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Modulation of the ligand-independent activation of the human estrogen receptor by hormone and antihormone

(dopamine/estradiol/antiestrogen/transactivation)

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ABSTRACT **It has been previously demonstrated that several members of the steroid receptor superfamily may be activated by the neurotransmitter dopamine in the apparent absence of cognate ligand. We have examined wild-type and mutant human estrogen receptors (ERs, [Gly^{400}]ER and [Val^{400}]ER, respectively) for their abilities to activate ER-dependent transcription of a transgene in a ligand-independent manner. In cells expressing the wild-type ER, dopamine was nearly as effective as 17β-estradiol at inducing the chloramphenicol acetyltransferase activity of the reporter gene in a dose-dependent manner; simultaneous addition of suboptimal concentrations of 17β-estradiol and dopamine stimulated transcription more than either compound alone. Dopamine alone was unable to induce gene expression in cells expressing [Val^{400}]ER mutant receptors, but concomitant treatment with 17β-estradiol produced a synergistic increase in transcription, suggesting that the ligand may alter the mutant receptor's conformation such that it can be activated subsequently by a dopaminergic signaling mechanism. In the presence of the antiestrogen ICI 164,384, dopamine-stimulated gene expression was undetectable in cells expressing either form of ER. However, simultaneous treatment of cells expressing wild-type ER with *trans*-4-hydroxytamoxifen and dopamine resulted in transgene expression that was additive in nature compared to either compound alone; similar treatment of cells expressing [Val^{400}]ER produced a synergistic increase. Our results suggest that ligand and ligand-independent activation of the ER initiate from distinct pathways and that the latter may occur in a variety of target tissues subject to modulation by receptor ligands.**

The estrogen receptor (ER) belongs to a superfamily of transcription factors that includes other steroid/thyroid/vitamin receptors in addition to the "orphan" receptors for which ligands have not yet been identified (1, 2). Receptors of the former category, such as the ER, bind their cognate ligand with high affinity, undergo a conformational change, dissociate from an inhibitory heteroligomeric complex of heat shock proteins, dimerize, and bind to specific DNA sequences typically found within the regulatory regions of steroid-sensitive genes (3–9). Receptors may be further modified by phosphorylation and, ultimately, interact with the transcriptional apparatus to alter gene transcription (10–12). In addition to agonistic ligands, synthetic antihormones have been developed that are bound with high affinity by steroid receptors and increase receptor binding to steroid response elements (8, 13, 14) but are unable to effectively activate receptors to stimulate gene transcription (13, 15).

In addition to classical ligand-dependent activation, we have observed that membrane-permeable agents that increase cellular phosphorylation levels (8-bromo-cAMP and okadaic acid) are also able to enhance progesterone-receptor-dependent gene transcription (16), presumably by inducing and/or increasing phosphorylation of receptor or some other rate-limiting cofactor. Furthermore, the catecholaminergic neurotransmitter dopamine appears to initiate a signal transduction pathway from its plasma membrane receptor that also gives rise to the ligand-independent activation of several orphan receptors (17, 18), the chicken progesterone receptor, the human ER, and certain other steroid receptors (19).

This unexpected ability to activate the ER in the apparent absence of its ligand (17β-estradiol, E_2) raises questions regarding the absolute control of ER function *in vivo* and has prompted our examination of the impact of agonistic and antagonistic ligands to further modulate ER activity stimulated by an intracellular signaling pathway initiated at the plasma membrane. In the present study, we have, therefore, examined the effect of the agonist E_2, the partial agonist/antagonist *trans*-4-hydroxytamoxifen (4HT; ref. 20), and the pure antagonist *N*-(*n*-butyl)-11-[3,17β-dihydroxyestra-1,3,5(10)-trien-7α-yl]*N*-methylundecanamide (ICI 164,384; ref. 21) on the dopaminergic activation of ER-dependent gene transcription in intact cells.

