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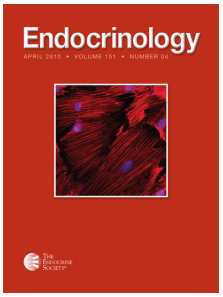

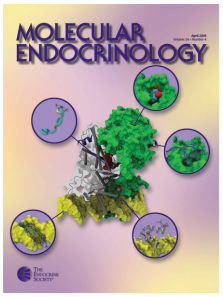

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Selective Modulation of Genomic and Nongenomic Androgen Responses by Androgen Receptor Ligands

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Steroids can induce both transcription-dependent (genomic) and independent (nongenomic) signaling. Here, several classical androgen receptor ligands were tested for their ability to modulate genomic and nongenomic responses, focusing on the role of the oocyte-expressed *Xenopus* classical androgen receptor (XeAR) in mediating these processes. Cellular fractionation and immunohistochemistry revealed that the XeAR was located throughout oocytes, including within the plasma membrane. RNA interference and oocyte maturation studies suggested that androgen-induced maturation was mediated in part by the XeAR in a transcription-independent fashion, perhaps by altering G protein-mediated signaling. While inducing minimal transcription in oocytes, all AR ligands promoted significant XeAR-mediated transcription in CV1 cells. In contrast, only testosterone and androstenedione potently induced oocyte maturation, whereas dihydrotestosterone and R1881 actually inhibited testosterone and human chorionic gonadotropin-induced maturation and signaling. These results suggest that the nature of a steroid-induced signal (genomic *vs.* nongenomic) may depend on the type of target cell, the receptor location within cells, as well as the ligand itself. The identification of molecules capable of selectively altering genomic *vs.* nongenomic signaling may be useful in delineating the roles of these pathways in mediating androgen responses and might lead to the development of novel compounds that specifically modulate these signals *in vivo*. (*Molecular Endocrinology* 17: 1106–1116, 2003)

STEROID HORMONES ARE traditionally known to mediate most of their signaling and subsequent biological activities by modulating transcription within target cells through interactions with receptors in the cytoplasm or nucleus. In recent years, however, increasing evidence has suggested that many steroid-induced signaling events are triggered independent of transcription. Further, some of these transcription-independent, or nongenomic, signaling events seem to be mediated by the same classical steroid receptors that modulate steroid-induced transcription. For example, classical estrogen receptors within the plasma membrane appear to be mediating estrogen-induced activation of Src in osteoblast (1) and breast cell lines (2, 3), as well as rapid increases in estrogen-induced phosphatidylinositol 3-kinase and nitric oxide synthase activities in vascular endothelial cells (4–7)

In addition to mediating nongenomic estrogen effects, classical steroid receptors may also be promoting nongenomic steroid-mediated signaling in *Xenopus laevis* oocytes. (8–11). Modulation of classical progesterone receptor (PR) levels in oocytes by overexpression of PR or through the use of antisense oligonucleotides to reduce PR expression modestly altered progesterone-mediated maturation of isolated oocytes, suggesting that the PR might be playing a role in mediating progesterone-induced signaling *in vitro* (9, 11). Furthermore, androgen-induced maturation, which may be the primary physiologic pathway mediating *Xenopus* oocyte maturation *in vivo*, was attenuated by classical androgen receptor (AR) antagonists, suggesting that androgen-induced signaling in oocytes may be in part mediated by a classical *Xenopus* androgen receptor (XeAR; Refs. 10 and 12). Interestingly, although testosterone and androstenedione (AD) are equally or more potent promoters of oocyte maturation than progesterone and are produced at significantly higher levels than progesterone *in vivo*, no studies to date have confirmed that androgen-induced oocyte maturation does indeed occur independent of transcription. Furthermore, the role of dihydrotestosterone (DHT), the most potent physiologic promoter of mammalian AR-mediated transcription, in mediating *Xenopus* oocyte maturation has yet to be determined.

The effects of AD, testosterone, DHT, and the AR ligand R1881 on both genomic and nongenomic signaling were examined to 1) characterize the signaling

Abbreviations: AD, Androstenedione; AR, androgen receptor; CMV, cytomegalovirus; DHT, dihydrotestosterone; DME/H/BSA, DMEM containing 20 mM HEPES and 1 mg/ml BSA; DNABD, DNA binding domain; hCG, human chorionic gonadotropin; HuAR, human AR; K_d, equilibrium constant; LBD, ligand binding domain; MBSH, modified Barth's solution; M2R, muscarinic receptor type 2; MMTV, mouse mammary tumor virus; PCOS, polycystic ovarian syndrome; PR, progesterone receptor; XeAR, *Xenopus* classical AR; XePR, *Xenopus* classical PR.

pathways involved in androgen-induced maturation; 2) further examine the role of the XeAR, in mediating androgen-induced maturation; and 3) compare the relative abilities of DHT and other compounds to promote oocyte maturation.

We found that androgen-induced maturation did in fact occur independent of transcription, and, like progesterone-induced signaling, could be inhibited by G$\beta\gamma$ signaling. Further, injection of double-stranded RNA oligonucleotides targeted against the XeAR into oocytes specifically attenuated AD-mediated maturation and activation of the MAPK signaling pathway, suggesting that the XeAR was playing a role in mediating these processes. Finally, whereas testosterone, DHT, and R1881 were all potent promoters of XeAR-mediated transcription, only AD and testosterone induced nongenomic signaling and maturation in oocytes. In fact, both DHT and R1881 served as antagonists of testosterone- and human chorionic gonadotropin (hCG)-induced oocyte maturation. The identification of AR ligands that differentially modulate genomic and nongenomic signaling may lead to the development of other selective AR modulators that can be useful both scientifically and clinically.

RESULTS

Androgen-Induced Maturation of *Xenopus* Oocytes Is Transcription Independent

Progesterone-mediated maturation is known to occur independent of transcription (13). To test the role of transcription in androgen-mediated maturation, oocytes were incubated with actinomycin D for 24 h before the addition of testosterone. As seen in Fig. 1A, testosterone-induced maturation was identical both in the absence and presence of actinomycin D. Under these conditions, actinomycin D was a potent inhibitor of transcription within the oocyte, reducing luciferase production by a nuclear-injected cytomegalovirus (CMV)-luciferase plasmid by greater than 99% (Fig. 1B). Actinomycin D had no effect on androgen-induced activation of the MAPK signaling pathway as well (data not shown), confirming that this earlier androgen-induced signaling event was also transcription independent.

