ABSTRACT

Androgens are potent differentiation agents that regulate prostate-specific antigen (PSA) gene expression via the androgen receptor (AR) that binds to androgen response elements (AREs) on the PSA gene to initiate transcription. However, in the absence of androgens, PSA gene expression can become elevated. This suggests that either the AR can be activated in the absence of androgen to elevate PSA gene expression through AREs on the PSA gene or that another transcription factor acting on the PSA promoter is stimulated. We have previously shown in vivo that butyrate, a differentiation agent that causes cell cycle arrest, increases serum PSA levels in castrated animals. Therefore, to determine the mechanism of butyrate induction of PSA, we used the LNCaP human prostate cancer cell line. Northern analyses and transfection experiments using a PSA reporter plasmid demonstrated induction of PSA gene expression by butyrate in LNCaP cells. Application of the antiandrogen bicalutamide blocked the induction of PSA mRNA by butyrate, suggesting a mechanism dependent on the AR. Consistent with this conclusion, electromobility shift assays showed increased AR-ARE complex formation with nuclear extracts from butyrate-treated cells. In addition, another reporter gene constructs that contain AREs were also induced by butyrate. Western blot analysis showed an increase in nuclear levels of AR protein in cells exposed to butyrate, whereas whole cell levels remained unchanged, suggesting that butyrate causes nuclear translocation of the AR. Thus, the differentiation agent butyrate causes ligand-independent activation of the AR to increase expression of the differentiation marker PSA in human prostate cancer cells.

INTRODUCTION

The AR is a ligand-mediated transcription factor that belongs to the superfamily of nuclear receptors that includes the estrogen receptor, glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor, thyroid receptor, and retinoic acid receptors (1). These receptors contain distinct functional domains that include an NH2-terminal region that is involved in transactivation, a highly conserved DNA-binding domain, a hinge region that contains a nuclear translocation signal, and a ligand-binding domain (2). Binding of ligand to the receptor results in the transformation or activation of the receptor such that it can effectively bind to its specific DNA element. The mechanism of ligand-induced transformation of the AR is not completely understood; however, it is known that the conformation of the AR becomes more compact on ligand binding, heat shock proteins are dissociated, and dimerization and phosphorylation occur before DNA binding (3). AR that is activated by ligand can stimulate or repress androgen-regulated genes. Recent evidence has shown that the human AR can be activated in the absence of androgen, its cognate ligand, by elevation of intracellular levels of cAMP or by exposure to specific growth factors (4–6). Whereas the mechanism of ligand-independent activation of the AR has not been clarified, it has been shown that the NH2 terminus of the AR is activated by the PKA pathway (6), but whether a change in phosphorylation or association with coactivators is involved is still unknown.

PSA belongs to the kallikrein-like serine protease family (7), is abundantly expressed in the prostate, and is considered a marker of differentiation. The serum level of PSA glycoprotein is important in the diagnosis of prostate cancer and the monitoring of hormonal progression (8, 9). PSA gene expression is regulated by androgen through several well-characterized AREs to which the AR binds to initiate transcription (10, 11). However, in patients with androgen-independent prostate cancer, regulation of PSA gene expression escapes androgen control and becomes elevated in the absence of androgen through a mechanism that is unknown. PSA gene expression can also become elevated in the absence of androgens by numerous compounds including butyrate (12, 13), phenylacetate (13), vasoactive intestinal peptide (14), retinoic acid (13, 15), vitamin D (13, 16), interleukin 6 (17), growth factors (4, 18), and activators of PKA (6).

The mechanism of androgen-independent induction of PSA by IGF-I and activators of the PKA pathway has been suggested to involve ligand-independent activation of the AR (4, 6). The mechanism underlying the in vivo increases of PSA gene expression by butyrate has not been determined.

