ANDROGEN RECEPTOR ACTIVATION BY Gs SIGNALING IN PROSTATE CANCER CELLS

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Running title: Gs activates AR in prostate cancer

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Prostate cancer, the most common noncutaneous malignant transformation in American men (1), starts as an androgen-dependent (AD) lesion in the prostate gland that can be successfully treated with surgical removal of the tumor or local radiation (2). Locally advanced and metastatic diseases are treated with endocrine therapies aimed to: [i] decrease circulating androgen levels via chemical or physical castration, [ii] block androgen receptor (AR) activation with antiandrogens, or [iii] achieve both (3). The hormonal therapies cause only a temporary shrinkage in tumor mass and the cancer invariably reappears in the form of androgen-insensitive (AI) disease. Despite decades of intense basic and clinical research, to date there is no cure for AI prostate cancer.

Accumulating evidence suggests that the AR itself is involved in the transition of prostate cancer from AD to AI (4). AI prostate tumors express AR-regulated genes, including human kallikrein 2 (hK2) and hK3 (Ref. 5), also known as prostate-specific antigen (PSA), suggesting the AR is activated in these tumors despite the continued presence of the hormonal therapies. In vitro, the AR can be activated by factors other than androgens (6,7) and, in animal models increased AR expression was reported to be the only change associated with progression to hormone refractory disease (8). In humans, the AR is overexpressed in up to one third of AI prostate carcinomas, suggesting it could respond to low serum levels of...
androgens (9). Also, AI lesions exhibit frequent mutations in the AR that may allow it to be activated by other androgens or even antiandrogens (10). Hence, AR is a focal point in progression of the prostate cancer to advanced stage. However, the mechanisms regulating AR activation in the presence of low androgen concentrations and their significance to the progression to AI disease remain unclear.

Transition of the prostate cancer to the AI stage is associated with increased expression of plasma membrane-localized guanine nucleotide-binding (G) protein-coupled receptors (GPCRs) (Ref. 11), which mediate cellular responses to a diverse array of extracellular molecules including lipid and peptide growth factors (12). Human prostate cancer biopsies show elevated expression of the GPCRs for endothelin (13), bradykinin (14) and lysophosphatidic acid (R.G. and Y.D., unpublished), compared to benign specimens. The cancerous prostate also expresses elevated levels of GPCR ligands, including endothelin-1, follicle stimulating hormone and several neuropeptides (11,13,15). Thus, prostate tumors express elevated levels of GPCRs as well as their ligands, suggesting these receptors are ‘on’ and, therefore, may contribute to progression of the disease. The GPCRs transduce their signals primarily via activation of heterotrimeric G proteins to produce Gα-GTP and Gβγ subunits. The G proteins are divided into four groups; Gs, Gi, Gq and G12. Gs and Gi regulate mainly adenylyl cyclases that produce adenosine 3',5'-cyclic monophosphate (cAMP), Gq regulates phospholipases that control intracellular Ca²⁺ levels, and G12 regulates low molecular weight GTPase Rho and other effectors. The Gβγ subunits regulate activity of many effectors, including phospholipase C and serine/threonine and tyrosine kinases (11,12).

In vitro, stimulation of endogenous GPCRs, including those for lysosphatidic acid (16,17), bombesin (18) and bradykinin (14), induces mitogenic signaling and growth of AI prostate cancer cells. In animal models, the expression of a Gβγ subunit inhibitor peptide attenuates prostate tumor growth (19). Here, we tested the hypothesis that G proteins exert their mitogenic signals in prostate cancer cells by activating the AR. We found that activation of Gs activates the AR and reduces the concentration of androgen necessary for maximal activation of the AR. Our data demonstrate that AR can be fully activated in the presence of castrate levels of androgen if Gαs is activated. These results predict failure of hormonal therapies in cancer patients in which the Gs signaling axis is activated.

