally stimulate wild-type AR in LAPC-4 cells, it fails to enhance androgen-mediated AR transactivation as observed with tumor-derived mutant ARs (Figs. 1 and 4).

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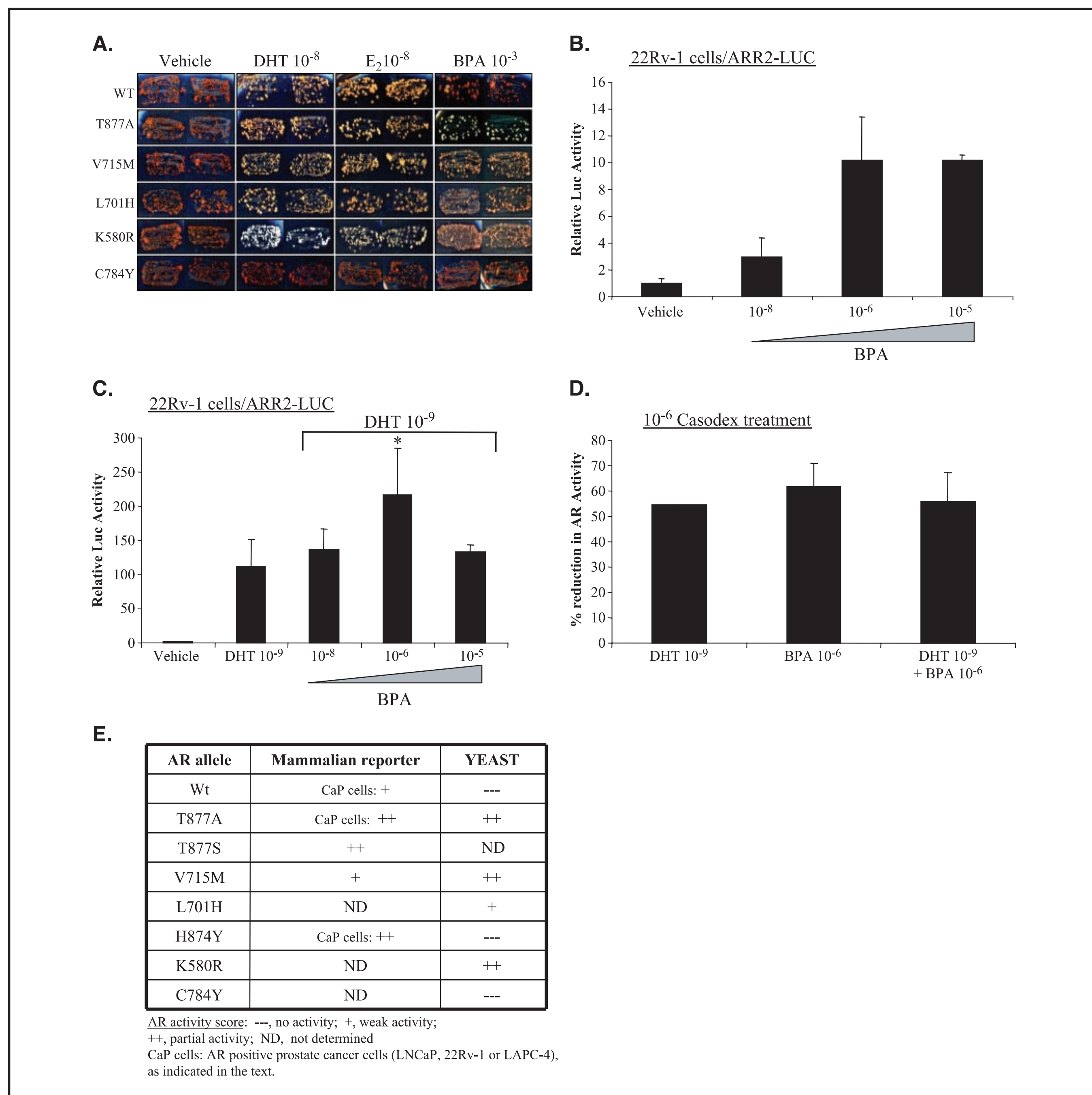

AR allele	Mammalian reporter	YEAST
Wt	CaP cells: +	---
T877A	CaP cells: ++	++
T877S	++	ND
V715M	+	++
L701H	ND	+
H874Y	CaP cells: ++	---
K580R	ND	++
C784Y	ND	---

AR activity score: ---, no activity; +, weak activity; ++, partial activity; ND, not determined
CaP cells: AR positive prostate cancer cells (LNCaP, 22Rv-1 or LAPC-4), as indicated in the text.