Butyrate is a short-chain fatty acid that is generated in the large intestine by bacterial fermentation of dietary fiber (19). The interest in butyrate for the treatment of malignancies in humans was initiated by the observation of its potent effects on differentiation and growth arrest of numerous cancer cell lines maintained in culture (20–23). In vivo studies of the butyrate analogue isobutyramide also showed a potent effect on differentiation, G1 cell cycle arrest, and increased expression of PSA in the LNCaP tumor model (12). Whereas butyrate has been shown to cause hyperacetylation of histones (24–27), increase the levels of WAF1/Cip1 protein (28–30), decrease the expression of c-myc (21), and activate protein phosphatase activities (31), the mechanism by which butyrate induces cellular differentiation (32, 33), suppresses growth (34, 35), and increases PSA gene expression has not been clarified.

With our observation that butyrate increases PSA gene expression in the LNCaP tumor model in the absence of androgen (12), the question of how butyrate mediates this effect was addressed. Because the AR can be activated in the absence of androgen by various growth factors and vitamin D and via cross-talk with the PKA pathway, the role of the AR in butyrate induction of PSA gene expression was investigated.

MATERIALS AND METHODS

Cell Culture. All chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise. Bicalutamide was a generous gift from Dr. Mark Zarenda (Zeneca Pharma Inc.). LNCaP cells between the 44th and 55th generation were maintained in RPMI 1640 supplemented with 5% FBS. PC3 cells between the 30th and 45th generation were maintained in DMEM supple-
mented with 5% FBS (Life Technologies, Inc., Burlington, Ontario, Canada). Du145 cells were maintained in DMEM supplemented with 10% FBS. When cells in the plates or wells reached 60–70% confluence, the culture medium was changed to serum-free medium containing R1881 or butyrate.

**Northern Blot Analysis.** Total RNA was extracted from LNCaP cells with Trizol (Life Technologies, Inc.) and fractionated by electrophoresis before blotting onto Hybond-N filters (Amersham, Oakville, Ontario, Canada). The 1.4-kb EcoRI fragments of the PSA cDNA and 18S RNA were labeled with [α-32P]dCTP using the Random Primers DNA Labeling kit (Life Technologies, Inc.). Hybridization was performed as reported previously (36). The mRNA bands were quantified with the STORM 860 PhosphorImager (Molecular Dynamics).

**Plasmid.** Full-length human AR cDNA was a gift from Dr. Brinkmann (Erasmus University, Rotterdam, the Netherlands). PSA-luciferase reporter (−630/+12) has been reported previously by us (6). The ARR-3-luciferase reporter construct consists of three congruent rat protein ARs (−244 to −96) ligated in tandem into the HindIII site of the pT81 luciferase vector (American Type Culture Collection, Manassas, VA) as described previously by us (37). The probasin (PB)-luciferase reporter (−386/+28) was constructed as reported previously (38).

**Transient Transfections and Luciferase Activity Assay.** LNCaP cells (3 × 10^5) were plated on 6-well plates and incubated in RPMI 1640 with 5% FBS before transfection as described previously (37). PC3 and Du145 cells (3 × 10^5) were transfected using FuGene6 (Roche Molecular Biochemicals). The total amount of plasmid DNA used was normalized to 3 μg/well by the addition of empty plasmid. For LNCaP cells, the medium was replaced after 24 h by RPMI 1640 (i.e., serum-free media) containing R1881 or butyrate. LNCaP cells were collected after 48 h of incubation. For PC3 and Du145 cells, after a 6-h transfection using FuGene6, the medium was replaced with serum-free medium containing R1881 or butyrate, and the cells were incubated for an additional 24 h before harvesting. Luciferase activities in cell lysates were measured using the Dual Luciferase Assay System (Promega) with the aid of the Berthold multplate luminometer. After a delay of 5 s, the light emission was recorded for 30 s. The protein concentration of the cell lysates was determined by the method of Bradford (39). Luciferase activities were normalized by protein concentrations of the samples. The results are presented as the fold induction that is the relative luciferase activity of the treated cells divided by that of the control. The control plasmid used for each experiment encoded the luciferase gene but lacked the promoter insert (e.g., for PSA-luciferase, the control was pGL2, whereas for PB- and ARR3-luciferase, the control plasmid was pXP2). All transfection experiments were carried out in triplicate wells and repeated two to eight times using at least two sets of plasmids prepared separately.