MATERIALS AND METHODS

Chemicals and Materials. E_2 and dopamine were obtained from Sigma. The antiestrogen ICI 164,384 was provided by Alan Wakeling (ICI, Macclesfield, England) and 4HT was from D. Salin-Drouin (Laboratoires Besins Iscovesco, Paris). [*ring*-3,5-^{3}H]Chloramphenicol (30–60 Ci/mmol; 1 Ci = 37 GBq) was purchased from DuPont/NEN. ^{125}I-labeled protein A (>30 μCi/μg) was from ICN.

DNA Constructs. pSVMT-wER, the human ER expression vector, contains the complete coding region of the wild-type human ER cDNA ([Gly^{400}]ER) downstream of the metallothionein promoter and simian virus 40 enhancer. To produce an equivalent expression vector for the mutant [Val^{400}]ER, an *Acc* I–*Sst* I fragment of the wild-type ER cDNA was removed and replaced with the corresponding region of the ΔHER expression vector (22). Plasmid phPRB contains the cDNA for the B form of the human progesterone receptor in the same vector backbone used for the ER constructs. The ER-responsive ERE-E1bCAT reporter plasmid contains a fragment of the vitellogenin A2 gene promoter (positions −331 to −87) upstream of the adenovirus E1b "TATA" box linked to a chloramphenicol acetyltransferase (CAT) gene.

Cell Culture Conditions and Transfections. ER-negative (23) HeLa cells (human epitheloid carcinoma) were routinely maintained in Dulbecco's modified Eagle's medium supple-

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Abbreviations: ER, estrogen receptor; E_2, 17β-estradiol; 4HT, *trans*-4-hydroxytamoxifen; CAT, chloramphenicol acetyltransferase.

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mented with 10% (vol/vol) fetal calf serum. Twenty-four hours prior to transfection, cells were seeded at a density of 10^6 cells per 100-mm dish and 4–6 h later were switched to phenol-red-free medium supplemented with Nutridoma-SR (Boehringer Mannheim). Cells were cotransfected with 2 μg of expression vectors encoding the progesterone receptor or wild-type or variant ER plus 5 μg of ERE-E1bCAT by the Polybrene method (16). Test compounds were added 24 h after transfection, 24 h thereafter cells were harvested, and CAT activity was measured in cell extracts containing 100 μg of protein by a phase-extraction method utilizing [^{3}H]chloramphenicol and butyryl-coenzyme A as substrates (24).

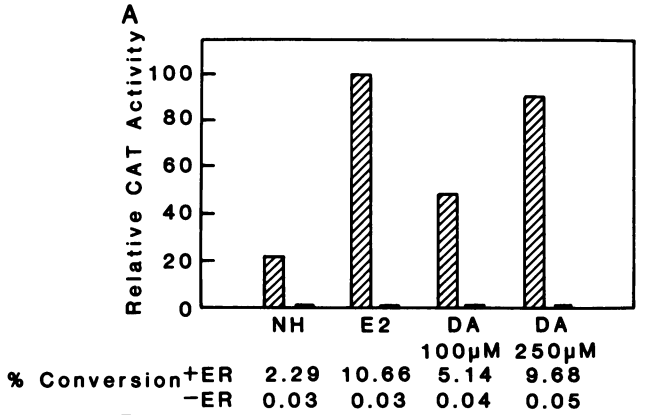
Immunoblots. HeLa cells were maintained and transfected as described for the CAT assays with the exception that 5 μg of [Gly^{400}]ER or [Val^{400}]ER expression vector was used. After 24 h of exposure to the test compounds, cells were harvested and resuspended in TESH (40 mM Tris·HCl, pH 7.7/4 mM EDTA/0.1% monothioglycerol) containing 0.5 M NaCl, and crude cell lysates were obtained by several cycles of rapid freezing/thawing, followed by centrifugation (Eppendorf centrifuge, model 5415). Cell extracts (250 μg of protein each) were resolved by SDS/PAGE on 7.5% gels by the method of Laemmli (25), electrotransferred to nitrocellulose, and probed with anti-human ER monoclonal antibodies (D75 and H226, each at 1 μg/ml) (26). Specific ER–antibody complexes were visualized by further incubating the filter with rabbit anti-rat IgG and then with ^{125}I-labeled protein A. The blots were subject to autoradiography and the resulting signals were quantified by densitometry (model 620 video densitometer, Bio-Rad).