Figure 4. Multiple tumor-derived mutant AR proteins are activated by BPA. *A,* yeast strains containing the *ARE*-driven *ADE2* reporter gene and expressing individual AR alleles were grown on selective medium plates as described in Materials and Methods, supplemented with either vehicle (0.1% ethanol), 10^{-8} mol/L DHT, 10^{-8} mol/L estradiol (E_2), or 10^{-3} mol/L BPA. AR-mediated transactivational activity was scored based on the color of the yeast colonies. *White* or *pink,* AR was either completely or partially activated, respectively; *red,* failure of AR activation. *B,* 22Rv-1 cells were transfected with probasin reporter (ARR2-LUC, 1.0 μg), β-galactosidase expression plasmid (0.5 μg), and an empty vector pcDNA3.1 (1.5 μg). Following transfection, cells were stimulated with either vehicle alone (0.1% ethanol) or increasing BPA concentrations as indicated for 48 hours. *Columns,* average relative luciferase activities from at least three independent experiments; *bars,* SD. *C,* 22Rv-1 cells were transfected as in *B* and treated with either vehicle (0.1% ethanol) or 10^{-9} mol/L DHT in the absence or presence of increasing concentrations of BPA for 48 hours, as indicated. Cells were harvested and monitored for luciferase activity as in *B*. *Columns,* average relative luciferase activities from at least three independent experiments; *bars,* SD. *, $P < 0.05$, compared with the DHT alone–treated cells. *D,* 22Rv-1 cells were transfected as in *B* and pretreated with 10^{-6} mol/L Casodex before addition of the indicated drugs. After 48 hours, cells were harvested and processed for luciferase activity. *Columns,* average percentage reduction in AR activity compared with the corresponding AR activity in the absence of Casodex; *bars,* SD. Experiments were performed at least in triplicate. *E,* summary panel of BPA as a partial agonist of multiple tumor-derived mutant AR proteins.

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Bisphenol A Uncouples AR-Mediated Transcription from AR-Mediated Mitogenesis in Androgen-Dependent Prostatic Adenocarcinoma Cells. Previously, we showed a biphasic pattern for BPA-mediated proliferative response in LNCaP cells under androgen ablation conditions, wherein 10^{-9} mol/L BPA maximally stimulated LNCaP cell proliferation, yet higher doses failed to induce mitogenesis (21). These findings are similar to those observed with DHT, wherein 10^{-10} mol/L concentration is optimal for LNCaP cell proliferation, whereas high doses halt cell growth (46). To determine if BPA affects DHT-mediated cellular proliferation, multiple cell models were used: LNCaP (AR-T877A, androgen-dependent), LAPC-4 (wild-type AR, androgen-dependent), and 22Rv-1 (AR-H874Y, androgen-independent). As shown in Fig. 6*A*, ~30% of LNCaP and ~23% of LAPC-4 cells (both androgen-dependent and AR-positive) incorporated BrdUrd in the presence of DHT, compared with only ~10% and ~15% BrdUrd incorporation, respectively, in the absence of androgen. These observations are consistent with findings published previously (21, 47). Strikingly, BPA at 10^{-5} mol/L concentration inhibited proliferation of both LNCaP and LAPC-4 cells, bringing BrdUrd incorporation levels down below basal levels (~2% and ~4%, respectively). These data indicate that high doses of BPA are growth inhibitory for AR-positive, androgen-dependent prostate cancer cells. To determine if BPA-induced cell cycle arrest could be reversed by treatment with androgen, experiments were repeated in the presence of DHT at concentrations that maximally induce proliferation in these cells (10^{-10} mol/L for LNCaP cells and 10^{-9} mol/L for LAPC-4 cells). As shown, DHT supplementation did not reverse inhibitory effects of BPA on LNCaP and LAPC-4 cell proliferation, as both cell lines showed only ~5% BrdUrd incorporation when treated with both DHT and 10^{-5} mol/L BPA. Thus, these data show that AR-positive, androgen-dependent prostate cancer cells are growth inhibited by high dose of BPA.