Nongenomic Androgen-Induced Maturation and Signaling Are Inhibited by G$\beta\gamma$ Signaling

Previous work has demonstrated that nongenomic progesterone-induced maturation and signaling in *Xenopus* oocytes might be occurring by a "release of inhibition" model, whereby addition of progesterone overcomes a constitutive G$\beta\gamma$ signaling that is inhibiting maturation (14). The nongenomic nature of androgen-induced maturation suggested that androgens might be functioning in a similar fashion. As seen with progesterone, overexpression of G$\beta\gamma$ in oocytes

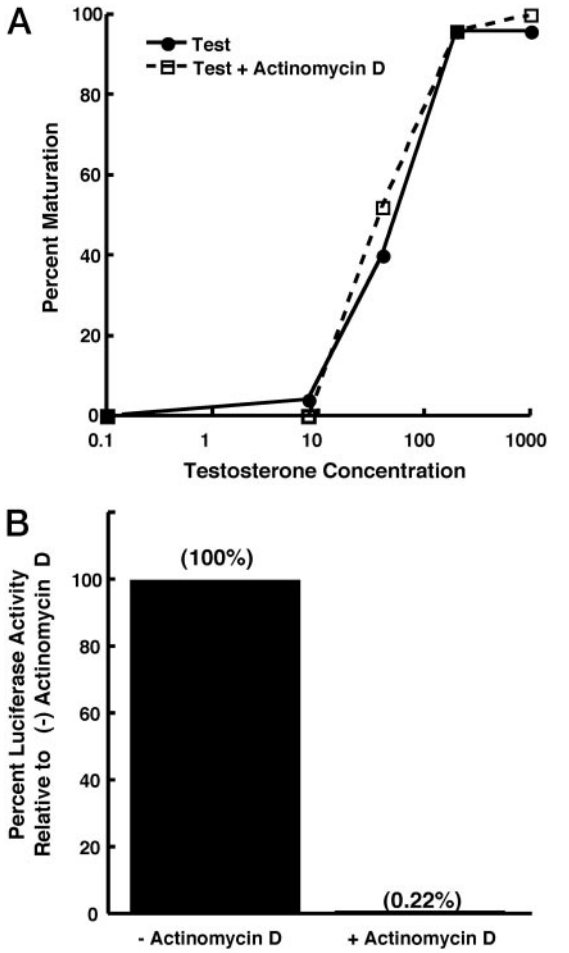

Fig. 1. Testosterone-Induced Oocyte Maturation Is Transcription Independent

Oocytes were treated with either ethanol or 10 μg/ml actinomycin D for 24 h. Oocytes were then injected with the CMV-luciferase expression vector and incubated overnight with either ethanol or testosterone at the indicated concentrations in the continued presence of actinomycin D. The percent maturation at each concentration of testosterone is indicated in A, whereas the percent of luciferase activity relative to ethanol-treated (without actinomycin D) oocytes is indicated in B. This experiment was performed three times with identical results.

significantly inhibited testosterone-induced maturation (Fig. 2A) and phosphorylation of p42 (90% inhibition; Fig. 2B). Further, activation of endogenous G$\beta\gamma$ signaling by overexpression of the Gα_i-coupled muscarinic receptor type 2 (M2R) and treatment with the M2R agonist carbachol inhibited both testosterone-induced maturation (Fig. 2C) and phosphorylation of p42 (90% inhibition; Fig. 2D).

Nongenomic Androgen-Mediated Signaling and Maturation Is Mediated in Part by the Classical XeAR

As mentioned in the *Introduction*, classical steroid receptors appear to play important roles in mediating

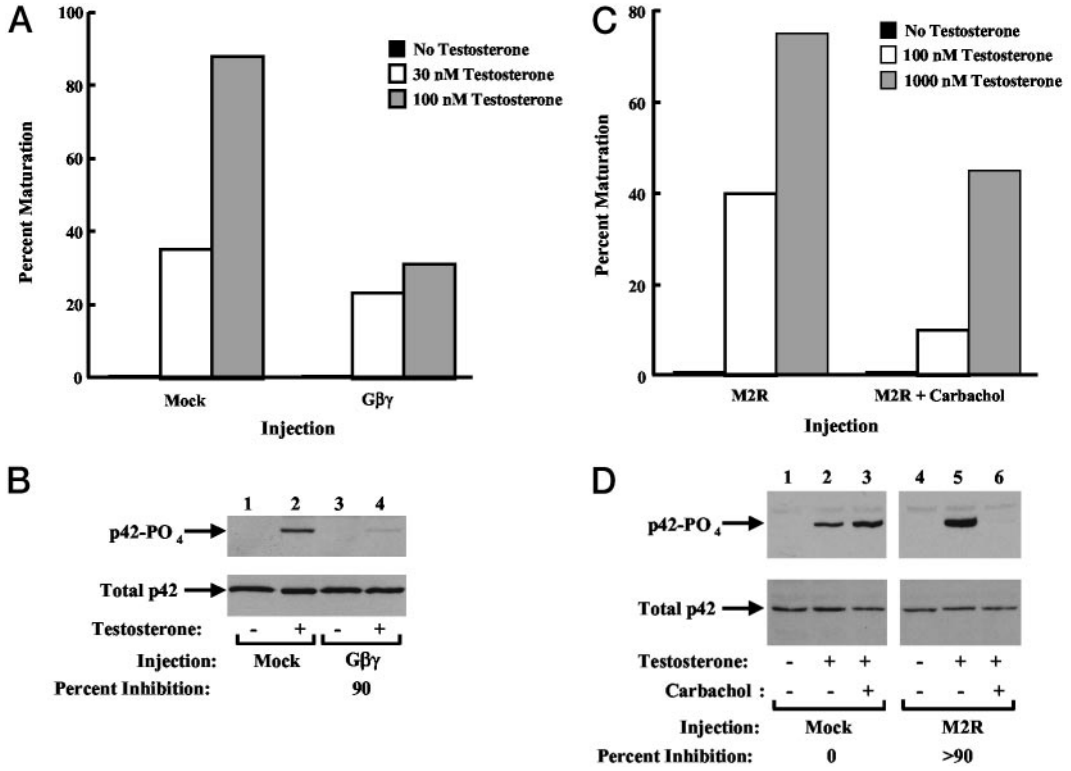

Fig. 2. Gβγ-Mediated Signaling Inhibits Testosterone-Induced Maturation and Phosphorylation of p42 ERK

Oocytes were injected with either 10 mM HEPES (Mock) or cRNAs encoding the Gβ and Gγ proteins (A and B), or the M2R (C and D). Maturation or MAPK assays were performed at 36 and 48 h, respectively. A, Oocytes were incubated overnight with either ethanol (no testosterone) or the indicated concentration of testosterone. Maturation was then determined visually. B, Oocytes were incubated for 4 h with either ethanol (−) or 500 nM testosterone (+), followed by Western blot analysis using the anti-phospho-p44/p42 (*top*) or the antitotal-p44/p42 antibodies (*bottom*). The percent decrease in phosphorylation relative to ethanol-treated cells was calculated using NIH Image Software and is noted underneath (90%). Note that *Xenopus* oocytes only express p42. C and D, Maturation and MAPK assays were performed as in A and B, only oocytes were treated with either water or 30 μM carbachol for 1 h before and throughout the incubations with testosterone (as indicated or at 100 nM for the MAPK assay). All experiments were performed at least three times with nearly identical results.

nongenomic steroid-induced signals in several tissues, including bone, breast, and endothelium (1, 2, 7). The ability of AR antagonists to specifically attenuate androgen-induced maturation in *Xenopus* oocytes (10) suggested that the classical XeAR might also be playing a role in nongenomic signaling in oocytes. We used RNA interference to determine the importance of the XeAR in androgen-induced maturation. Oocytes were injected with either buffer or double-stranded RNA oligonucleotides targeted against the XeAR. Because endogenous XeAR expression in oocytes could not be consistently measured by Western blot (see Fig. 4A), immunohistochemistry was used to detect changes in XeAR levels, revealing a qualitative decrease in AR expression within oocytes of approximately 30–50% (data not shown). Sixty hours after injection, oocytes were treated with either progesterone or AD, as both steroids are equally potent promoters of oocyte maturation and therefore are most easily compared. Injection of the XeAR-targeted double-stranded RNA oligonucleotides had no effect on progesterone-induced maturation when compared with mock-injected oocytes (Fig. 3A); however, AD-induced maturation was significantly inhibited (Fig. 3B) in the oocytes injected with the RNA oligonucleotides. Higher concentrations of AD (1000 nM) overcame the inhibitory effects of the RNA oligonucleotides. This rescue by saturating concentrations of AD may have been due to multiple factors, including AD stimulation of the remaining endogenous XeAR, or AD stimulation of endogenous PR receptors within the oocytes [AD binds to the XeAR and XePR with equilibrium constants (K_ds) of 44 nM (Table 1) and 1400 nM (data not shown), respectively]. To test an earlier nongenomic signaling event triggered by steroids, activation of p42 ERK was similarly examined. Injection of the XeAR-targeted double-stranded RNA oligonucleotides into oocytes inhibited AD-induced phosphorylation of p42 by approximately 80% in comparison to mock-injected cells (Fig. 3C, lanes 5 and 6) but did not reduce either progesterone-induced (Fig. 3C, lanes 3 and 4) or insulin-induced (Fig. 3C, lanes 7 and 8) activation of p42. Notably, 100 nM AD was used in these p42 ERK phosphorylation assays, which is an order of magnitude below the