RESULTS

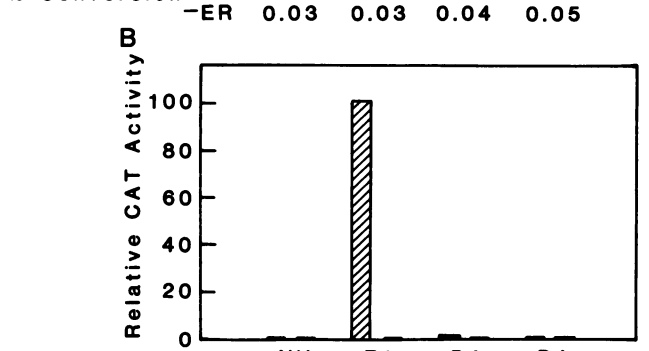
The wild-type human ER ([Gly^{400}]ER) was examined for its ability to stimulate CAT gene expression from an estrogen-response-element-containing reporter plasmid when HeLa cells were treated with either E_2 or the neurotransmitter, dopamine (Fig. 1*A*). Estradiol induced the expression of CAT activity maximally at 1 nM, and treatment with 250 μM dopamine, a concentration similar to that previously utilized to stimulate adenylyl cyclase in cultured cells (27), increased CAT gene transcription to a similar extent. To ensure that changes in gene expression were dependent on ER expression and were not the result of an overall increase in basal transcription, cells transfected with the reporter alone (Fig. 1*A*) or the reporter gene plus a human progesterone receptor expression vector (data not shown) were treated with E_2 and dopamine, and in no instance was transcription of the ERE-E1bCAT target gene stimulated.

The first human ER cDNA isolated (23) contains, within its ligand binding domain, a point mutation relative to wild-type ER that results in a Gly → Val substitution at residue 400 ([Val^{400}]ER). The binding affinity of [Val^{400}]ER for E_2 is reduced in comparison to [Gly^{400}]ER (28) as is its ability to bind DNA (13) and its stability in cytoplasmic extracts incubated *in vitro* (28). We therefore compared the ligand-independent activation of this mutant receptor to the wild-type receptor (Fig. 1 *A* and *B*). Both forms of ER were activated at physiological concentrations of E_2, but as reported (28), the level of gene expression in the absence of hormonal stimulation varied between wild-type and mutant receptors. Little basal CAT activity was detected in HeLa cells transfected with [Val^{400}]ER (Fig. 1*B*), whereas in cells expressing wild-type receptor, basal transcription was ≈20% of the activity achieved after treatment with 1 nM E_2 (Fig. 1*A*). Wild-type ER was activated by dopamine in a dose-dependent fashion (Fig. 1*A*), but surprisingly, dopamine was unable to transcriptionally activate [Val^{400}]ER at any concentration tested (Fig. 1*B*).

To examine the effects of simultaneously activating the ER by ligand-dependent and ligand-independent mechanisms,



% Conversion	NH	E2	DA 100μM	DA 250μM
+ER	2.29	10.66	5.14	9.68
−ER	0.03	0.03	0.04	0.05



% Conversion	NH	E2	DA 100μM	DA 250μM
+ER	0.11	26.40	0.25	0.14
−ER	0.03	0.03	0.04	0.05

FIG. 1. Wild-type ER, but not mutant ER, is able to mediate ER-dependent gene transcription stimulated by both E_2 and dopamine. HeLa cells were transfected with ERE-E1bCAT and either [Gly^{400}]ER (hatched bars) (*A*) or [Val^{400}]ER (hatched bars) (*B*) expression vectors. For comparison, cells transfected with only ERE-E1bCAT (solid bars) are shown in *A* and *B*. Measurements of CAT activity are given as the average of values determined for samples prepared from duplicate plates, expressed relative to the CAT activity induced by 1 nM E_2 (100%), and are representative of at least five experiments. The percent conversion of [^{3}H]chloramphenicol to acylated [^{3}H]chloramphenicol for samples with (+ER) or without (−ER) expressed receptor is indicated. Cells were treated with the following hormones for 24 h: no hormone added (NH), 1 nM E_2 (E2), or dopamine (DA), as indicated.