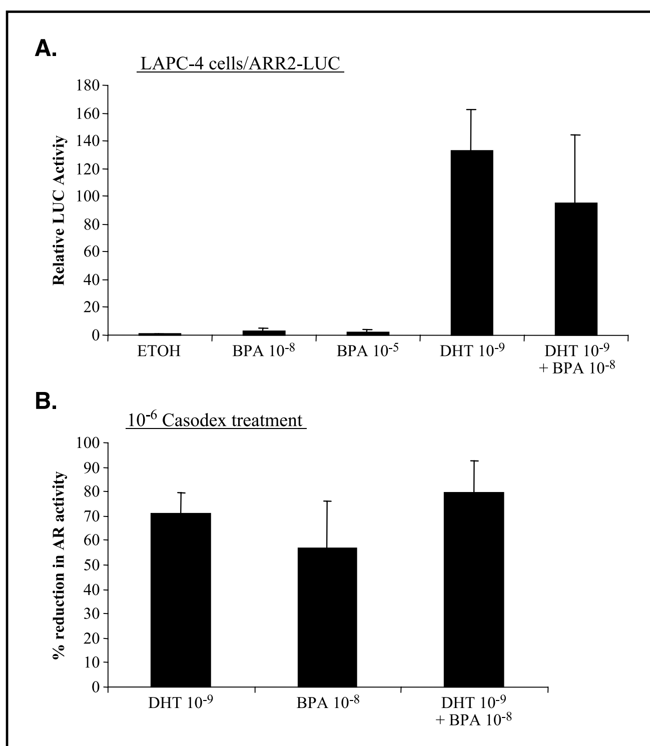

Figure 5. BPA has a marginal effect on wild-type AR and fails to enhance its androgen-mediated transcriptional activity. *A,* LAPC-4 cells were transfected as 22Rv-1 cells in Fig. 4*A*. Following transfection, cells were stimulated with either vehicle (0.1% ethanol), 10^{-8} mol/L BPA, 10^{-5} mol/L BPA, 10^{-9} mol/L DHT, or DHT + 10^{-8} mol/L BPA. After 48 hours of treatment, cells were harvested and processed for luciferase activity. Vehicle-treated AR activity was set to 1. *Columns,* average relative luciferase activities from at least three independent experiments; *bars,* SD. *B,* LAPC-4 cells were transfected as in *A* and pretreated with 10^{-6} mol/L Casodex for 4 hours prior to the addition of indicated treatments. *Columns,* average percentage reduction in AR activity compared with the corresponding AR activity in the absence of Casodex; *bars,* SD. Experiments were performed at least twice in triplicates.