EXPERIMENTAL PROCEDURES

Reagents: The mammalian expression plasmids were kindly provided as follows: probasin-luciferase reporter, Z. Culig (University of Innsbruck); ARR2-luciferase reporter, R. Matusik (Vanderbilt University); SV40-Renilla luciferase reporter, H. Matsunami (Duke University); constitutively activated Gα, R.T. Premont (Duke University) and the Guthrie cDNA Resource Center (Sayre, PA); phosphodiesterase (PDE) 4D and Flag-epitope tagged vasodilator-stimulated phosphoprotein (VASP), R.J. Lefkowitz (Duke University) and wild type and mutated (S650A) AR, M. J. Weber (University of Virginia). Flag-tagged Gαs-ct was generated by PCR
amplification of a 310-bp fragment that encodes amino acids 303-410 of human Ga\(\alpha\)s and was cloned into EcoRI-XhoI sites in pcDNA3.1 vector. The 5 \(\alpha\)-dihydrotestosterone (DHT) and isoproteinerol (ISO) were purchased from Sigma (St. Louis, MO). The synthetic androgen R1881 was purchased from PerkinElmer (Boston, MA) and the anti-androgen bicalutamide was the generous gift of Astra Zeneca (Wilmington, DE). The androgens DHT and R1881 were used interchangeably throughout the study and gave similar results in all assays used. Antibodies were obtained as follows: anti-AR, BioGenex (San Ramon, CA); anti-PSA, Dako (Carpinteria, CA); anti-Ga\(\alpha\)s, Calbiochem (San Diego, CA); M2 anti-Flag, Sigma; anti-enolase, NeoMarkers (Fremont, CA); anti-actin, Transduction Labs (Lexington, KY); and anti-mouse IgG-CY3, Jackson ImmunoResearch Labs (West Grove, PA).

**Cell Culture:** Androgen-dependent LNCaP cells were obtained from ATCC (Manassas, VA) and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 10 mM HEPES buffer, pH 7.5, 1 mM sodium pyruvate, and 1.26 g/L glucose. The androgen-dependent LAPC4 cells were generously provided by C.L. Sawyers (University of California).

**Luciferase assay:** Cells were seeded in 100-mm dishes and transfected at 70% confluence using DMRIE-C (Invitrogen; Carlsbad, CA). Transfections were carried out with cDNAs encoding the probasin or AR2 luciferase (1 \(\mu\)g) and SV40-Renilla luciferase (10 ng) together with empty vector or the indicated gene of interest using a total of 6 \(\mu\)g DNA/plate. At the end of transfection, cells were equally divided into 6-well plates and allowed to attach. The culture medium was replaced after 24 hr with starvation medium (phenol red-free RPMI 1640 containing 5% charcoal-stripped FBS, 1% penicillin-streptomycin and 10 mM HEPES buffer, pH 7.5) and identical cell populations, in duplicate, were stimulated with androgen, ISO or vehicle for additional 24 to 48 hr. Luciferase activities in cell lysates were measured using the Dual Luciferase assay system (Promega; Madison, WI) and were normalized by the Renilla activities and protein concentrations of the samples. Results are presented as fold increase over baseline by dividing the relative luciferase activity of the treated cells over the value obtained for unstimulated cells.

**cAMP assay:** Accumulation of cAMP was determined using the direct cyclic AMP assay kit (Correlate-EIA\(^{\text{TM}}\) kit) from Assay Designs, Inc. (Ann Arbor, MI). Briefly, LNCaP cells (10\(^6\) cells/well in 24-well plates) were stimulated with ISO (10 \(\mu\)M) or testosterone (1 \(\mu\)M) for 10 min at 37 \(^\circ\)C, lysed using 0.1 N HCl and lysates processed according to the manufacturer’s instructions. The assay was performed in duplicate and repeated three times.

**Western blot analysis:** Appropriately treated cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 1 mM EDTA, 0.25% (w/v) sodium deoxycholate, 0.1% (v/v) Nonidet P-40, 1 mM NaF, 1 mM sodium pyrophosphate, 100 \(\mu\)M Na\(_3\)VO\(_4\), 1 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml leupeptin, 10 \(\mu\)g aprotonin and 0.7 \(\mu\)g/ml pepstatin) and analyzed by SDS-PAGE and Western blotting (14,16).

**Immunostaining:** Cells on glass coverslips were labeled with mouse monoclonal anti-AR antibodies at a
dilution of 1:200, washed with PBS then mounted and inspected under a Zeiss AxioVision 3.1 fluorescence microscope (14,19). The 4′-6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining.