**EMSA.** Nuclear extracts from LNCaP cells were used for EMSA studies. Nuclear extracts were prepared from cells (40) that had been treated with compounds, whole cell lysates and nuclear extracts were prepared as described previously (41). Western blots were performed with 40 μg of total protein from nuclear extracts in a total volume of 60 μl containing DNA binding buffer [10 mM HEPES (pH 7.9), 10% (v/v) glycerol, 100 mM KC1, 1 mM EDTA, 5 mM MgCl2, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 2 μg of poly[d(oxynosinic-oxycytidylic acid)] (Pharmacia Biotech Inc., Piscataway, NJ) with approximately 1.5 fmol of double-stranded 32P-labeled PSA-ARE oligonucleotide (5′-TTCGAGAACGACAGGTGCTAGCTC-3′) or PSA mutant ARE (5′-TTCGAAAAAGACAAAGTGCTAGCTC-3′). Protein-DNA complexes were separated under nondenaturing conditions in a 4% polyacrylamide gel (29:1) containing 2.5% glycerol and run in 0.5× Tris-borate EDTA [1× Tris-borate EDTA = 89 mM Tris-borate, 89 mM boric acid, and 2 mM EDTA (pH 8.3)] at 200 V. Protein-DNA complexes were quantified with a STORM 860 PhosphorImager (Molecular Dynamics).

**Immunoblots.** LNCaP cells were incubated in RPMI 1640 (serum-free medium) for 24 h before the addition of R1881 or butyrate. After incubation with compounds, whole cell lysates and nuclear extracts were prepared as described previously (41). Western blots were performed with 40 μg of total protein per lane. Immunoblots were blocked overnight in 5% milk (w/v) in 20 mM Tris-HCl (pH 7.4) containing 500 mM NaCl (Tris-buffered saline). Blots were incubated for 4 h with antibodies to the AR (2 μg/ml; PA-1–11A; Affinity Bioreagents Inc., Golden, CO). The blots were washed and incubated for 1 h with the secondary antibody (1:5000). Antibodies were diluted in 5% milk/Tris-buffered saline solution. AR protein was detected using the enhanced chemiluminescence kit (Amersham). Densitometric analyses of protein bands from scanned X-ray films were performed using the Personal Densitometer (Molecular Dynamics).

**RESULTS**

**Butyrate Increases PSA mRNA in LNCaP Cells.** Androgen regulation of PSA gene expression occurs through the AR that binds to AREs on the PSA promoter. However, recent in vivo studies have shown elevation of PSA gene expression by butyrate in the absence of androgen (12). Therefore, we investigated whether PSA gene expression could be elevated by butyrate in the absence of androgen in LNCaP cells maintained in culture. These cells were used because they express endogenous PSA and AR. As a positive control, all experiments were performed in parallel with R1881, a synthetic androgen that induced PSA mRNA levels (Fig. 1A). LNCaP cells exposed for 16 h to various concentrations of butyrate (0.01–5 mm) showed a maximum increase in the levels of PSA mRNA between 0.2 and 0.5 mM butyrate (Fig. 1A). An increase in PSA mRNA was shown to be dose dependent over the range of 0.01–0.2 mM butyrate, with an estimated EC50 of approximately 0.08 mM (Fig. 1B). Levels of PSA mRNA plateaued between 0.2 and 0.5 mM butyrate (approximately

**Fig. 1.** Northern blot analysis of PSA mRNA isolated from LNCaP cells. A. Northern blot of PSA mRNA in cells treated with R1881 (10 nM) and various concentrations of butyrate (0.01–5 μM) for 16 h. B. Concentration dependence of butyrate induction of PSA mRNA normalized to 18S rRNA levels. C. Time course of PSA mRNA induction by butyrate (0.5 mM). RNA bands corresponding to PSA at 1.5 kb were quantified by scanning with a PhosphorImager. Each lane contains 20 μg of total RNA.