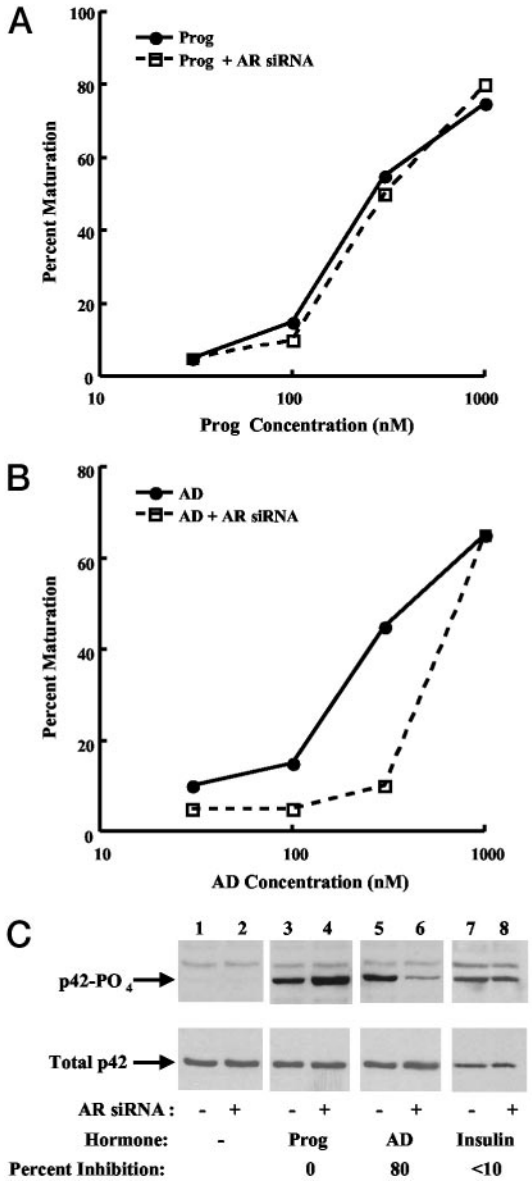

Fig. 3. Injection of Double-Stranded RNA Oligonucleotides Directed Against the XeAR Specifically Attenuates AD-Induced Maturation and Phosphorylation of p42

Oocytes were injected with either 10 mM HEPES or the AR-specific double-stranded RNA oligonucleotides (siRNA). After 50 h, maturation assays using progesterone (A) or AD (B) were performed. p42-phosphorylation studies were also performed as described (C) using 100 nM progesterone (Prog), 100 nM AD, or 200 nM insulin (gift from Robert Dobbins, UTSW). The percent inhibition of phosphorylation is shown underneath. These experiments were performed at least three times with similar results.

aforementioned K_d for AD binding to the XePR; therefore, significant AD signaling through the PR seems unlikely under these conditions. Injection of nonspecific double-stranded RNA oligonucleotides had no effect on AD, progesterone, or insulin-induced maturation (data not shown). Together, these data suggest that the XeAR is playing at least a partial role in nongenomic androgen-induced signaling in *Xenopus* oocytes.

The XeAR Is Expressed in the Nucleus, Cytoplasm, and Plasma Membrane of *Xenopus* Oocytes

Because nongenomic estrogen-induced signaling appears to be mediated primarily by classical ERs that are associated with the plasma membranes of target cells (1, 2, 7), the subcellular localization of the XeAR within oocytes was determined. Western blot analysis of extracts from fractionated oocytes injected with cRNA encoding the XeAR revealed that a majority of the XeAR was expressed in the nuclear/cytoplasmic fractions (Fig. 4A, lanes 3 and 4); however, a small but significant amount of XeAR was also detected in the membrane fraction, both in the presence or absence of testosterone (Fig. 4A, lanes 7 and 8). These results are similar to those reported for the *Xenopus* PR, where approximately 5% of receptors were reported in the membrane (15). Similar analysis of mock-injected cells revealed virtually no detectable XeAR expression by Western blot (Fig. 4A, lanes 1, 2, 5, and 6), suggesting that endogenous XeAR levels are below the level of detection by this method.

To examine endogenous XeAR expression, immunohistochemistry was performed on sections from uninjected oocytes using two different anti-XeAR rabbit polyclonal antibodies that recognized either the amino (Fig. 4B) or carboxyl termini (Fig. 4C) of the protein. Oocytes from albino frogs were used in these studies to eliminate background signal from melanin around the cell surface of the animal pole. These studies confirmed that endogenous XeAR was expressed throughout the oocyte, with significant staining in the cytoplasm and nucleus, as well as within the plasma membrane. Addition of testosterone had no effect on receptor localization (data not shown). In contrast, XeAR expressed in COS cells was found in both the cytoplasm and nucleus in the absence of steroid but localized primarily to the nucleus upon stimulation with testosterone (Fig. 4D). Unlike in *Xenopus* oocytes, no significant membrane expression of the XeAR was observed in these or nonpermeabilized COS cells overexpressing the XeAR (data not shown). These results indicate that the subcellular localization of the XeAR may be dependent upon the cell type, and they also suggest that the AR expression pattern within a given cell might predict the nature (genomic *vs.* nongenomic) of its response to androgens.

Testosterone, DHT, and R1881 Are Potent Promoters of XeAR-Mediated Transcription in CV1 Cells

To begin studying XeAR-mediated signaling, androgen-induced activation of transcription was examined in *Xenopus* oocytes and CV1 cells. Nuclear injection of