cells transfected with either [Gly^{400}]ER or [Val^{400}]ER were treated with dopamine and a suboptimal concentration of E_2 (0.1 nM). In cells expressing wild-type ER, this resulted in increased gene expression that appeared to be greater when compared to the effect of either agent alone (Fig. 2*A*) and also yielded CAT activity greater than that maximally induced by 1 nM E_2 (data not shown). However, when dopamine and 0.1 nM E_2 were used to activate [Val^{400}]ER, the resulting ER-dependent gene transcription was greater than additive when compared to that achieved by either compound alone (Fig. 2*B*). These results prompted us to examine the impact of dopaminergic signaling pathways on the antiestrogenic effects of ER antagonists.

Two classes of antiestrogens were examined for their ability to modulate the dopaminergic activation of [Gly^{400}]ER and [Val^{400}]ER. At 100 nM, 4HT increased gene expression over unstimulated levels, thus exhibiting partial agonist activity (Fig. 3). Nevertheless, this concentration of 4HT was able to block the estrogenic activation of both forms of ER and maintained CAT activity at levels approximately equivalent to antihormone alone. In contrast, 4HT was unable to block the transactivation of dopamine-stimulated ER-dependent gene transcription in cells expressing wild-type ER (Fig. 3*A*), and in

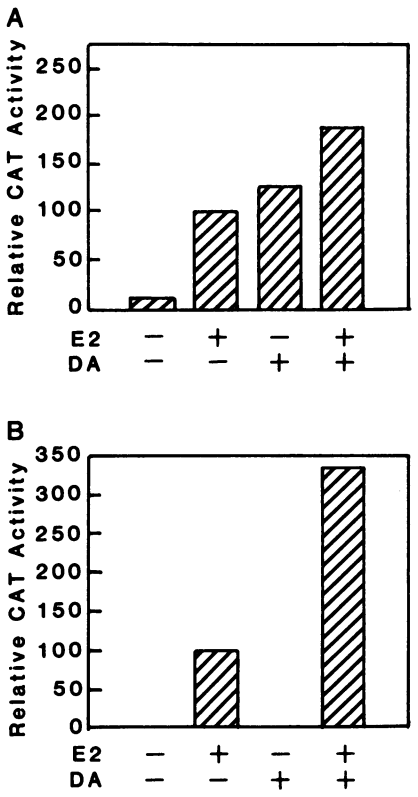


FIG. 2. Dopamine and E_2 can simultaneously activate [Gly^{400}]ER and [Val^{400}]ER. Wild-type (*A*) or mutant (*B*) ER expression vectors were cotransfected with ERE-E1bCAT reporter plasmid. Bars represent the average of duplicate plates and values are expressed relative to CAT activity induced by 0.1 nM E_2 (100%). These results are representative of at least three experiments. Cells were treated for 24 h with 0.1 nM E_2 (E2) or 200 μM dopamine (DA).