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4-fold higher than the control levels). Further increases in the concentration of butyrate (>0.5 mM) were shown to decrease PSA mRNA levels when compared with the maximum induced levels. As shown in Fig. 1C, the optimal time for maximum induction of PSA mRNA in LNCaP cells was 8 h after the addition of butyrate; after this time, the levels of PSA mRNA decreased. The poor induction of PSA mRNA by butyrate at later time points may reflect the short half-life of this compound.

Butyrate Induction of PSA Promoter Activity (−630/+12) and other Androgen-responsive Reporters. To determine whether the induction of PSA mRNA involves changes in the activity of the PSA promoter as opposed to posttranscriptional regulation, we transfected LNCaP cells with the PSA promoter (−630/+12)-luciferase reporter plasmid. This region of the PSA promoter has been partially characterized and contains several AREs that are required for androgen induction (10, 42). The optimal concentration of R1881 has previously been shown to be 10 nM (37). Butyrate induction of PSA-luciferase activity was dose dependent, with an optimal concentration of 2 mM (data not shown). PSA promoter activity was increased 21-fold by R1881, 34-fold by butyrate, and 109-fold by a mixture of R1881 and butyrate (Fig. 2A). These results illustrate the independent and synergistic stimulating effects of butyrate on PSA promoter activity in LNCaP cells.

Because this region of the PSA promoter contains two AREs, we next examined whether other reporter constructs that contain AREs could also be induced by butyrate in LNCaP cells. The first of these was the PB-luciferase reporter that contains a naturally occurring androgen-responsive promoter specific to the rat prostate. As shown in Fig. 2A, the PB-luciferase reporter was induced 311-fold by R1881, 4-fold by butyrate, and 1550-fold by a mixture of R1881 and butyrate. These results show a synergistic effect of butyrate on the induction of the PB-luciferase reporter by R1881.

The second of these reporters was the ARR3-tk-luciferase reporter, which contains three repeats of the PB ARE1 and ARE2 region ligated in tandem with a luciferase reporter (43). This reporter construct was induced 301-fold by R1881, 4-fold by butyrate, and 911-fold by a mixture of R1881 and butyrate. Similar to the results obtained with PSA- and PB-luciferase reporters, a synergistic effect was observed for butyrate on the induction of the ARR3-luciferase reporter by R1881.

Next we investigated the effects of butyrate on two other human prostate cancer cell lines, PC3 and Du145, that were transfected with an expression vector for the wild-type AR because these cell lines express little or no AR protein. The results for PC3 cells are shown in Fig. 2B. In this figure, the PSA promoter was poorly induced by R1881 (2.6-fold), butyrate (2.8-fold), and a mixture of the two compounds (3.6-fold). However, when the PB reporter was transfected into PC3 cells, the luciferase activity was increased 3.2-fold by R1881, 6.3-fold by butyrate, and 17.8-fold by a mixture of the two compounds. PC3 cells transfected with the ARR3 reporter had luciferase activities that were induced 41-fold by R1881, 2-fold by butyrate, and 141-fold by a mixture of the two compounds. Thus, in the presence of butyrate, the induction of PB and ARR3 reporters by R1881 was synergistic in PC3 cells.

The results obtained using Du145 cells transfected with the various reporter plasmids are shown in Fig. 2C. PSA promoter activity was induced 2-fold by R1881, 10-fold by butyrate, and 14-fold by a mixture of the two compounds. The induction of the PB reporter was 5-fold by R1881, 4-fold by butyrate, and 49-fold by a mixture of the two compounds. The induction of the ARR3 reporter was 8-fold by R1881, 5-fold by butyrate, and 264-fold by a mixture of the two compounds. Thus, in Du145 cells, butyrate caused a synergistic increase in the induction of PB and ARR3 reporters by R1881 in a pattern similar to that obtained with LNCaP and PC3 cells. All three reporters were induced to variable extents when butyrate was added to each of the cell lines in the absence of R1881.