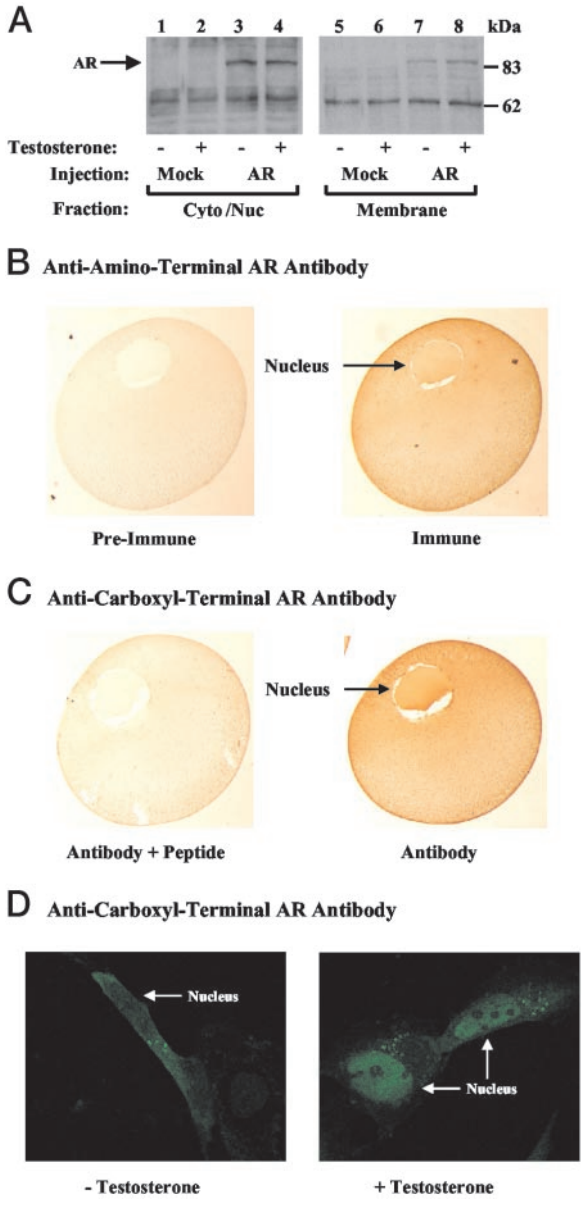

Fig. 4. The XeAR Is Expressed throughout the *Xenopus* Oocyte

A, Oocytes were injected with either 10 mM HEPES (Mock) or cRNA encoding the XeAR (AR). After 48 h, oocytes were treated with either ethanol (− testosterone) or 100 nM testosterone (+ testosterone) for 1 h. Membranes were then separated from the cytoplasm and nuclei (Cyto/Nuc), and both fractions were analyzed by Western blot using a rabbit anti-amino-terminal XeAR antibody. Equal amounts of protein were added to each lane. The location of the XeAR is indicated (AR). B, Sections from uninjected albino *Xenopus* oocytes were probed with rabbit serum containing an anti-amino-terminal XeAR antibody (immune) or its corresponding prebleed serum (preimmune). C, Albino oocyte sections were probed with a rabbit anti-carboxyl-terminal AR antibody (Antibody) or the same antibody pretreated with a neutralizing peptide (Antibody + Peptide). D, COS cells overexpressing the XeAR were treated with either ethanol (− testosterone) or 100 nM testosterone (+ testosterone) for 1 h followed by fluorescent staining using the anti-carboxyl-terminal AR antibody.

both double- and single-stranded plasmids containing either the mouse mammary tumor virus (MMTV) promoter or an androgen response element driving luciferase expression resulted in very high constitutive luciferase activity that was not enhanced by addition of testosterone, AD, DHT, or R1881 (data not shown). These results are consistent with earlier studies demonstrating constitutive activation of these promoters in oocytes (16). Interestingly addition of flutamide often resulted in a 2- to 3-fold reduction in this constitutive activity (data not shown), suggesting that endogenous XeAR might be mediating this constitutive transcriptional activity.

Because the high constitutive activity of the AR-promoters precluded studying XeAR-mediated transcription in oocytes, transcriptional studies of the XeAR were instead performed in parallel with the human AR (HuAR) using CV1 cells, which do not express endogenous AR. As noted in Table 1, testosterone, DHT, and R1881 were all potent promoters of XeAR-mediated transcription, with EC_{50} values of 32 nM, 14 nM, and 44 pM, respectively. By comparison, the EC_{50} values for testosterone, DHT, and R1881-induced transcription via the HuAR were approximately 10-fold lower, at 4.2 nM, 1.7 nM, and 5.5 pM, respectively. Notably, although the EC_{50} values varied, the maximum signals induced by all three ligands through both the human and *Xenopus* ARs were virtually identical (data not shown). Additionally, AD-mediated transcription in CV-1 cells could not be examined due to their innate ability to rapidly convert AD to testosterone (data not shown).

The Dissociation Rates of Ligands from the AR Correlate with Ligand Sensitivity

The 10-fold higher potency of the HuAR relative to the XeAR could be due to differences in receptor interactions with DNA, ligands, or transcription cofactors. To determine which region of the AR was most involved in regulating the potency of steroid-induced transcription, the ligand-binding domain (LBD) of the XeAR was replaced with the homologous region from the HuAR to make a Xe/Hu chimera (Fig. 5). The LBDs of the human and *Xenopus* ARs share approximately 90% identity, whereas the DNA binding domains (DNABD) and A/B regions are 100% and 35% identical, respectively. Surprisingly, testosterone and DHT responses by the Xe/Hu chimera were similar to those of the wild-type HuAR (6.9 nM and 4.3 nM), suggesting that receptor-ligand interactions may be playing important roles in regulating the potency of *Xenopus* and human AR-mediated transcription.

Interestingly, the importance of the receptor-ligand interaction in mediating the potency of the transcriptional response was supported by examination of the ligand dissociation rates from the different androgen receptors (Table 1). Although the K_d values for *Xenopus* and human AR binding to testosterone, DHT, and R1881 were all similar (Table 1), their ligand dissocia-

Table 1. Comparison of Activation and Binding Properties of the *Xenopus* and Human ARs

	Steroid	XeAR	HuAR	Xe/Hu Chimera
EC_{50}	Test (nM)	32 ± 13 (n = 9)	4.2 ± 2.3 (n = 3)	6.9 ± 3.6 (n = 4)
	DHT (nM)	14 ± 6.1 (n = 7)	1.7 ± 0.5 (n = 3)	4.3 ± 1.5 (n = 4)
	R1881 (pM)	44 ± 27 (n = 3)	5.5 ± 3.0 (n = 3)	33 ± 2.0 (n = 3)
Dissociation rate relative to HuAR	Test	3.8 ± 1.4 (n = 3)	1	1 ± 0.05 (n = 3)
	DHT	5.5 ± 1.9 (n = 2)	1	0.7 ± 0.01 (n = 2)
	R1881	19 ± 5.5 (n = 3)	1	7.1 ± 4.9 (n = 3)
K_d (nM)	Test	0.82 ± 0.07 (n = 3)	1.03 ± 0.4 (n = 3)	
	DHT	0.64 ± 0.07 (n = 3)	0.45 ± 0.16 (n = 3)	
	R1881	0.73 ± 0.06 (n = 3)	0.8 ± 0.22 (n = 3)	
	AD	44 ± 24 (n = 3)	24 ± 13 (n = 2)	

Transcriptional activity was measured in CV1 cells transfected with the cDNAs encoding the various ARs. Cells were incubated with the indicated steroids for 48 h and luciferase activity assayed. Results represent the average of the indicated number of experiments (n) ± SD. Dissociation rates of the indicated steroids were measured in COS cells transfected with the cDNAs encoding the indicated ARs. Each experiment was performed in triplicate and repeated at least two times. Results are shown as the average dissociation rate relative to the human AR ± SD; thus, higher values indicated more rapid dissociation of the steroid from the receptor. K_d values for the indicated steroids were measured in COS cells transfected with a cDNA encoding the XeAR. Each experiment was performed in triplicate, and results are shown as the average K_d ± SD (n as indicated).