RESULTS

Expression of activated Gα proteins activates the AR. Activation of endogenous GPCRs induces mitogenic signaling and growth of prostate cancer cells, and inhibition of G protein signaling attenuates the growth of prostate tumors in xenograft animal models (11). We tested the hypothesis that G proteins are capable of being mitogenic via activation of the AR. We examined the effects of G proteins on AR activation using androgen receptor response element (ARE)-regulated luciferase reporters and constitutively activated Gα subunits (Gαq Q209L, Gαz Q205L, Gαi-1 Q204L, Gα12 Q231L, Gα13 Q226L or Gαs Q227L) in LNCaP cells that endogenously express the AR. Cells were incubated in phenol red-free culture medium supplemented with 5% charcoal stripped serum (starvation medium) and reporter activity determined after overnight stimulation with DHT (10 nM). Expression of activated Gαq and Gαs, but not Gαz, Gαi-1, Gα12 or Gα13 resulted in an increase in basal AR activity (Figure 1A). On average, expression of constitutively active Gαq and Gαs induced 4- and 10-fold increases in AR activity, respectively. We observed that treatment with DHT promoted about 20-fold increase in reporter activity, compared to untreated cells (Figure 1A). The transient expression of activated Gαs, Gαq, Gαi-1, Gα12 or Gα13 did not significantly impact on the androgen-mediated increases in AR activity, while the expression of activated Gαz reduced AR activation by roughly 30% (Figure 1A).

Stimulation of endogenous β2AR activates the AR. Because its effect was the greatest, we focused our attention on Gs, which becomes activated in response to stimulation of a number of GPCRs (11,12). Gene expression analysis of GPCR signaling pathways using GEArray filters revealed that LNCaP cells express about 100-fold more of the Gs-coupled β2 adrenergic receptor (β2AR) than β1AR (data not shown). Therefore, we used the β2AR as a model Gs-coupled receptor to test the hypothesis that receptor-mediated activation of Gs can activate the AR. Stimulation with ISO induced the dose-dependent increase in ARE-regulated reporter activity (Figure 1B). Maximal response was achieved at an ISO concentration of 10^-6 M with an EC50 of 10^-7 M. The ISO-stimulated AR activation is mediated by β2AR, because addition of propranolol, a β2AR antagonist, abrogated the ligand-mediated increase in activated AR (data not shown). Maximal induction of reporter activity by ISO was roughly 40-50% of that achieved by androgen (Figure 1B), implying an important physiologic function for β2AR in the activation of AR. The increase in ARE-luciferase reporter activity was dependent on AR because treatment of cells with the AR antagonist bicalutamide attenuated the ISO- and DHT-mediated effects. Interestingly, treatment of cells with ISO enhanced activity of the AR partial antagonist hydroxyflutamide; a combination of ISO and hydroxyflutamide activated AR more than either reagent alone (data not shown).
To further validate the finding of ISO-induced activation of the AR, we used two additional assays; nuclear expression of endogenous AR and expression of the ARE-regulated PSA protein. In the inactive state, AR forms complex with heat shock proteins and is mostly sequestered in the cytoplasm (20). Upon activation, the AR is stabilized in the nucleus. We determined the effect of ISO treatment on subcellular localization of endogenous AR in LNCaP cells using immunofluorescence, and observed that treatment with ISO induced time-dependent nuclear import of the AR (Figure 1C). Once in the nucleus, active AR binds AREs in promoter and enhancer regions of specific genes to control their transcription (20). We examined whether ISO treatment could induce expression of the ARE-regulated PSA gene product using Western blot analysis. ISO, like DHT, induced a time-dependent increase in PSA expression (Figure 1D). To demonstrate these responses are not restricted to the LNCaP cells, we used another AD prostate cancer cells, LAPC4 (21). Stimulation with ISO induced the activation of ARE-regulated reporter in these cells as well (data not shown), affirming generality of the response.

**Synergy between Gs and androgen to activate the AR.** Prostate cancer patients undergoing endocrine ablative therapy have reduced, but not eliminated circulating androgens as the adrenal glands can still produce androgens that bind to and activate the AR (22). Therefore, we examined whether expression of activated Gαs influences the androgen-induced activation of the AR. Mock-transfected LNCaP and LNCaP cells transiently expressing activated Gαs were treated with increasing concentrations of DHT, and ARE-regulated reporter activity determined. DHT promoted the concentration-dependent increase in AR reporter activity with an EC50 of 10^-9 M (Figure 2A), and maximal response was achieved at a DHT concentration of 10^-8 M. Importantly, expression of activated Gαs resulted in increased basal AR transcriptional activity, as well as reduced the DHT concentration required to achieve the maximal (10^-9 M) response (Figure 2A).