To investigate the mechanism behind the inhibitory effects of BPA on androgen-dependent prostate cancer cell growth, we examined the effect of this compound on known androgen-responsive cell cycle regulatory proteins. It has been shown by our group and others that androgen induces accumulation of cyclin D1, cyclin A, and $p21^{cip1}$ in LNCaP cells; in contrast, $p27^{kip1}$ levels are reduced on androgen treatment (48–51). Previously, we have shown that a low BPA dose induces a similar cell cycle profile, consistent with its ability to stimulate prostate cancer cell growth in the absence of androgens (21). Here, we investigated antimitogenic effects of 10^{-5} mol/L BPA on LNCaP cells. For these experiments, cells were propagated in steroid-free medium supplemented with either vehicle (0.1% ethanol), 10^{-10} mol/L DHT, 10^{-5} mol/L BPA, or DHT plus BPA, as indicated in Fig. 6*B*. As expected, LNCaP cells cultured in androgen-free conditions failed to proliferate, resulting in very low levels of cyclin A and cyclin D1, but cells supplemented with 10^{-10} mol/L DHT exhibited a marked increase in both G_1 cyclins (Fig. 6*B*, *lanes 1* and *2*, respectively). In agreement with our BrdUrd data (Fig. 6*A*), where LNCaP cells treated with 10^{-5} mol/L BPA alone or together with 10^{-10} mol/L DHT failed to proliferate, levels of cyclin A and cyclin D1 were also strongly attenuated (Fig. 6*B*, *lanes 3* and *4*, respectively). Next, we examined expression of both $p21^{cip1}$ and $p27^{kip1}$ in LNCaP cells treated either with 10^{-5} mol/L BPA alone or in combination with 10^{-10} mol/L DHT. In agreement with previously published literature (48–51), $p27^{kip1}$ levels were down-regulated and $p21^{cip1}$ expression was induced on androgen treatment (Fig. 6*B*, compare *lanes 5* and *6*). Treatment of LNCaP cells with either 10^{-5} mol/L BPA alone or together with 10^{-10} mol/L DHT also resulted in significant accumulation of $p27^{kip1}$, as expected (*lanes 7* and *8*, respectively). Interestingly, $p21^{cip1}$ levels remained elevated in response to BPA treatment, especially in the presence of DHT (*lanes 7* and *8*). This observation likely reflects the ability of these agents to activate AR-T877A, as $p21^{cip1}$ is a direct AR target gene (50, 51). These data indicate that BPA-mediated inhibition of androgen-dependent prostate cancer cell growth is molecularly distinct from that observed on androgen ablation.

To determine if the antimitogenic effect of BPA was conserved in other AR-positive prostate cancer cells, the 22Rv-1 cell line was used. Although these cells express AR (mutant AR-H874Y), they no longer depend on androgens for growth. For these experiments, 22Rv-1 cells were cultured in phenol red–free, steroid-free medium supplemented with indicated treatments for 48 hours and then pulsed with BrdUrd. These cells robustly proliferated in the absence of steroids (~60% BrdUrd-positive cells), and DHT supplementation did not further stimulate proliferation (Fig. 6*C*). These results agree with previously published results (reviewed in ref. 52). High doses of BPA (up to 10^{-5} mol/L), either in the presence or in the absence of 10^{-9} mol/L DHT, had a slight (but significant, $P < 0.05$) effect on 22Rv-1 cell proliferation, as only ~45% of cells continued to incorporate BrdUrd in the presence of 10^{-5} mol/L BPA. Addition of DHT did not reverse BPA-induced inhibition, as ~40% of cells were BrdUrd positive when treated with both DHT and BPA (Fig. 6*C*). These results indicate that although the antiproliferative

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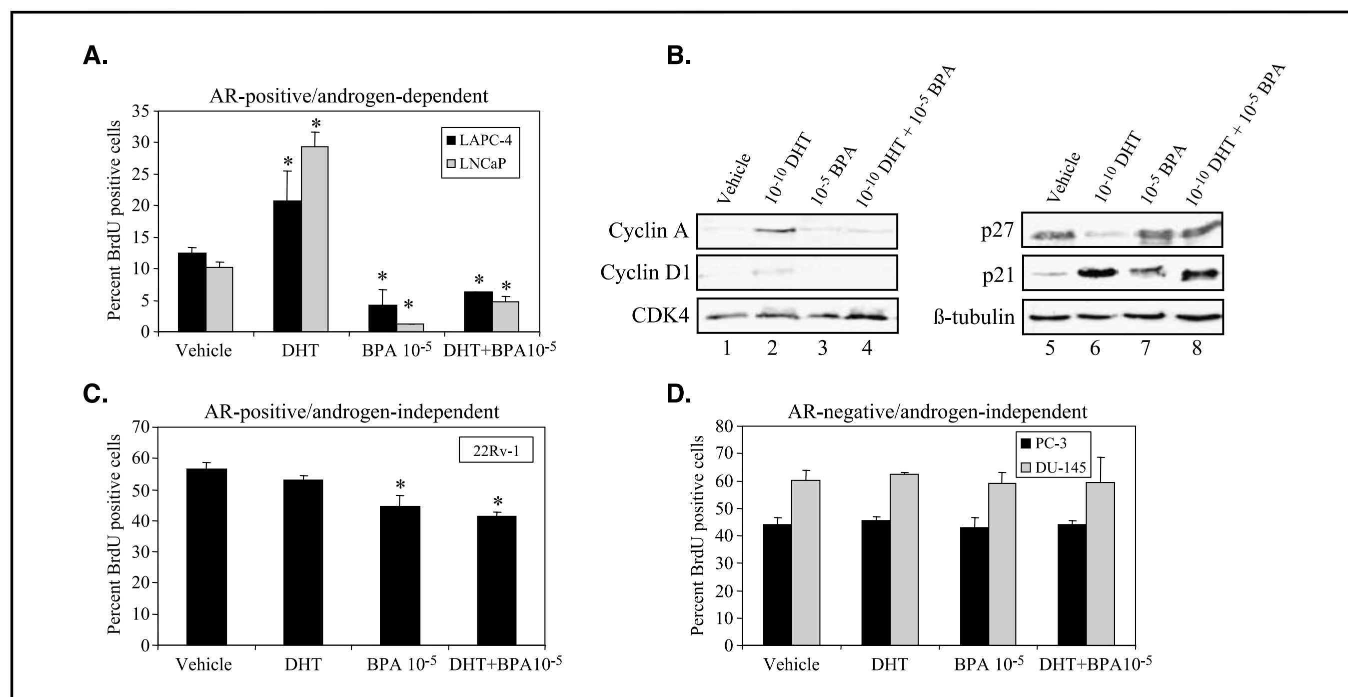