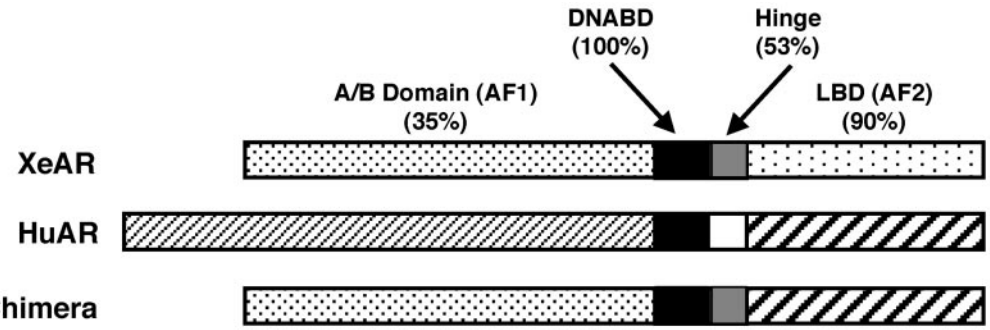

Fig. 5. Schematic Comparison of XeAR, HuAR, and Xe/Hu Chimera Proteins

The percent identity of the A/B domain, DNABD, hinge domain, and LBD are indicated in *parentheses*. The A/B domain contains the AF1 regions, whereas the LBD contains the AF2 region. The chimera consists of the XeAR protein containing the HuAR LBD in place of its own LBD.

tion rates differed considerably, correlating very well with their relative potencies. For example, DHT and testosterone dissociated from the XeAR approximately four times faster than from the HuAR or Xe/Hu chimera, consistent with the higher EC_{50} values (lower potency) for DHT and testosterone-induced transcription by the XeAR. Similarly, R1881 dissociated from the XeAR and Xe/Hu chimera significantly faster than from the HuAR, which correlated with the higher EC_{50} values for R1881-induced transcription by the XeAR and Xe/Hu chimera. Perhaps differences in the stability of ligand binding to the AR partially explain the observed differences in potencies of the various ligands on transcriptional activation of the human and *Xenopus* ARs. This concept would be consistent with earlier work demonstrating the importance of the stability of ligand-receptor binding in regulating human AR-mediated transcription (17).

R1881 and DHT Antagonize Nongenomic Testosterone-Mediated Signaling in Oocytes

Having determined that all of the AR ligands tested were potent promoters of XeAR-mediated transcrip-

tion, we next examined their effects on nongenomic signaling. Initial studies revealed that all ligands bound to and were absorbed by *Xenopus* oocytes equally (data not shown). As previously shown (10), testosterone (Fig. 6A) and AD (Fig. 3B) were potent promoters of oocyte maturation. Surprisingly, DHT, an important androgen in mammalian male sexual development and a potent promoter of XeAR-mediated transcription in CV-1 cells (Table 1), was a poor promoter of maturation, with an EC_{50} more than 10-fold higher than that of testosterone (Fig. 6A). Additionally, R1881, the most potent promoter of XeAR-mediated transcription (Table 1), did not induce oocyte maturation at concentrations of up to 1 μM. Instead, R1881 was a potent antagonist of testosterone-induced maturation (Fig. 6B) and almost completely blocked testosterone-induced phosphorylation of p42 (95% inhibition; Fig. 6C). These results suggest that R1881 is acting as an inhibitor of nongenomic testosterone-induced signaling, perhaps through binding to the XeAR. DHT also inhibited testosterone-induced maturation and phosphorylation of p42 (Fig. 6, B and C), though to a lesser extent than R1881 due to the lower concentration of DHT used to avoid its partial agonist qualities. As seen previously with flutamide (10), the R1881 and DHT inhibitory effects on maturation were specific to androgen-induced signaling, as they did not attenuate progesterone-induced events in oocytes (data not shown). In addition, the inhibitory effects of R1881 and DHT appeared to occur independent of transcription, as they blocked testosterone-induced maturation similarly both in the presence and absence of actinomycin D (data not shown). Furthermore, 1 μM R1881 and 100 nM DHT had minimal effects on both constitutive MMTV-luciferase activity (3.2- and 1.2-fold induction over baseline, respectively) and MMTV-luciferase

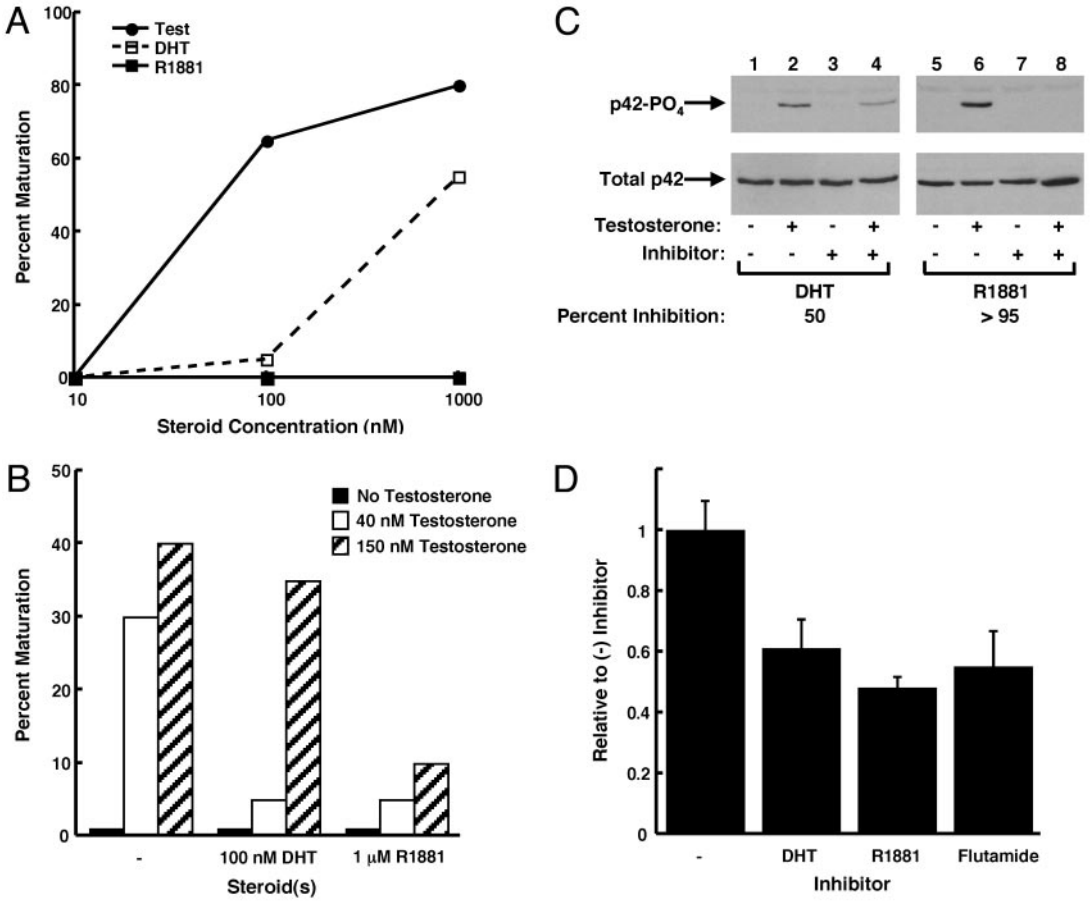

Fig. 6. DHT and R1881 Inhibit Nongenomic Signaling in Oocytes

A, Maturation assays were performed on the same preparation of oocytes using testosterone, DHT, or R1881. B, Oocytes were treated with ethanol, 100 nM DHT, or 1 μM R1881 for 1 h before addition of 40 or 150 nM testosterone. The percent maturation was determined after incubating for 16 h at 16 C with testosterone and the inhibitors. C, Oocytes were treated with 100 nM DHT or 1 μM R1881 for 1 h before addition of testosterone (300 nM and 100 nM for the DHT and R1881 experiments, respectively). Western blot analysis for phosphorylation of p42 was performed after 4 h. The percent inhibition of phosphorylation is shown underneath. D, Ovarian fragments were pretreated for 1 h with ethanol, 100 nM DHT, 1 μM R1881, or 20 μM flutamide, followed by addition of 100 U/ml hCG. After 12 h, ovaries were teased apart and the percent of mature oocytes determined. The fraction of mature oocytes relative to those treated with ethanol (maximum maturation = 30% of stage V and VI oocytes) is indicated on the y-axis ± SD (n = 3). All experiments were performed at least three times with nearly identical results.

activity in the presence of testosterone (1.5- and 1.4-fold induction above activity with testosterone alone, respectively) in oocytes. Given its lower affinity for the XeAR and its own high potency for promoting maturation, we were unable to find a concentration of AD that inhibited testosterone-induced oocyte maturation.