To further examine the possible crosstalk between Gαs and the AR, we asked whether stimulation with ISO could cooperate with low concentrations of androgen (like those found in patients undergoing androgen ablative therapy) to fully activate the AR. Cells were exposed to fixed concentrations of DHT (10^-10 or 10^-11 M) that only minimally activate the AR (see Figure 2A). As expected from the studies described above, ISO alone could induce the dose-dependent increase in reporter activity (Figure 2B). Remarkably, in the presence of 10^-10 M DHT, ISO treatment activated the AR to similar levels as those achieved by saturating concentrations of androgen. Together, these data demonstrate that simulation of endogenous β2AR-Gs signaling cooperates with castrate levels of androgen to maximally activate the AR.

Expression of activated Gαs (Figure 1A) as well as stimulation of β2AR (Figure 2B) promotes the activation of the AR. To establish a role for Gs in the β2AR-mediated activation of the AR, we employed a peptide inhibitor of receptor-mediated Gs signaling, Gαs-ct (23). Expression of the Gαs-ct peptide (Figure 2C) obliterated the ISO-mediated activation of the AR (Figure 2D). Remarkably, the expression
of Gs-ct peptide also significantly impacted the direct activation of AR by androgen (Figure 2D). These data demonstrate involvement of Gs in the β2AR-mediated activation of AR and suggest that some low level of Gs signaling is permissive for androgen-mediated activation of the AR as well.

cAMP regulates AR activation.

Stimulation of the β2AR induces the Gs-mediated activation of the AR. The best-studied signaling pathway engaged by activated β2AR involves the Gs-mediated activation of adenylyl cyclases that synthesize cAMP. Stimulation of the LNCaP cells with ISO induced the robust accumulation of intracellular cAMP and, unexpectedly, treatment with androgen also promoted the modest, but significant accumulation of cAMP (Table 1). To implicate cAMP in the signaling pathway from activated β2AR to AR, we examined effect of the cAMP-degrading enzyme phosphodiesterase (PDE) on AR activation. Ectopic expression of the PDE4D isoform (24) almost completely suppressed the ISO-induced activation of the AR (Figure 3A). The expression of PDE4D also significantly attenuated the androgen-mediated activation of the AR (Figure 3A), again arguing for a permissive role for cAMP in the androgen-mediated activation of the AR. Together with the studies described above, these data establish existence of a β2AR → Gαs → cAMP → AR signaling pathway.

Modulations in cellular levels of cAMP control activity of several target proteins, including protein kinase A (PKA), Rap exchange factor activated by cAMP (Epac), and cyclic nucleotide-gated ion channels. The most widely-studied of these target proteins is PKA; hence we tested ability of ISO to activate PKA in LNCaP cells by measuring the phosphorylation state of VASP, a known substrate of PKA (25). In the absence of β2AR stimulation, VASP is unphosphorylated, but becomes phosphorylated in response to stimulation with ISO (Figure 3B). Next, we tested whether PKA plays a role in the activation of the AR. First, we determined optimal concentration of the specific PKA inhibitor isouquinoline H89 (Ref. 26) needed to block PKA activation in the LNCaP cells. Pretreatment with H89 resulted in the dose-dependent decrease in phosphorylated VASP following stimulation with ISO (Figure 3B) with essentially complete inhibition of PKA activation at H89 concentration of 30 µM. A similar treatment of the LNCaP cells with H89 resulted in the dose-dependent reduction of ISO-induced activation of the AR (Figure 3C). The treatment with androgen also promoted the modest phosphorylation of VASP (data not shown) and H89 inhibited, in a dose-dependent manner, the androgen-mediated activation of the AR (Figure 3D). These results are consistent with data showing that inhibition of PKA activity with the peptide PKI attenuates the androgen-induced activation of AR in monkey kidney CV1 cells (27).