Fig. 2. Induction of the activities of androgen-responsive promoters by butyrate. Plasmids (1 μg) encoding PSA (−630/+12), PB (−286/+26), or ARR3-tk-luciferase reporters were transfected into (A) LNCaP cells, (B) PC3 cells cotransfected with 0.5 μg of wild-type AR, or (C) Du145 cells cotransfected with 0.5 μg of wild-type human AR. Cells were incubated with R1881 (10 nM), butyrate (2 mM), or a mixture of R1881 (10 nM) and butyrate (2 mM) for 48 h (LNCaP cells) or 24 h (PC3 and Du145 cells) under serum-free conditions, harvested, and luciferase activities were measured. The normalized luciferase activities were divided by the normalized activity of control-treated cells. Values are expressed as the mean ± SD of triplicate samples. Butyrate treatment had no effect on the empty plasmid.
Bicalutamide Blocks Induction of PSA mRNA by Butyrate. A possible role for the AR in the induction of PSA by butyrate has been suggested in the above-mentioned experiments. Therefore, to provide evidence in support of this hypothesis, we next performed experiments using the antiandrogen bicalutamide, which is known to specifically inhibit the DNA-binding activity of the AR (44–46). Northern blot analysis of RNA isolated from LNCaP cells showed that bicalutamide blocked the induction of PSA mRNA by butyrate (Fig. 3, compare Lane 3 with Lane 6). Bicalutamide also prevented R1881 induction of PSA mRNA, as expected (compare Lane 4 with Lane 5). These results confirm that butyrate induction of PSA mRNA requires a functional AR that is able to bind to DNA.

**Butyrate Treatment Causes Binding of the AR to the PSA-ARE.** The above-mentioned data suggest that butyrate induction of PSA gene expression may occur through the AR and the PSA-ARE. To further examine this possibility, we investigated whether butyrate had an effect on the DNA-binding activity of AR by EMSAs using radiolabeled oligonucleotides of the PSA-ARE (−170/−156) and nuclear extracts from LNCaP cells. The results in Fig. 4 show the formation of a distinct band obtained with nuclear extracts from cells treated with either R1881 (Lane 1) or butyrate (Lane 3). As expected, this band was not formed when an identical oligonucleotide containing a mutation in the ARE was used (Lanes 5–8), which is consistent with a previous report showing that this mutation substantially decreases AR binding to the ARE (11). The AR-ARE complex can be supershifted with an antibody to the NH2 terminus of the AR (see Lanes 12–14). These data are consistent and agree with the above-mentioned results identifying the AR as a possible mediator of butyrate induction of PSA gene expression.

**Effects of Butyrate on Whole Cell and Nuclear Levels of AR.** To test the possibility that butyrate may alter the cellular levels and/or localization of the AR, we determined the whole cell lysate and nuclear levels of AR protein by Western blots. LNCaP cells were incubated with butyrate, and then whole cell lysates and nuclear extracts were prepared from cells harvested at the 1.5 and 3 h time points. The AR was barely detectable in nuclear extracts obtained from control cells (Fig. 5, Lanes 2 and 5). Butyrate treatment resulted in an increase in the nuclear levels of AR protein. The nuclear levels of AR protein in extracts prepared from butyrate-treated cells were 2.7-fold after 90 min and 9.6-fold after 3 h as compared with those in controls. Whole cell levels of AR protein were slightly increased in butyrate-treated cells as compared with controls at 24 h. R1881 also slightly increased the levels of AR in whole cell lysates after 24 h of incubation, as described previously (6).