Flutamide, R1881, and DHT Block hCG-Induced Maturation of Oocytes in Intact Ovaries

The ability of DHT, R1881, and flutamide to inhibit testosterone-induced oocyte maturation *in vitro* afforded the opportunity to test the importance of testosterone and AD in mediating oocyte maturation in an intact organ under physiologic conditions. *Xenopus* ovaries were treated with hCG in the presence of ethanol, DHT, R1881, or flutamide. After 12 h, DHT, R1881, and flutamide had reduced the number of mature oocytes in the ovaries relative to ethanol-treated oocytes by 40%, 52%, and 45%, respectively (Fig. 6D). Under these conditions, steroid production was nearly identical in all samples, with very high release of testosterone (~50 ng/g ovarian tissue) and AD (~25 ng/g ovarian tissue) in the setting of virtually undetectable production of progesterone (~1 ng/g ovarian tissue). Notably, DHT production was also nearly undetectable under these conditions (data not shown). The high levels of androgen production by hCG-stimulated ovaries likely explain the incomplete inhibition by DHT, flutamide, and R1881, as they cannot completely antagonize endogenous androgen binding to the XeAR at the concentrations produced over the course of 12 h. In fact, addition of micromolar amounts of AD or testosterone does not significantly increase hCG-induced oocyte maturation in the frog ovary (12), suggesting that saturating amounts of androgens are produced under these conditions.

DISCUSSION

Regulation of steroid-mediated transcription involves many factors, including the levels of specific steroid receptors and transcription cofactors expressed within the target cell. As more examples of transcription-independent, or nongenomic, steroid-induced signaling are described, the complexity of steroid-mediated actions becomes even greater. Here we have demonstrated that certain androgens can differentially affect genomic *vs.* nongenomic androgen-induced signaling. We have shown that R1881, the most potent activator of XeAR-induced transcription tested, was in fact a strong antagonist of nongenomic androgen-induced signaling in oocytes (Fig. 6). In addition, DHT, another potent promoter of transcription, served as either a weak agonist or antagonist of nongenomic signaling, depending upon the concentration used. The discovery of androgens capable of selectively altering genomic *vs.* nongenomic signaling may lead to the development of useful reagents for teasing apart the roles of these two pathways in mediating various steroid-induced actions. Similar to the synthetic estrogen receptor agonist estren, which appears to prevent bone loss in mice through a nongenomic mechanism (18), the development of selective genomic or nongenomic androgens may eventually also prove useful clinically to modulate various physiologic responses to androgens.

One example of a physiologic response that can be modulated by selective inhibition of nongenomic androgen-mediated signaling is the marked inhibition of hCG-induced oocyte maturation by R1881 and DHT in intact *X. laevis* ovarian follicles (Fig. 6). These functional studies are also significant in that they confirm the physiologic importance of androgens in mediating oocyte maturation in the *Xenopus* ovary (10, 12). Although the direct role of androgens in mammalian oocyte maturation has yet to be determined, androgens do appear to play important roles in normal mammalian ovarian and oocyte development (19), as well as in ovarian disease states such as polycystic ovarian syndrome (PCOS; Ref. 20). If these androgen-mediated effects are similarly transcription-independent, then the development of molecules capable of specifically modulating nongenomic signaling in the ovary might be useful in treating PCOS or other forms of infertility. Notably, the relatively weak AR antagonist flutamide improves infertility in some women with PCOS (21, 22); however, the large-scale use of flutamide in treating PCOS will likely be limited, given its ability to block both genomic and nongenomic AR-mediated signaling in a number of different tissues.

How are androgens mediating nongenomic signaling in oocytes? These and other data suggest that they may be signaling at least in part through the classical XeAR expressed in oocytes. First, we have shown here that oocytes injected with XeAR-targeted double-stranded RNA oligonucleotides were less responsive to AD-induced maturation and phosphorylation of p42 than mock-injected cells but were equally sensitive to progesterone and insulin-induced signaling (Fig. 3). The specificity of these XeAR-targeted RNA oligonucleotides was further supported the inability of several nonspecific RNA oligonucleotide pairs to alter maturation induced by any of the agonists tested (data not shown). Second, several known inhibitors of AR-mediated transcription, including flutamide, hydroxflutamide, and bicalutamide, significantly reduced AD- and testosterone-induced signaling and maturation in *Xenopus* oocytes (10), as did the known AR-binding proteins DHT and R1881 (Fig. 6). Together, these data suggest that flutamide, hydroxyflutamide, bicalutamide, R1881, and DHT may be inhibiting maturation by blocking testosterone and AD binding to the XeAR. Although plausible, in order for an AR other than the XeAR to be the primary mediator of androgen-induced oocyte maturation, this novel receptor would have to

bind to the same array of AR ligands as the classical XeAR. Further, given the results of the RNA interference studies (Fig. 3), the activity of this alternative androgen receptor would have to be regulated in part by endogenous XeAR expression levels within the oocyte.

It is still possible, however, that one or more molecules are playing additional role(s) in mediating androgen-induced oocyte maturation. Recent work has described a novel family of high affinity membrane steroid receptors with structure and signaling similarities to G protein-coupled receptors that may be involved in mediating progesterone-induced oocyte maturation in fish (23). When expressed in somatic cells, the spotted seatrout membrane progesterone receptor appeared to modulate a pertussis-sensitive decrease in intracellular cAMP in response to progesterone, suggesting a role for $G\alpha_i$ in the maturation process. Similar membrane steroid receptors might be involved in frog oocyte maturation as well; however, as shown here and elsewhere (14, 24), frog oocyte maturation is not dependent on $G\alpha_i$ signaling; in fact, activation of a $G\alpha_i$-coupled G protein-coupled receptor (M2R) inhibits androgen induced signaling and maturation in *Xenopus* oocytes (Fig. 2). Notably, this inhibitory effect by M2R signaling does not appear to be due to changes in endogenous XeAR-mediated transcription, as MMTV-luciferase activity in oocytes treated with or without testosterone is minimally affected by M2R signaling ($<$2-fold changes in activity compared with baseline, data not shown). If a member of this novel family of steroid receptors were involved in *Xenopus* oocyte maturation, it would likely be signaling through different G proteins and would need to bind to androgens as well as progesterone.

Given that a population of XeARs appeared to be associated with the plasma membrane (Fig. 4), one possible model to reconcile much of the existing data might be that the classical and novel steroid receptors are acting in concert in the membrane, much like the classical ER and GPR30 (2, 3, 25) may be doing in breast cells. Such a model might explain why overexpression of the XeAR alone is not sufficient to significantly enhance androgen-mediated signaling and maturation (10). Furthermore, this model adds another level of complexity to androgen-mediated signaling, suggesting that the complete actions of an individual steroid may depend not only on the expression levels of classical steroid receptors and cofactors within the cell, but also on the subcellular location of the classical steroid receptors, the presence of other signaling cofactors or steroid binding proteins in the membrane, and the binding properties of the ligand itself.