Figure 6. High dose bisphenol A uncouples ligand-dependent transactivation from mitogenesis in prostatic adenocarcinoma cells. *A*, AR-positive/androgen-dependent (LAPC-4 and LNCaP) prostate cancer cell lines were propagated in phenol-red free medium in the absence of steroids and treated with either vehicle (0.1% ethanol), DHT alone (10^{-10} mol/L for LNCaP cells and 10^{-9} mol/L for other cell lines), and 10^{-5} mol/L BPA in the absence or presence of DHT for 72 hrs. Cells were then labeled with BrdUrd for a period of 16 hrs, and BrdUrd incorporation was monitored via indirect immunofluorescence. *B*, LNCaP cells were treated as in *A* for 72 hrs in steroid-free medium. Cells were then harvested and subjected to SDS-PAGE followed by immunoblotting for Cyclin A, Cyclin D1, b27kip1 and p21cip1. Levels of CDK4 and β-tubulin served as loading controls. *C,* AR-positive/androgen-independent (22Rv-1) and *D,* AR-negative/androgen-independent (PC-3 and DU-145) were treated and analyzed as described in *A*. *Columns*, average of at least two independent experiments in duplicate (300 cells per experiment analyzed); *bars*, S.D.; *, $P < 0.05$, compared with the vehicle-treated cells.

effect of BPA is common to prostate cancer cells expressing a functional AR, this effect is much more pronounced in androgen-dependent prostate cancer cells.

To examine the requirement of the AR for BPA-induced inhibition of cell cycle progression in prostate cancer cells, we used AR-negative, androgen-independent prostate cancer cell lines (PC-3 and DU-145). For these experiments, cells were cultured in phenol red–free medium in the absence of steroids and treated with either vehicle alone or the indicated drugs for 48 hours. Consistent with previous findings (reviewed in ref. 52), growth of these cells was not affected by the absence of androgens (Fig. 6*D*). Additionally, supplementation with either 10^{-9} mol/L DHT, 10^{-5} mol/L BPA, or combination did not have an effect on BrdUrd incorporation in these prostate cancer cells, indicating that the antiproliferative action of BPA on prostate cancer cells requires a functional AR.

A summary of our findings of BPA action in prostate cancer cells is presented in Table 1. Collectively, these data indicate that low-dose BPA exposure results in enhancement of androgen-mediated mutant AR activity, whereas high-dose BPA exposure leads to pharmacologic uncoupling of transcriptional response from mitogenesis (both events are mediated by AR) in AR-positive prostate cancer cells.