**Blocking the PKA Pathway Inhibits Butyrate Induction of PSA Gene Expression.** Recently, we have shown that PSA gene expression can be induced through activation of the PKA pathway with forskolin by a mechanism dependent on a functional AR (6). Therefore, to determine whether butyrate induces PSA gene expression via the PKA pathway, we used the sodium salt of the adenosine 3’5’-cyclic monophosphorothioate 8-bromo-Rp-isomer, which shows preferential inhibition of PKA type I (47). Application of this inhibitor of PKA reduced the induction of PSA-luciferase activity by 68% and 83% for butyrate and forskolin (positive control), respectively (Fig. 6). These data suggest that butyrate induces PSA gene expression by a mechanism that may be dependent on the PKA pathway.

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**Fig. 3**. Inhibitory effect of bicalutamide on the induction of PSA mRNA by butyrate. LNCaP cells were preincubated with bicalutamide (BIC; 50 μM) for 2 h before the addition of R1881 (10 nM) or butyrate (0.5 mM) and then incubated for an additional 16 h. At the end of the incubation period, cells were harvested, RNA was isolated, and Northern blots were performed using radiolabeled probes for PSA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RNA bands corresponding to PSA at 1.5 kb were quantified by phosphorImager. Each lane contains 20 μg of total RNA.

**Fig. 4**. Formation of AR-ARE complexes in the presence of nuclear extracts from LNCaP cells treated with butyrate. EMSAs were performed using radiolabeled PSA-ARE of PSA-mARE oligonucleotides with nuclear extracts isolated from LNCaP cells incubated with compounds for 3 h. Lanes 1, 5, 9, and 12 contain nuclear extracts from cells treated with R1881; Lanes 2, 6, 10, and 13 contain nuclear extracts from control-treated cells; Lanes 3, 7, 11, and 14 contain nuclear extracts from cells treated with butyrate; and Lanes 4 and 8 contain no nuclear protein, only BSA (10 μg). Lanes 1–4 and 9–14, PSA-ARE; Lanes 5–8, mutated PSA-ARE (PSA-mARE). Lanes 12–14 contain nuclear extracts incubated with an antibody to the NH2 terminus of the AR.
DISCUSSION

The AR belongs to the superfamily of steroid hormone receptors considered to be ligand-activated transcription factors. Over the past few years, evidence has been provided that some of these receptors can mediate extracellular signals in the absence of their cognate ligand by dopamine, epidermal growth factor, heregulin, gonadotrophin-releasing hormone, tumor growth factor $\alpha$, insulin and IGF-I, cAMP, okadaic acid, and vanadate (48–53). Such activation of steroid hormone receptors in the absence of their cognate ligand has been termed "ligand-independent activation" or "steroid-independent activation."

Ligand-independent activation of the human AR has been suggested to occur in androgen-deprived cells and androgen-independent prostate cancer cells. Measurement of the activities of androgen-responsive reporters transfected into various cell lines has shown that the human AR can be activated in the absence of androgen by IGF-I, keratinocyte growth factor, epidermal growth factor, and compounds that elevate cAMP (4–6). Increased endogenous expression of the androgen-responsive PSA gene by ligand-independent activation of the AR in human prostate LNCaP cells is mediated by cross-talk with the PKA pathway (6). Endogenous induction of PSA gene expression by butyrate has also been reported to occur in LNCaP cells grown as xenographs in castrated animals (12). Therefore, the present studies investigated the mechanism of increased PSA gene expression by butyrate in human prostate cancer cells maintained in culture in the absence of androgens. These studies are the first to reveal the following: (a) induction of PSA and other androgen-responsive genes by butyrate is promoter and cell specific; (b) induction of PSA by butyrate is mediated through a pathway dependent on the AR; and (c) induction of PSA by butyrate partially involves the PKA pathway.