To further confirm a role for PKA in the Gs-mediated AR activation, we tested the effect of H89 on agonist-stimulated expression of endogenous PSA protein. LNCaP cells were pretreated with H89 (30 µM) followed by stimulation with ISO or R1881. We observed that exposure to H89 attenuated both ISO- and R1881-induced expression of PSA (Figure 3E). Together, these data demonstrate that PKA regulates both the transactivation of AR by ISO and the activation of AR by androgens.
The AR is a phosphoprotein and reversible phosphorylation of AR is required for its activity (28). A recent study showing treatment of cells with the adenylyl cyclase activator forskolin promotes phosphorylation of the AR on residue S650 led to the conclusion that PKA phosphorylates AR only on S650 to regulate its activity (29). To assess whether this event could account for our observations of PKA-dependent activation of the AR, we examined ability of the AR S650A mutant to be transcriptionally active. Human embryonic kidney (HEK)-293 cells, which do not express endogenous AR, were transiently transfected with cDNAs encoding ARE-reporter alone, or together with wild-type or S650A forms of AR. Treatment with either ISO or DHT was required for the ectopically expressed AR to promote increases in the ARE-regulated reporter activity (Figure 4A), demonstrating the utility of this heterologous system. Importantly, both wild-type and S650A AR could equally support the ISO- and DHT-mediated increase in reporter activity, implying that PKA-induced phosphorylation of the AR on S650 is not required for ISO- or androgen-regulated activation of the AR.

**PKA regulates the subcellular localization of AR.** The data described above indicate that PKA plays a central role in ISO- and androgen-mediated activation of AR. To begin to address just how PKA regulates AR activity, we examined effect of PKA inhibition on subcellular localization of the AR following agonist treatment. Both ISO and R1881 caused the import of AR into the nucleus of LNCaP cells (Figure 4B). Treatment with H89 inhibited both the ISO- and androgen-induced nuclear translocation of the AR (Figure 4B,C), demonstrating PKA regulates AR function, at least in part, by affecting its nucleocytoplasmic distribution.

**DISCUSSION**

Prostate cancer patients with locally advanced or metastatic disease are commonly treated with endocrine therapies aimed at inhibiting AR function, which cause temporary shrinkage in tumor mass (3,5). However, the cancer invariably reappears as androgen-insensitive lesion characterized by aggressive growth and ability to colonize distal organs; this is the reason for majority of prostate cancer-related deaths. Androgen insensitive cancer cells express AR and require a functioning AR to grow (4). Hence, identification of means by which cancer cells activate the AR, especially in the presence of low concentrations of androgen, is critical for improving outcome of late-stage disease. In this study we identified a new, GPCR-mediated pathway to activate the AR. We found that activation of Gs signaling pathways activates the AR and synergizes with low concentrations of androgen to fully activate the AR. The data presented are consistent with the model depicted in Figure 4D. Stimulation with ISO induces AR activation via Gs (inhibited by Gαs-ct), cAMP (inhibited by PDE4D) and PKA (inhibited by H89). Signal relay from stimulated β2AR to PKA is well established and involves Gs, adenylyl cyclase and cAMP (30). Less clear is how PKA regulates AR activation. The AR is a phosphoprotein and a recent study suggested it may serve as a direct substrate of PKA (29). However, mutation of the only identified PKA phosphorylation site (S650) does not
affect the AR activation, suggesting PKA regulates AR function by an alternate mechanism. PKA may mediate extracellular signal-regulated kinase (ERK) activation (31), which was reported to directly phosphorylate the AR and to control its function (7). However, neither androgen nor ISO could induce the detectable activation of ERK in the LNCaP cells (data not shown). Also, treatment of LNCaP cells with epidermal growth factor does not activate the AR, in agreement with published data (32,33), but promotes the robust phosphorylation of ERK. Hence, it does not appear that ERK is involved in the pathway via which PKA activates the AR. Rather, our data indicate that PKA controls AR function, at least in part, by regulating the nucleocytoplasmic shuttling of the AR, since the inhibition of PKA led to sequestration of AR in the cytoplasm. Regulation of the subcellular localization of AR is likely controlled by receptor coregulatory factors. Indeed, it is widely accepted that the cytoplasmic retention of AR is mediated by its binding to heat shock protein 90 (HSP90) (Ref. 34). In this regard, it is intriguing to note that a recent report showed that PKA can directly phosphorylate HSP90 in vitro (35). Thus, it is possible that PKA-mediated phosphorylation of HSP90 serves to uncouple it from AR thereby allowing the receptor to migrate into the nucleus to become transcriptionally competent. Alternatively, PKA may act as a general regulator of nucleocytoplasmic shuttling of proteins (36), possibly via the phosphorylation of nuclear localization signal-binding proteins (37,38).