Discussion

We have shown previously that BPA can activate a specific tumor-derived AR allele (AR-T877A), leading to inappropriate proliferation of androgen-dependent prostate cancer cells (21). However, the effect of BPA on other tumor-derived AR alleles or its effects in the presence of androgens were not examined previously. Here, we show that BPA action on AR alleles is dose dependent and the biological outcome on BPA exposure depends on both AR and androgen dependence status of prostate cancer cells. First, we showed that BPA enhances transcription of an androgen-activated mutant AR (*T877A*) using reporter assay readouts and reverse transcription-PCR measuring levels of an endogenous AR target (Figs. 1 and 2). These data are consistent with the observation that BPA is a noncompetitive inhibitor of DHT for binding to the AR-T877A (Fig. 3). Strikingly, the ability of BPA to activate tumor-derived AR is conserved across multiple mutant ARs, as shown by both yeast-based and mammalian cell assays (Fig. 4). By contrast, BPA exhibits little effect on wild-type AR (Fig. 5). Lastly, we show that higher doses of BPA repress cellular proliferation of prostate carcinoma cells, and this antimitogenic action is correlated with the presence of a functional AR (Fig. 6). Taken together, these data indicate that BPA can significantly modify both androgen signaling and cellular proliferation in prostate cancer cells at different stages of the disease.

It is well established that mutations in the ligand binding domain of the AR can alter its function. For example, AR-T877A is activated not only by androgens but also by non-androgenic steroids, such as estrogen, progestins, glucocorticoids, and an anti-androgen hydroxyflutamide (53, 54). *T877A* mutation was identified in a prostate cancer patient who received combined androgen blockade with flutamide, an effective wild-type AR antagonist (17, 55). In this study, we investigated BPA action on multiple tumor-derived AR proteins. *V715M*, *H874Y*, *T877S*, and *L701H* mutations in the AR were

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Table 1. Summary panel: BPA uncouples androgen-mediated AR transactivation from mitogenesis in prostate cancer cells

		Transcription		Mitogenesis	
	DHT	−	+	−	+
AR+, AD CaP (LNCaP or LAPC-4)	BPA, low dose	↑	↑↑↑ LNCaP	↑ LNCaP	No effect
	BPA, high dose	↑	↑↑ LNCaP	↓↓↓	↓↓
AR+, AI CaP (22Rv-1)	BPA, low dose	↑	↑↑	No effect	No effect
	BPA, high dose	↑↑	↑↑	↓	↓
AR−/−, AI CaP (PC-3, DU-145)	BPA, low dose	N/A	N/A	No effect	No effect
	BPA, high dose	N/A	N/A	No effect	No effect

NOTE: Low dose 10^{-9} μ/L, 10^{-8} μ\L, as indicated in the text; high dose 10^{-6} μ/L, 10^{-5} μ/L, as indicated in the text. Transcriptional activity score: ↑, partial activity; ↑↑, moderate activity; ↑↑↑, enhanced activity. Effect on mitogenesis: ↑, partial induction; ↓, partial inhibition; ↓↓, moderate inhibition; ↓↓↓, complete inhibition.
Abbreviations: AD, androgen dependent; AI, androgen independent; N/A, not applicable.

identified in patients with metastatic androgen-independent disease following androgen ablation therapy (56, 57). These AR mutants respond to multiple non-androgenic steroids, and both AR-T877A and AR-L701H are also activated by glucocorticoids (33). The AR-K580R is a gain-of-function mutant and it is sensitive to low levels of androgens. Similarly, this mutant AR was derived from a metastatic prostate cancer patient (18). In contrast to promiscuous mutant ARs, AR-C784Y (derived from a patient with a complete androgen insensitivity syndrome) lost the ability to be activated by androgens (58). Using a yeast assay of AR activity, we showed that all examined tumor-derived AR mutant proteins, but not wild-type or androgen-insensitive AR-C784Y, respond to BPA. Perhaps due to differential uptake of the drug (59) and/or lack of steroid receptor coregulators in yeast, higher BPA concentrations (10^{-3} mol/L) were required to observe its effects in this system. It will be of interest in future studies to examine contributions of AR cofactors to BPA-mediated transactivation of mutant ARs. In fact, it has been reported that specific cofactors can enhance AR transactivation in the presence of non-androgenic steroids (60) and deregulation of AR coactivator expression had been implicated in prostate cancer transition to androgen independence (14–16). The ability of BPA to stimulate tumor-derived mutant AR (AR-V715M, AR-T877S, and AR-H874Y) was confirmed in a mammalian cell–based reporter assay demonstrating that low concentrations of BPA can indeed transactivate these alleles (Fig. 4; data not shown).