Induction of PSA gene expression by butyrate in LNCaP cells deprived of androgens was shown by both Northern blot analysis and measurement of the activity of a PSA-reporter gene construct (−630/+12) that contains the well-characterized ARE (11). Increased levels of PSA mRNA in LNCaP cells exposed to butyrate were shown to be transient and dose dependent, with an optimal time and concentration of 8 h and 0.5 mM, respectively. Higher concentrations of butyrate did not result in a plateau in the induction of PSA mRNA levels but rather caused a decrease. A similar biphasic dose-dependent curve for PSA mRNA was shown in LNCaP cells exposed to forskolin (6). Concentrations of butyrate above 2 mM resulted in a decrease in mRNA levels below that of the control. Such a decrease at these high concentrations may be due to apoptosis because this has been reported previously at 5 mM concentrations of butyrate (12). Dose-dependent decreases in PSA mRNA levels have been reported previously in LNCaP cells exposed to 12-O-tetradecanoylphorbol 13-acetate, which induces apoptosis in these cells (37, 54, 55).

Induction of PSA-luciferase activity in LNCaP cells exposed to butyrate was consistent with the induction of PSA mRNA levels by butyrate shown here and with previously reported in vivo data (12). Whereas the synthetic androgen R1881 was a more powerful inducer of PSA mRNA as compared with butyrate, the opposite was true for the induction of this PSA-luciferase reporter in LNCaP cells maintained in culture. This suggests not only that the induction of PSA gene expression by butyrate can be mapped at least in part to this region of the promoter (−630/+12), but that other regions or mechanisms may be involved in the induction by the endogenous PSA gene by R1881 and/or butyrate. Such a mechanism may involve the upstream ARE in the enhancer region (−4148/−4134) of the PSA gene (42).

Examination of the induction of androgen-responsive reporters by butyrate and R1881 in three human prostate cancer cell lines emphasizes the importance of gene and cell specificity in the regulation of PSA gene expression. The PSA reporter was induced to a greater extent by butyrate than R1881 in DU145 cells, whereas in LNCaP and PC3 cells, the induction achieved by these two compounds was relatively equal. The PB reporter was strongly induced by R1881 in LNCaP cells, whereas in PC3 and DU145 cells, the differences between the induction achieved by R1881 and butyrate were less pronounced. Induction of the AR reporter by R1881 was consistently greater than that of butyrate in all three of the cell lines. In DU145 cells, the differences in the level of induction achieved between these two compounds were less pronounced. Butyrate mediated a greater response than R1881 when examining the induction of the PSA reporter in DU145 and LNCaP cells or the induction of the PB reporter in PC3 cells. In contrast, when compared with R1881, butyrate was a relatively poor compound with which to mediate the induction of the PB reporter in LNCaP cells and the AR reporter in LNCaP and PC3 cells. Such differences in the measured responses of these reporters to butyrate, as compared with R1881, in the various cell lines demonstrate gene specificity and cell specificity that may reflect differences.
in the recruitment of coregulators and/or signaling pathways. This is in agreement with a previous report showing promoter specificity when comparing transcription mediated by AR activated by ligand and transcription mediated by AR activated by the PKA pathway (6).

Synergistic increases in activities of the three androgen-responsive reporters were consistently observed in all three prostate cancer cell lines that were exposed to a mixture of R1881 and butyrate, with the exception of PSA in PC3 cells. Because the optimal concentration of R1881 was used (i.e., higher concentrations of R1881 do not mediate additional increases in the induction of the reporter), the involvement of additional mechanism(s) besides those used by R1881 is suggested for butyrate. Such a mechanism does not include butyrate altering the levels of AR protein in whole cell lysates as indicated in the Western blot shown in Fig. 5. Synergistic increases in the activities of androgen-responsive reporters by ligand-mediated rat AR in the presence of modifiers of phosphorylation have been shown previously (3). However, in those studies, there was no observation of ligand-independent activation of the rat AR by these modifiers (3). The synergistic increases observed with the rat AR were suggested to involve changes in its phosphorylation state that altered interaction of the ligand-activated AR with proteins in the transcriptional machinery and was not due to altered levels of AR (3).