The use of low concentrations of androgen to recapitulate the situation in prostate cancer patients undergoing endocrine therapies helped to reveal the presence of a positive cooperative crosstalk between Gαs signaling and androgen to activate the AR. Co-exposure of prostate cancer cells to low concentrations of androgen that alone are insufficient to elicit measurable AR activation and ISO promoted the full activation of the AR. This finding may have important clinical implications, as many patients that become refractory to endocrine therapies still express normal levels of AR-regulated genes (5), suggesting the AR is fully functional. The results, therefore, predict that prostate cancer cells in which the Gαs-PKA axis is activated would be resistant to endocrine therapies.

An unexpected finding in our studies was that androgen-mediated activation of the AR is attenuated in the presence of inhibitors of Gs signaling pathway. The inhibition of Gs, cAMP or PKA all obliterated activation of the AR by low androgen concentration or ISO, and also significantly reduced AR activation by the high androgen concentration alone. Two possibilities exist to explain these results. In the first scenario, a tonic level of Gs signaling is required for the efficient activation of the AR by androgens. For example, cAMP is a known regulator of the AR coactivator cAMP response element-binding protein-binding protein (CBP), which possesses AR and histone acetylase activity and relaxes chromatin to allow formation of transcription initiation complexes (39). Also, activated PKA may phosphorylate the AR coactivator GT198 to enhance AR transcriptional activity (40), or proteins involved in nucleocytoplasmic shuttling (35-38). In the second scenario, Gs becomes activated in response to stimulation of cells with androgen.
Indeed, our data show that stimulation with androgen induced about two-fold increase in cAMP levels, compared to unstimulated samples, and recent reports showed progestin (41) and thyroid (42) hormones activate G proteins via the binding to plasma membrane-anchored receptors. Hence, it is possible to envision a similar situation for androgen; the stimulation of prostate cells with androgen initiates Gs-cAMP signaling to activate PKA. Importantly, PKA is activated in human prostate tumors (data not shown) and appears to be required for the full genomic action of the AR by androgen.

ABBREVIATIONS

AR, androgen receptor; ARE, AR response element; cAMP, adenosine 3',5'-cyclic monophosphate; DHT, 5α-dihydrotestosterone; GPCR, G protein-coupled receptor; ISO, isoproterenol; PKA, protein kinase A; PSA, prostate specific antigen.

ACKNOWLEDGMENTS

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REFERENCES

Table 1: Agonist-induced cAMP accumulation in LNCaP cells.

<table>
<thead>
<tr>
<th>Condition</th>
<th>cAMP Concentration (nM)</th>
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<tr>
<td>Basal</td>
<td>0.396 ± 0.092</td>
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<tr>
<td>ISO (10 µM)</td>
<td>16.153 ± 1.584</td>
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<tr>
<td>Testosterone (1 µM)</td>
<td>0.975 ± 0.216</td>
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\[cAMP production was measured by EIA using LNCaP cells grown for 24 hr in serum-free medium and then stimulated for 10 min with ISO or testosterone. Values represent mean ± SEM of three experiments performed in duplicate.\]

**FIGURE LEGENDS**

**Figure 1.** Activated Ga proteins transactivate AR. (A) Effect of activated Ga proteins on AR-responsive promoter activity. LNCaP cells were cotransfected with firefly and Renilla luciferase genes driven by probasin and SV40 promoters, respectively, alone or together with Ga\(q\) Q209L, Gaoz Q205L, Ga\(i\)-1 Q204L, Ga\(a\)12 Q231L, Ga\(a\)13 Q226L or Ga\(s\) Q227L. Cells were incubated in starvation medium in the presence, or absence, of DHT (10 nM). Each point represents the mean ± S.D. of the normalized luciferase activities obtained from five independent experiments performed in duplicate. *P < 0.01 compared to unstimulated samples. **P < 0.05 compared to DHT-treated wild type cells. NS, control not stimulated cells; CN, cells not transfected with cDNAs encoding activated Ga proteins. (B) ISO transactivates AR. Cells were transfected with the luciferase genes and treated with increasing concentrations of DHT (10\(^{-13}\) to 10\(^{-7}\) M) or ISO (10\(^{-10}\) to 10\(^{-7}\) M). Normalized luciferase activities are presented as mean ± S.D of three experiments done in duplicate. (C) ISO promotes the nuclear import of AR. Cells were stimulated with DHT (10 nM) or ISO (10 µM) and endogenous AR localization
determined by immunofluorescence staining. (D) ISO induces expression of PSA protein. Cells were stimulated with DHT (10 nM) or ISO (10 μM), lysed, and PSA expression determined by immunoblotting.