Several recent studies showed that BPA can antagonize wild-type AR in both yeast-based assays (61, 62) and mammalian cells (63, 64). These studies examined BPA action on the AR in non–prostate cancer cell background, either transiently or stably expressing the wild-type receptor with the exposure to high (micromolar or higher) concentrations of BPA. In agreement with these findings, we also observed an anti-androgenic action of BPA at micromolar concentrations in non-prostate cells (i.e., CV-1 cells). BPA acted as an anti-androgen on all mutant AR proteins at high doses, including the wild-type, as measured by reporter assays (data not shown) in CV-1 cells. However, we believe this outcome of BPA action holds little biological relevance, because in prostate cancer cells we observed that BPA stimulated and further enhanced androgen-mediated activity of endogenously expressed AR (AR-T877A and AR-H874Y, as shown in Figs. 1, 2, and 4). Thus, our findings indicate that the effect of endocrine-disrupting compounds on AR can vary dramatically depending on cellular context. Differential outcomes of BPA action in prostate cancer cells versus non-prostate cancer cell types possibly implicates involvement of cell type–specific coregulatory molecules in mediating biological outcomes on exposure to BPA. Given the ability of BPA to stimulate AR-T877A activity in a prostate cancer cell line, we assessed the effect of BPA on DHT binding to this allele. We showed that BPA alters androgen binding to AR-T877A through noncompetitive inhibition, suggesting that BPA action on this allele possibly occurs through an alternative binding site (Fig. 3). It has been shown that other environmental estrogenic compounds, such as metabolites of pesticides vinclozolin and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, possess strong anti-androgenic activity by competitively inhibiting androgen binding to the AR (65, 66). However, noncompetitive inhibition of ligand binding to steroid receptors has also been reported. For example, an anti-arrhythmic drug amiodarone and unsaturated fatty acids affect the binding of endogenous ligands to the thyroid hormone receptor type b1 and glucocorticoid receptor, respectively (67, 68). In addition, an antiprogestin RU486 is an effective noncompetitive inhibitor of the ER (69). It has been proposed that these alternative ligands bind to their perspective receptors at sites different from native ligand binding sites. Thus, our data support the hypothesis that BPA induces an active AR conformation through interaction outside the AR-T877A ligand binding pocket.

It is of note that cooperative action of BPA on activated AR-T877A decreases in the presence of 10^{-5} mol/L BPA, which is the concentration that inhibits DHT binding to AR-T877A in *in vitro* assay. These observations may reflect the presence of alternative binding sites on AR-T877A. Such function has recently been proposed for the ER antagonist 4-OH-tamoxifen. It has been suggested that when present at high concentrations, the drug uses an alternative binding site on the ER-β, thus acting as an agonist.[5] At present, the location of a potential BPA binding site(s) on AR-T877A remains elusive, but an influence from an indirect BPA action also cannot be ruled out. Supporting this, no significant

[5] T.P. Burris, personal communication.

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displacement of the labeled ligand was observed in the presence of low BPA concentrations (Fig. 3), wherein biological effects in prostate cancer cells are seen (21). In fact, recent report by Jansen et al. showed that certain environmental contaminants can dramatically affect the function of steroid receptors without direct binding. This group showed that a pervasive xenobiotic methoxyacetic acid enhances nuclear receptor activity by activating mitogen-activated protein kinase pathways and inhibiting activity of histone deacetylases (70). Thus, it is possible that low-dose BPA action on the AR-T877A may not be through simple binding to the receptor, and its mode of action on AR may also depend on the level of exposure to this compound.