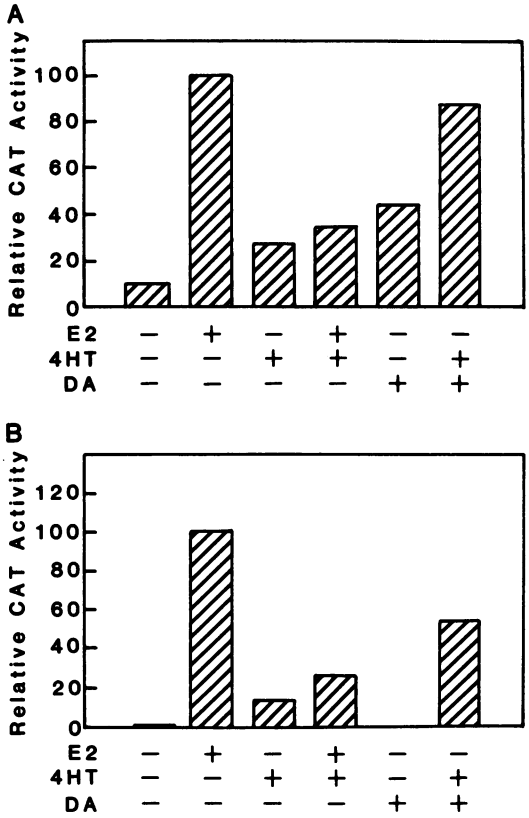


FIG. 3. 4HT is not an antagonist of the dopaminergic activation of the ER. Expression vectors for [Gly^{400}]ER (*A*) or [Val^{400}]ER (*B*) were cotransfected with the target construct ERE-E1bCAT. Bars represent the average of duplicate plates and values are expressed relative to the CAT activity induced by E_2 (100%). Similar results were obtained for three experiments. Cells were treated with 1 nM E_2 (E2), 100 nM 4HT, or 200 μM dopamine (DA).

cells expressing the [Val^{400}]ER mutant, 4HT and dopamine markedly increased gene expression (Fig. 3*B*).

Unlike 4HT, the pure steroidal estrogen antagonist ICI 164,384 showed a markedly different effect on the dopaminergic activation of ER (Fig. 4). This synthetic compound was able to completely block E_2-induced CAT activity in cells expressing either form of receptor, and in contrast to 4HT, it effectively blocked the dopaminergic activation of wild-type ER (Fig. 4*A*) and did not facilitate activation of the mutant receptor by dopamine (Fig. 4*B*).

Since a previous report (28) indicated that [Val^{400}]ER *in vitro* was unstable at 25°C in the absence of ligand, extracts prepared from HeLa cells transfected with either wild-type or mutant ER expression vectors were subjected to Western blot analysis (Fig. 5). A single ER band with a mass of ≈66 kDa was detected in extracts of HeLa cells transfected with cDNAs for either wild-type or mutant ER and comigrated with the endogenous ER detected in a similarly prepared MCF-7 cell extract. Dopamine treatment (200 μM) reduced wild-type and mutant ER levels to 30 and 33% of control levels, respectively, in much the same manner as did treatment with 1 nM E_2 (46 and 50%, respectively). Treatment of cells expressing [Gly^{400}]ER with 100 nM 4HT or 100 nM ICI 164,384 increased and decreased receptor levels by 373 and 15%, respectively, and [Val^{400}]ER levels were increased and decreased by 328 and 38%, respectively.

DISCUSSION

Previous studies of the dopaminergic activation of several steroid receptor superfamily members utilized green monkey kidney epithelial (CV1) cells (17–19), and we now extend our previous observations to HeLa cells. There are two classes of dopaminergic receptor: those of the D_1 subtype stimulate adenylyl cyclase and phospholipase C activities (29, 30) and those of the D_2 subtype inhibit the production of cAMP by adenylyl cylase (30). We exposed HeLa cells *in vitro* to increasing dopamine concentrations and observed a dose-dependent augmentation of cAMP production (data not shown), suggesting that these cells express dopamine receptors of the D_1 subtype, as was shown for CV1 cells (19). Thus, dopaminergic activation of the human ER is not cell specific and suggests that apparent ligand-independent activation may occur in other cell types that express functional dopamine receptors of the general D_1 subtype. It is important to note that our definition of ligand-independent activation relates to receptor-dependent target gene transcription in the absence of exogenous ligand in cells that are not known to produce detectable amounts of ligand. We cannot rule out the possibility that certain cells could produce a low-affinity substance that crossreacts with the ER ligand binding site and acts as a weak agonist.

Although [Val^{400}]ER was activated by E_2, as had been shown by others (15, 28, 31, 32), dopamine alone could not induce [Val^{400}]ER-dependent transcription. This inability was unlikely to be due to the relative instability of mutant receptor expressed in dopamine-treated cells since the steady-state levels of wild-type and mutant receptor were similar after treatment with either E_2 or dopamine in comparison to receptor levels present in control cells. This suggests that a specific functional property intrinsic to the ER is required for the receptor to mediate ligand-independent activation and that at least a portion of the ligand binding domain (i.e., aa 400) is required to mediate this effect. When