A common mechanism that appears to be involved in the induction of PSA gene expression by both R1881 and butyrate in LNCaP cells is dependent on the AR. In addition to the above-mentioned experiments describing the measurement of multiple androgen-responsive reporters, further evidence supporting the role of AR in this mechanism can be drawn from the following: (a) increased AR-ARE complex formation occurred when using nuclear extracts from cells treated with butyrate with oligonucleotides encoding this high-affinity ARE region of the PSA promoter in EMSAs; (b) the induction of PSA mRNA levels by butyrate was blocked by application of an antiandrogen; and (c) Western blots showed an increase in the levels of AR protein in nuclear extracts prepared from LNCaP cells exposed to butyrate.

The PSA promoter contains a high-affinity ARE at $-176/-150$ that binds AR in nuclear extracts from LNCaP cells treated with androgen (11). EMSAs using oligonucleotides encoding this ARE ($-170/-156$) with nuclear extracts from LNCaP cells exposed to butyrate resulted in AR-ARE complex formation that was comparable to that achieved with nuclear extracts from R1881-treated cells. These complexes were not formed when the half-site for the AR was mutated, which was previously shown to prevent AR-ARE formation (6, 11). Thus, butyrate causes an increase AR-ARE complex formation.

Further evidence that the AR is involved in the induction of PSA gene expression by butyrate in LNCaP cells involves the application of the antiandrogen bicalutamide. This antiandrogen has been used in numerous studies to determine the role of AR in the increased expression of various genes (4–6). Bicalutamide is thought to block the AR by inhibiting the dissociation of heat shock proteins, thereby preventing DNA-binding activity and possibly preventing AR nuclear translocation (44–46). In this study, application of bicalutamide completely blocked the induction of PSA mRNA levels by butyrate in LNCaP cells, thereby providing evidence that butyrate increases PSA mRNA through an AR-dependent pathway.

If butyrate activates the AR to increase transcription of androgen-responsive promoters, one would expect butyrate to increase the levels of AR protein in the nucleus. In the absence of androgens, the AR is principally located in the cytoplasm of LNCaP cells (6, 37). In these studies, we showed that the addition of butyrate to LNCaP cells caused an increase in the nuclear levels of AR protein. This suggests that butyrate alters the shuttling of the AR between the cytoplasm and nucleus and supports the role of the AR in the mechanism of the increased transcription of the PSA gene by butyrate. Such changes in the nuclear levels of AR protein were also shown for LNCaP cells treated with forskolin, which stimulates PKA activity to activate the AR (6).

Recently, we have reported cross-talk between the AR and PKA signal transduction pathways in LNCaP cells (6). Therefore, in the present studies, we investigated whether activation of the AR by butyrate to increase PSA gene expression was dependent on the PKA pathway. Through the use of an inhibitor to PKA, we showed that the induction of PSA reporter activity by butyrate could be partially blocked. This suggests that stimulation of the PKA pathway to activate the AR may represent one mechanism involved in the induction of PSA gene expression by butyrate. Additional experiments using point mutations of the putative PKA phosphorylation sites in the AR are under way to more definitively evaluate the role of PKA phosphorylation.

In summary, the data presented here provide evidence that the differentiation agent butyrate causes ligand-independent activation of the AR to increase expression of the differentiation marker PSA in human prostate cancer cells. Vitamin D, which is also a differentiation agent, has similarly been reported to increase PSA gene expression through an AR-dependent pathway (16). Thus, it is not inconceivable that in the absence of androgens, the AR may play a role in the differentiation of prostate cancer cells. In addition, an improved understanding of the pathways controlling transactivation of the AR and the consequent downstream changes in the expression of genes regulated by this transcription factor will provide insights into the molecular events involved not only in differentiation but possibly also in the progression of prostate cancer to androgen independence.

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Ligand-independent Activation of the Androgen Receptor by the Differentiation Agent Butyrate in Human Prostate Cancer Cells

Marianne D. Sadar and Martin E. Gleave


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