Ultimately, examining the effect of BPA exposure on androgen-dependent proliferation is critical in elucidating xenoestrogen effect on prostate cancer growth. Few studies have evaluated the average levels of human exposure to BPA, although this compound is present at significant concentrations in the environment (22). Thus, we examined the effect of a range of doses on cellular proliferation of prostate cancer cells. We show that although BPA cooperates with DHT to enhance AR activation, it antagonizes androgen-mediated proliferation. This action of BPA was observed in AR-positive prostate cancer cells and was not conserved in either AR-negative prostate cancer cells (Fig. 6) or breast cancer cells (data not shown). At the molecular level, high-dose BPA inhibited expression of key G_1 cyclins (D1 and A) and induced $p27^{kip1}$ levels (Fig. 6*B*). Strikingly, $p21^{cip1}$ expression, which typically correlates with androgen stimulation, remained elevated in growth-inhibited LNCaP cells treated with 10^{-5} mol/L BPA either in the absence or in the presence of androgen (10^{-10} mol/L DHT). This likely reflects the ability of BPA to stimulate AR-T877A activity, because $p21^{cip1}$ is a known target gene (48, 50, 51). Thus, these data indicate that the molecular mechanism behind BPA-induced cell cycle arrest is distinct from that observed on androgen ablation in LNCaP cells.

Interestingly, the ability of BPA to antagonize proliferation of prostate cancer cells included those that are no longer androgen dependent or androgen responsive (22Rv-1) but still express a functional AR (Fig. 6*C*). However, antimitogenic effect of BPA on these cells was modest by comparison with prostate cancer cells that are dependent on androgen for growth (Fig. 6*A* and *C*). These data suggest that prostate cancer progression to androgen independence uses modified ligand-mediated proliferative response, including potential exposure to nonsteroidal environmental compounds. Mounting evidence suggests that prostate cancer androgen-independent state is associated with abnormal coactivator expression in prostate cancer (14–16). Therefore, it is plausible that 22Rv-1 cells have a modified expression profile of coregulatory molecules, resulting in an altered proliferative response of these cells to androgens (they are no longer dependent on androgen for growth) and a moderate response to an antimitogenic action of BPA. In fact, it has been shown that the AR-H874Y mutation possibly affects binding of coactivator proteins, resulting in altered activity of this mutant (71). Strikingly, the antimitogenic effect of BPA was also observed in LAPC-4 cells that express wild-type AR (Fig. 6*A*). In contrast, BPA did not exert a cooperative effect on androgen-activated wild-type AR (Fig. 5). This observation establishes a further distinction of BPA action on the wild-type versus tumor-derived mutant AR. Collectively, these data show that antimitogenic action of BPA correlates with the presence of functional AR in prostate cancer cells and we also show that the effect of this compound on AR transcriptional activity is distinct from its action on AR-dependent mitogenesis.

Cumulatively, our study lays another level of complexity in steroid hormone and receptor function, indicating that exposure to low, environmentally relevant doses of an endocrine disruptor BPA can result in altered activity of multiple tumor-derived AR alleles. In this study, we showed that BPA enhances transcription of androgen-activated, tumor-derived AR-T877A and AR-H874Y. We also showed that the ability of BPA to activate mutant AR is conserved across multiple AR alleles. Moreover, we found that BPA alters androgen binding to AR-T877A in a noncompetitive manner, either using a different binding site or modifying conformation of the AR complex indirectly. Lastly, we show that higher doses of BPA repress cellular proliferation of prostate carcinoma cells, and this action correlates with the presence of a functional AR. Therefore, the importance of our findings is 2-fold. First is that BPA (especially lower doses) can serve as a potential "hormone sensitizer" of the mutant ARs present in advanced prostate adenocarcinomas, thereby possibly contributing toward therapeutic relapse in prostate cancer patients and supporting the notion that nonsteroidal environmental compounds can dramatically alter the function of nuclear receptor complexes. Secondly, this study lays a foundation for understanding pharmacologic uncoupling of two distinct molecular events: androgen-mediated transcription and AR-dependent mitogenesis. Together, these data suggest that a therapeutic outcome in prostate cancer patients with an advanced disease can be influenced by the exposure to ubiquitous environmental contaminants such as BPA. Thus, this study provides the impetus to monitor the influence of BPA and other endocrine disruptors on development of androgen-independent prostate tumors *in vivo*.

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