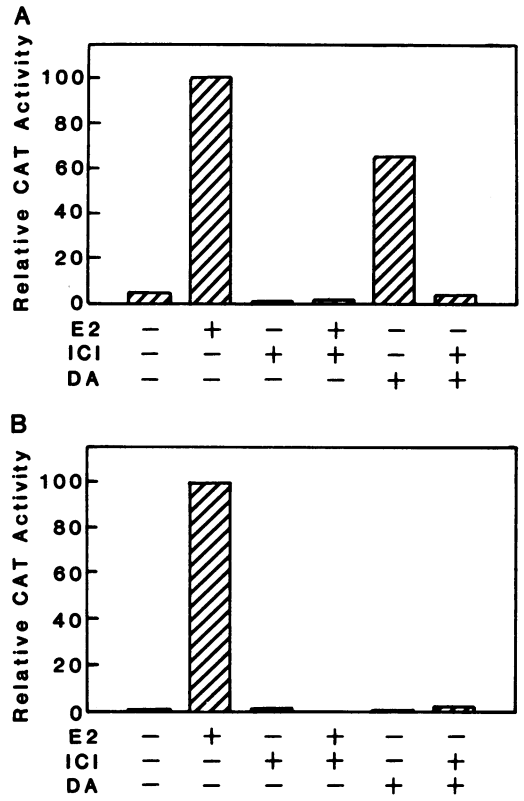


FIG. 4. Antiestrogen ICI 164,384 blocks dopaminergic activation of the ER. [Gly^{400}]ER (*A*) or [Val^{400}]ER (*B*) plasmid (2 μg) was transfected with 5 μg of ERE-E1bCAT into HeLa cells. Results are representative of three experiments and are given as the average obtained for duplicate plates expressed relative to the CAT activity measured for samples prepared from E_2-treated cells (100%). Transfected cells were treated with the following compounds: 1 nM E_2 (E2); 100 nM ICI 164,384 (ICI), or 200 μM dopamine (DA).

[Val^{400}]ER was expressed transiently in HeLa cells, the basal transcription of ER-dependent reporter genes was low to nondetectable whereas the transcriptional activity of cells expressing [Gly^{400}]ER in the absence of exogenous ligand was relatively high. This activity has been attributed (15, 28) to residual estrogen contamination of medium containing charcoal-stripped serum, but our experiments suggest an alternative hypothesis. The appreciable level of basal [Gly^{400}]-ER-dependent transcription may be due to receptor activation by an extracellular factor or factors present in stripped serum or serum substitutes that are able to initiate a second messenger pathway(s) and our prior results with a point mutant in the avian progesterone receptor support this possibility (19).

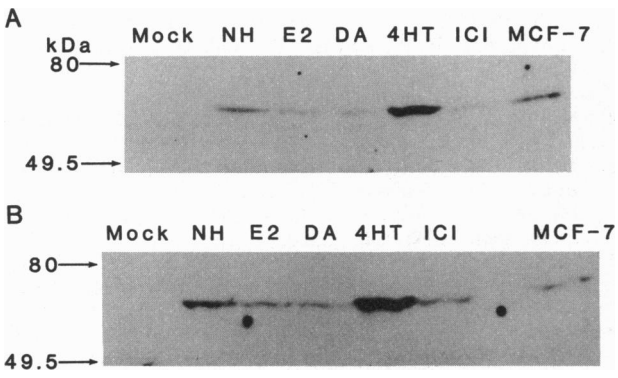


FIG. 5. Western blot analysis of ER expressed in HeLa cells. Extract (250 μg) from cells transfected with [Gly^{400}]ER (*A*) or [Val^{400}]ER (*B*) expression vectors was subjected to Western blot analysis using D75 and H226 as primary antibodies. For comparison, extracts from untransfected cells were also analyzed (mock) as was 50 μg of a similarly prepared MCF-7 cell extract. Cells were treated for 24 h with no hormone added (NH), 1 nM E_2 (E2), 200 μM dopamine (DA), 100 nM 4HT, or 100 nM ICI 164,384 (ICI). Molecular mass markers are shown on the left.