Figure 2. **Synergy between activated Gs and androgen to activate AR.** (A) Gαs Q227L increases the efficacy of androgen-regulated activation of AR. Cells were cotransfected with firefly and Renilla luciferase genes driven by probasin and SV40 promoter, respectively, alone or together with Gαs Q227L. Cells were treated with increasing concentrations of DHT. Each point represents the mean ± S.D. of the normalized luciferase activities obtained from five independent experiments performed in duplicate. CN, wild type cells. (B) ISO potentiates AR activation. Cells were treated with DHT (10^{-11} or 10^{-10} M), or not, and increasing concentrations of ISO. Each point represents the mean ± S.D. of the normalized luciferase activities obtained from three independent experiments performed in duplicate. (C) A representative immunoblot of total LNCaP cell lysates to demonstrate the expression of Gαs-ct peptide. NT, not transfected, Gs-ct, cells transfected with pcDNA3.1-Flag-Gαs (aa 303-410). Arrow indicates Gαs-ct peptide and double arrows indicate endogenous Gαs proteins. (D) Gαs-ct inhibits the ISO- and androgen-mediated activation of AR. Cells were cotransfected with luciferase reporter genes alone or together with Gαs-ct and treated with R1881 (5 nM) or ISO (10 μM). Each point represents the mean ± S.D. of the normalized luciferase activities obtained from four independent experiments performed in duplicate. *P < 0.05 compared to stimulated cells. NS, control not stimulated cells; NT, cells not expressing Gαs-ct peptide.

Figure 3. **Mechanisms of ISO-regulated transactivation of AR.** (A) PDE4D inhibits the ISO- and androgen-mediated activation of AR. Cells were cotransfected with firefly and Renilla luciferase genes, alone or together with PDE4D. Cells were treated with R1881 (5 nM) or ISO (10 μM). Each point represents the mean ± S.D. of the normalized luciferase activities obtained from four independent experiments performed in duplicate. *P < 0.01 compared to unstimulated samples. **P < 0.05 compared to stimulated cells. NS, control not stimulated cells; NT, cells not transfected with PDE4D gene. (B) H89 inhibits the ISO-mediated phosphorylation of VASP. Cells were transfected with a gene encoding Flag-VASP, pretreated with the indicated concentration of H89 for 30 min and stimulated with ISO (10 μM) for 5 min. Total cell lysates were immunoblotted with M2 anti-Flag antibody. (C, D) H89 inhibits the ISO- and DHT-regulated transactivation of AR. Cells were pretreated with the indicated concentration of H89 for 30 min prior to stimulation with increasing concentrations of ISO or DHT. Each point represents the mean ± S.D. of the normalized luciferase activities obtained from five independent experiments performed in duplicate. (E) H89 inhibits the ISO- and R1881-mediated expression of endogenous PSA protein. LNCaP cells were pretreated with H89 (30 μM) for 30 min prior to daily stimulation with ISO (10 μM) or R1881 (5 nM) for two days. Total cell lysates were immunoblotted with anti-PSA antibody (upper panel). The filter was stripped of IgG and reblotted with anti-actin antibody (lower panel) to show the equal protein loading in each lane.
Figure 4. **PKA regulates the nuclear expression of AR.** (A) S650A AR is transcriptionally competent. HEK-293 cells were cotransfected with firefly and Renilla luciferase genes, alone or together with wild type or S650A AR. Cells were treated with DHT (10 nM) or ISO (10 µM). Each point represents the mean ± S.D. of the normalized luciferase activities obtained from five independent experiments performed in duplicate. NT, control cells not transfected with AR; NS, cells not treated with agonist. (B) H89 inhibits nuclear expression of AR. LNCaP cells were pretreated with H89 (30 µM) for 30 min prior to stimulation with ISO (10 µM) or R1881 (5 nM). Subcellular localization of endogenous AR was determined by immunofluorescence. Data are presented as percent AR in the nucleus and were obtained from at least 100 cells for each treatment group. (C) A representative immunofluorescence image. (D) A model for signal relay from Gs-coupled receptors to AR. See text for more details. H, hormone; AC, adenylyl cyclase.
Figure 1

A. Graph showing RLU values for different treatments.

B. Graph showing RLU values as a function of log10 ligand concentration.

C. Images showing cellular responses over time.

D. Western blot analysis showing PSA expression over time.