MATERIALS AND METHODS

Oocyte Preparation

Oocytes were harvested from female *X. laevis* (Nasco, Fort Atkinson, WI) and treated as described (26, 27). Briefly, follicular cells were removed by incubation of the oocytes for 4 h at room temperature with 1 mg/ml collagenase A (Roche Applied Sciences, Indianapolis, IN) in modified Barth's solution (MBSH) without Ca^{2+}. Oocytes were then washed and incubated overnight at 16 C in MBSH with 1 mg/ml BSA, 1 mg/ml Ficoll, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Stage V–VI oocytes were selected and maturation assays were performed on each preparation to determine its sensitivity to steroids. Due to the variability in steroid sensitivity between preparations, experiments were done using at least three different preparations.

Steroid Maturation Assays

Oocytes were washed with MBSH and incubated with the individual steroid [progesterone from Sigma (St. Louis, MO); AD, testosterone, and DHT from Steraloids (Newport, RI); and R1881 from Perkin-Elmer Corp. (Boston, MA)] for 16 h. Twenty oocytes were used per condition. The ethanol concentration was kept constant. Maturation was detected by visualizing germinal vesicle breakdown (13). For the maturation inhibition experiments, oocytes were pretreated for 1 h with 30 μM carbachol (Sigma), 100 nM DHT, or 1 μM R1881, before addition of steroid and throughout the signaling or maturation assay. Again, ethanol concentrations were kept constant.

MAPK Assay

Activation of the MAPK cascade was measured by examining p42 phosphorylation (14). Twenty oocytes per condition were preincubated with or without the inhibitors for 1 h, followed by incubation for 4 h with the inhibitors and the indicated steroids. Steroid concentrations were used at or near the EC_{50} for maturation of the oocyte preparation used for each experiment. Oocytes were then solubilized in lysis buffer, lysates were resolved by electrophoresis on 10% polyacrylamide gels, and proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Membranes were probed with a rabbit anti-phospho-p44/42 MAPK antibody (Cell Signaling Technology, Beverly, MA), stripped, and reprobed using a corresponding rabbit anti-p44/42 MAPK polyclonal antibody that binds all p44/p42 regardless of phosphorylation status.

RNA Synthesis and Injection

cDNAs encoding the XeAR (10), the $G\beta$ and $G\gamma$ proteins (14), and the M2 muscarinic receptor (gift from L. Jan, University of California, San Francisco, CA) were cloned into the *Xenopus* oocyte expression vector pGEM HE (L. Jan). All pGEM constructs were linearized and transcribed *in vitro* with T7 RNA polymerase (Promega Corp., Madison, WI). Stage V and VI oocytes were injected with 50.6 nl cRNA at a concentration of approximately 200 ng/μl using a Drummond automatic injector and injected oocytes were incubated 36–48 h in MBSH before maturation or MAPK assays were performed. Oocytes were incubated for 48 h before membrane preparations were prepared.

RNA oligonucleotides were purchased from Dharmacon (Lafeyette, CO). The coding sequence for the RNA oligonucleotide was AAGCAGAAGCAGCGCCGCAAA. Stage V and VI oocytes were injected with 50.6 nl of a 30 μM solution of the double-stranded oligonucleotides and incubated for approximately 50 h before the maturation or MAPK assays were performed.

For the actinomycin D experiment, oocytes were pretreated with either 10 μg/ml actinomycin D (Sigma) or an equal volume of dimethylsulfoxide for 24 h. Oocyte nuclei were then injected with 23 nl (20 pg) of a CMV-luciferase expression plasmid [gift from D. Mangelsdorf, University of

Texas Southwestern (UTSW), Dallas, TX] and immediately incubated overnight with either ethanol or testosterone at the indicated concentrations in the continued presence of either ethanol or actinomycin D. Oocytes were lysed in Reporter Lysis buffer (Promega Corp.) and luciferase assays performed as described (10).

XeAR Western Blot, Immunohistochemistry, and Immunofluorescence

Oocytes were injected with either 10 mM HEPES or XeAR cRNA as described (10). After 48 h, oocyte membrane and nuclear/cytoplasmic fractions were isolated (14), and XeAR was detected by Western blot (10) using a rabbit anti-amino-terminal antibody (Bio-Synthesis, Inc., Lewisville, TX).

For the immunohistochemistry studies, oocytes were isolated from albino *X. laevis* frogs (Nasco, Fort Atkinson, WI), fixed in paraffin, sectioned, and mounted on slides (Molecular Pathology Core Facility, UTSW). Slides were incubated overnight with a rabbit anti-carboxyl-terminal XeAR antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) that had been previously treated with either PBS or excess neutralizing peptide (Santa Cruz Biotechnology, Inc.). Alternatively, slides were incubated with serum containing the rabbit anti-amino-terminal antibody or its corresponding prebleed serum. XeAR was then detected using the Vectasin ABC kit (Vector Laboratories, Inc., Burlingame, CA), and slides were viewed and photographed using a Nikon (Kanagawa, Japan) stereoscope and digital camera.

For the immunofluorescence studies, COS cells were transfected with the XeAR cDNA as described (10). After 48 h, cells were treated with either ethanol or 100 nM testosterone for 1 h at 37 C. Cells were then treated as described (28). Briefly, cells were fixed for 15 min with 3% paraformaldehyde in PBS and permeabilized for 10 min on ice with 0.1% TX-100 in PBS. Slides were then incubated with the rabbit anti-carboxyl-terminal AR antibody, followed by incubation with a fluorescein isothiocyanate-conjugated antirabbit antibody (DAKO Corp., Carpinteria, CA). Cells were then examined and photographed using a fluorescent microscope (Carl Zeiss, Jena, Germany).

Transcription and Ligand Dissociation Assays

Cells were grown in complete medium consisting of DMEM, 10% fetal bovine serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin.

The Xe/Hu chimera cDNA was created by PCR. The A/B, DNABD, and hinge regions of the XeAR were cloned using the primers ATGGAGGTGCACATAGGGCTCGGC (corresponding to the start codon of the XeAR) and AATGGCTTCCAGGACATTCAGAAAGAT (corresponding to 1627–1653 in the XeAR coding sequence). The LBD of the HuAR was cloned using the primers ATCTTTCTGAATGTCCTGGAAGCCATT (corresponding to 2008–2034 in the HuAR coding sequence) and TCACTGGGTGTGGAAATAGATGGG (corresponding to the stop codon of the HuAR). The two PCR fragments were then added together, and a final PCR was performed using the two outside primers. The chimera was sequenced to confirm its identity.

For the transcription assays, CV1 cells were transfected by calcium phosphate precipitation with an MMTV-luciferase plasmid and cDNAs encoding either the XeAR, HuAR, or the Xe/Hu chimera. Cells were then incubated in complete medium containing 5% charcoal-stripped fetal bovine serum and the indicated steroids for 48 h, and luciferase expression was measured as described (10).