Simultaneous treatment with E_2 and dopamine resulted in enhanced activation of [Gly^{400}]ER. Similar treatment of cells expressing [Val^{400}]ER led to the synergistic induction of CAT activity, indicating that ligand is able to convert [Val^{400}]ER to a dopamine-activatable form whereas the aporeceptor is unresponsive to the catecholamine. This suggests that the interaction between E_2 and this receptor may induce a conformational change and/or stabilize this molecule and allow it to respond to the signal transduction pathway initiated by dopamine. Secondary structure predictions of the region surrounding Gly^{400} of the wild-type ER suggest that this amino acid is located in a β-turn between an α-helix and a β-strand, and it is possible that substitution of a valine for the more compact glycine residue may destabilize the β-turn and surrounding local conformation (28).

The antiestrogen 4HT increased levels of mutant and wild-type ER expression in HeLa cells in a manner similar to that previously observed for endogenous receptor expressed in breast cancer cells (13), and this may contribute to the partial agonist activity of this antihormone. Nonetheless, 4HT was an effective blocker of the estrogenic activation of [Gly^{400}]ER and [Val^{400}]ER. 4HT was not, however, able to attenuate the ligand-independent activation of the wild-type ER and behaved only as an agonist in dopamine-treated cells expressing either form of receptor. Indeed, in this context, 4HT appeared to stabilize [Val^{400}]ER in much the same way as E_2. The ability of 4HT to block ligand-dependent but not ligand-independent ER activation suggests these two events differ mechanistically. The C-terminal ligand-activatable transcriptional activation function (TAF-2) is thought to be blocked by 4HT, leaving the N-terminal transactivation function (TAF-1) able to initiate gene transcription in a promoter-context-dependent cell-specific fashion (15, 33). Expression of CAT activity in [Gly^{400}]ER-expressing cells treated with 4HT and dopamine should, therefore, represent the combined activity of TAF-1 and that induced by dopamine.

The mechanism by which ICI 164,384 blocks ER-dependent gene transcription is not clear (13, 34–37) and the reduction in ER levels in our ICI 164,384-treated HeLa cells may contribute to the very low ER-dependent gene expression measured in this system. Nevertheless, this antagonist was able to effectively block ER-dependent CAT gene expression stimulated by either E_2 or dopamine, and it is, therefore, appropriate that ICI 164,384 remain classified as a pure antiestrogen (21, 38).

The physiological relevance of the dopaminergic activation of ER in HeLa cells is not yet clear; it may simply represent the preferential activation of a signaling pathway able to communicate with receptor or some requisite transcriptional coactivator. In consideration of this, it is possible that other extracellular molecules that transduce their signal by initiating signaling pathways that activate protein kinases (or inhibit phosphatases) may activate steroid receptors such as the ER. Indeed, it has been demonstrated that epidermal growth factor and insulin-like growth factor I increase progesterone receptor synthesis, which is classically thought to be an estrogen-regulated process (39–41), and antiestrogens such as ICI 164,384 block these effects (40, 41). We have also found that epidermal growth factor is able to stimulate ER-dependent gene transcription in this HeLa cell test system (unpublished observations). Furthermore, epidermal growth factor has been shown to mimic estrogen-induced growth in mouse uterus and biochemical changes in mouse

uterine ERs *in vivo*, such as increased DNA binding and production of heterogenous forms of nuclear receptor (42); this suggests that growth factors could influence ERs in living animals. These observations are of particular interest since estrogen has been reported to induce growth factors (e.g., epidermal growth factor, insulin-like growth factor I, and transforming growth factor α) and their receptors in breast and/or uterine tissues (43–48).

Thus, these data further substantiate our hypothesis that steroid receptor function in target tissues may be regulated via pathway "cross talk" from membrane receptors (17). Although ligand-dependent and ligand-independent pathways may act in concert under most physiological situations, it is possible under certain conditions that the dominant activation mechanism for selected steroid receptor family members may be initiated by a membrane-receptor transduced signal. The events subsequent to this are largely unknown but are likely to be complex and involve multiple downstream kinases and/or phosphatases. The implication of these observations for the management of hormone-dependent cancers, such as breast and endometrial cancers (49), could be important and in the future it may be necessary to consider both the ligand-dependent and ligand-independent mechanism of ER activation in the management of such diseases.

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