For the ligand binding and dissociation assays, COS cells were plated into 12- or 24-well plates and transfected with the empty pcDNA3.1 vector or cDNAs encoding either the XeAR, HuAR, or Xe/Hu chimera (10). For the binding assays, transfected cells were washed once at 4 C with DMEM containing 20 mM HEPES and 1 mg/ml BSA (DME/H/BSA) and incubated for 1 h at 4 C with DME/H/BSA containing varying concentrations of [1, 2, 6, 7-^{3}H(N)]-testosterone, [1, 2-^{3}H(N)]-DHT, or 17α-methyl-^{3}H]-R1881 (Perkin-Elmer Corp.). Cells were then washed three times with cold DME/H/BSA and steroids extracted from cells by incubating with 100% ethanol for 30 min. The total counts bound to cells and in the supernatants were measured by liquid scintillation and the K_ds calculated using Scatchard plots. AD binding was studied similarly, only transfected cells were treated with 1 nM radiolabeled testosterone and increasing concentrations of unlabeled AD. K_d values were then determined using the Prism software (GraphPad Software, Inc., San Diego, CA). For the dissociation assays, transfected cells were washed once at 4 C with DME/H/BSA and incubated for 1 h with DME/H/BSA containing either radiolabeled 1 nM testosterone, 2 nM DHT, or 1 nM R1881. Cells were then washed three times with cold DME/H/BSA and incubated with 10 μM unlabeled steroid at either room temperature (DHT and testosterone) or 37 C (R1881) for 0, 30, 60, 120, 180, 240, or 300 min. At the appropriate time points, cells were washed three times with DME/H/BSA and steroids extracted. The total counts bound were measured by liquid scintillation and the specific counts bound calculated by subtraction of the background counts from the pcDNA3.1-transfected cells. Dissociation rates were then calculated from plots of time (x-axis) *vs.* the natural log of the percent of specific counts bound relative to t = 0 (y-axis).

hCG-Mediated Maturation of Oocyte in Ovarian Fragments

Ovarian fragments of approximately 100–200 mg were washed in MBSH and treated for 1 h in 2 ml MBSH with either ethanol, 20 μM flutamide, 100 nM DHT, or 1 μM R1881. Ethanol concentrations were kept constant. hCG was then added at a concentration of 100 U/ml, and the ovarian fragments were incubated at 16 C for approximately 12 h. The MBSH was removed, and steroids were extracted and analyzed by RIA (10). Oocytes were manually removed from the ovarian fragments, and maturation was determined by visualization of a white spot on the animal pole.

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REFERENCES

1. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC 2001 Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors:

dissociation from transcriptional activity. Cell 104: 719–730
2. Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER 2002 ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. Mol Endocrinol 16:100–115
3. Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R, Santen RJ 2002 Linkage of rapid estrogen action to MAPK activation by ERα-Shc association and Shc pathway activation. Mol Endocrinol 16:116–127
4. Simoncini T, Fornari L, Mannella P, Varone G, Caruso A, Liao JK, Genazzani AR 2002 Novel non-transcriptional mechanisms for estrogen receptor signaling in the cardiovascular system. Interaction of estrogen receptor α with phosphatidylinositol 3-OH kinase. Steroids 67: 935–939
5. Chambliss KL, Yuhanna IS, Mineo C, Liu P, German Z, Sherman TS, Mendelsohn ME, Anderson RG, Shaul PW 2000 Estrogen receptor α and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. Circ Res 87:E44–E52
6. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW 1999 Estrogen receptor α mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. J Clin Invest 103:401–406
7. Shaul PW 2002 Regulation of endothelial nitric oxide synthase: location, location, location. Annu Rev Physiol 64:749–774
8. Bayaa M, Booth RA, Sheng Y, Liu XJ 2000 The classical progesterone receptor mediates Xenopus oocyte maturation through a nongenomic mechanism. Proc Natl Acad Sci USA 97:12607–12612
9. Tian J, Kim S, Heilig E, Ruderman JV 2000 Identification of XPR-1, a progesterone receptor required for Xenopus oocyte activation. Proc Natl Acad Sci USA 97:14358–14363
10. Lutz LB, Cole LM, Gupta MK, Kwist KW, Auchus RJ, Hammes SR 2001 Evidence that androgens are the primary steroids produced by *Xenopus laevis* ovaries and may signal through the classical androgen receptor to promote oocyte maturation. Proc Natl Acad Sci USA 98:13728–13733
11. Boonyaratanakornkit V, Scott MP, Ribon V, Sherman L, Anderson SM, Maller JL, Miller WT, Edwards DP 2001 Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol Cell 8:269–280
12. Yang WH, Lutz LB, Hammes SR 2003 *Xenopus laevis* ovarian CYP17 is a highly potent enzyme expressed exclusively in oocytes: evidence that oocytes play a critical role in Xenopus ovarian androgen production. J Biol Chem 278:9552–9559
13. Maller JL, Krebs EG 1980 Regulation of oocyte maturation. Curr Top Cell Regul 16:271–311
14. Lutz LB, Kim B, Jahani D, Hammes SR 2000 G protein $\beta\gamma$ subunits inhibit nongenomic progesterone-induced signaling and maturation in *Xenopus laevis* oocytes. Evidence for a release of inhibition mechanism for cell cycle progression. J Biol Chem 275:41512–41520
15. Bagowski CP, Myers JW, Ferrell Jr JE 2001 The classical progesterone receptor associates with p42 MAPK and is involved in phosphatidylinositol 3-kinase signaling in Xenopus oocytes. J Biol Chem 276:37708–37714
16. Huang ZQ, Li J, Wong J 2002 AR possesses an intrinsic hormone-independent transcriptional activity. Mol Endocrinol 16:924–937
17. Chang CY, McDonnell DP 2002 Evaluation of ligand-dependent changes in AR structure using peptide probes. Mol Endocrinol 16:647–660
18. Kousteni S, Chen JR, Bellido T, Han L, Ali AA, O'Brien CA, Plotkin L, Fu Q, Mancino AT, Wen Y, Vertino AM, Powers CC, Stewart SA, Ebert R, Parfitt AM, Weinstein RS, Jilka RL, Manolagas SC 2002 Reversal of bone loss in mice by nongenotropic signaling of sex steroids. Science 298:843–846
19. Hillier SG 2001 Gonadotropic control of ovarian follicular growth and development. Mol Cell Endocrinol 179:39–46
20. Nelson VL, Legro RS, Strauss 3rd JF, McAllister JM 1999 Augmented androgen production is a stable steroidogenic phenotype of propagated theca cells from polycystic ovaries. Mol Endocrinol 13:946–957
21. Rittmaster RS 1999 Antiandrogen treatment of polycystic ovary syndrome. Endocrinol Metab Clin North Am 28: 409–421
22. Eagleson CA, Gingrich MB, Pastor CL, Arora TK, Burt CM, Evans WS, Marshall JC 2000 Polycystic ovarian syndrome: evidence that flutamide restores sensitivity of the gonadotropin-releasing hormone pulse generator to inhibition by estradiol and progesterone. J Clin Endocrinol Metab 85:4047–4052
23. Zhu Y, Rice CD, Pang Y, Pace M, Thomas P 2003 Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci USA
24. Sadler SE, Maller JL, Cooper DM 1984 Progesterone inhibition of Xenopus oocyte adenylate cyclase is not mediated via the Bordetella pertussis toxin substrate. Mol Pharmacol 26:526–531
25. Filardo EJ, Quinn JA, Frackelton Jr AR, Bland KI 2002 Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol Endocrinol 16: 70–84
26. Vu T-KH, Hung DT, Wheaton VI, Coughlin SR 1991 Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 64:1057–1068
27. Williams JA, McChesney DJ, Calayag MC, Lingappa VR, Logsdon CD 1988 Expression of receptors for cholecystokinin and other Ca2+-mobilizing hormones in Xenopus oocytes. Proc Natl Acad Sci USA 85:4939–4943
28. Hein L, Ishii K, Coughlin SR, Kobilka BK 1994 Intracellular targeting and trafficking of thrombin receptors. A novel mechanism for resensitization of a G protein-coupled receptor. J Biol Chem 269:27719–27726