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The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signaling pathways

(progesterone receptor/phosphorylation/signal transduction)

CANDACE A. BECK*, NANCY L. WEIGEL†, MARISSA L. MOYER*, STEVEN K. NORDEEN*, AND DEAN P. EDWARDS*‡

*Department of Pathology and Molecular Biology Program, University of Colorado Health Sciences Center, Denver, CO 80262; and †Cell Biology Department, Baylor College of Medicine, Houston, TX 77030

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ABSTRACT **The protein kinase A stimulator cAMP can potentiate the ability of progestins to induce the trans-activation function of the human progesterone receptor (hPR). We questioned in the present study whether cAMP could functionally cooperate with the progestin antagonist RU486. In T47D human breast cancer cells, RU486 behaves as a pure antagonist with respect to induction of the progesterone-responsive mouse mammary tumor virus chloramphenicol acetyltransferase (MMTV-CAT) reporter gene. It fails to stimulate MMTV-CAT expression and completely inhibits induction by the synthetic progestin R5020. However, when RU486 is combined with 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), MMTV-CAT is induced to levels approaching that stimulated by R5020 alone. Also, RU486 in the presence of 8-Br-cAMP is only partially effective in antagonizing R5020 action. The agonist activity exhibited under these conditions appears to be due to RU486 acting through hPR as evidenced by the fact that 8-Br-cAMP alone has no effect on MMTV-CAT, whereas induction by the combination of 8-Br-cAMP and RU486 is dose responsive to RU486 in a saturable manner and can be inhibited by the type I antiprogestin (prevents hPR-DNA binding) ZK98299, which does not exhibit positive functional cooperation with cAMP. Acquisition of agonist activity in the presence of 8-Br-cAMP also extends to the type II antiprogestin (permits hPR-DNA binding) ZK112993. Since RU486 is also a type II antagonist, these results suggest that detection of functional synergism between cAMP and antiprogestins may require binding of the hPR–antagonist complex to DNA. We propose that cross-talk between second messenger and steroid receptor signal transduction pathways may be one mechanism for resistance to steroid antagonists that frequently develops in breast cancer.**

Progesterone receptors (PRs) are members of the steroid receptor superfamily of ligand-inducible transcriptional activators that mediate the actions of their respective hormonal ligands by binding to specific hormone response elements of target genes (1). Steroid receptors are phosphoproteins and several have been shown to exhibit hyperphosphorylation in response to hormone binding, suggesting a link between phosphorylation and production of the fully activated form of the receptor (see review, ref. 2). Although direct studies demonstrating a functional role for steroid receptor phosphorylation are generally unavailable, several correlative studies have suggested that phosphorylation may be involved in modulating either steroid binding, DNA binding, or trans-activation (2–6).

As one line of evidence that protein phosphorylation has a role in modulating transcriptional enhancement function, various steroid receptors, including chicken PR, human estrogen receptor (ER), vitamin D receptors, human thyroid receptor β, and a nuclear orphan receptor, have been shown to induce trans-activation (or become activated by other criteria) of their cognate target genes in the absence of ligand in response to various agents that stimulate protein phosphorylation signaling pathways (7–11). Ligand-independent activation is a potentially important finding since it also provides evidence for cross-talk between second-messenger signal transduction pathways and nuclear steroid receptors, suggesting the existence of alternate pathways for regulation of receptors that may be operative in nonclassical target tissues or under certain pathological conditions. Not all members of the steroid receptor family, however, appear to exhibit ligand-independent activation in this manner. For example, it has not been possible to activate human PR (hPR) or glucocorticoid receptors (GRs) to a significant extent in the absence of ligand. However, steroid hormones and other agents that stimulate protein phosphorylation have been shown to functionally synergize to enhance trans-activation functions of hPR and GR (5, 12, 13). Why hPR and GR do not exhibit ligand-independent activation is not known. One possibility for hPR is that it binds to specific DNA sequences in a strictly hormone-dependent manner (5, 14, 15), whereas various other steroid receptors have been reported to bind to their cognate DNA elements *in vitro* in the absence of hormone, with hormone addition showing little or no effect (16–18). This suggests that the effects of modulators of protein phosphorylation on trans-activation may only be detected when receptor is bound to DNA.

The steroid analog RU486 is a potent antagonist of progesterone and glucocorticoids and it binds with high affinity to either PR or GR (19). The precise molecular mechanisms responsible for RU486 antagonism remain unknown (see review, ref. 20). Several studies with hPR have shown that RU486 efficiently promotes the receptor activation steps of dissociation of the inactive oligomeric complex, dimerization, and binding to specific response element DNA (15, 21–24). This implies that RU486 impairs a receptor activation step(s) downstream of DNA binding, presumably communication with the transcriptional machinery through protein–protein interactions. Because RU486 promotes efficient binding of hPR to DNA, we questioned in the present study whether RU486 would antagonize or permit functional synergism between second-messenger signal transduction path-

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Abbreviations: 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; MMTV, mouse mammary tumor virus; CAT, chloramphenicol acetyltransferase; PR, progesterone receptor; hPR, human PR; ER, estrogen receptor; GR, glucocorticoid receptor; OA, okadaic acid; PMA, phorbol 12-myristate 13-acetate; PRE, progesterone response element; GRE, glucocorticoid response element.

‡To whom reprint requests should be addressed at: Department of Pathology (B216), University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262.

ways and the DNA-bound PR. Results of this study demonstrate a positive functional cooperation between cAMP and the hPR–RU486 complex that results in RU486 exhibiting substantial agonist activities with respect to induction of the progesterone responsive mouse mammary tumor virus chloramphenicol acetyltransferase (MMTV-CAT) reporter gene in breast cancer cells.

MATERIALS AND METHODS

Materials. RU486 (mifepristone), unlabeled and radiolabeled (17β-hydroxy-11β-[4-dimethylaminophenyl]-17α-prop-1-ynylestra-4,9-dien-3-one), was provided by Roussel-UCLAF; ZK112993 (11β-[4-acetylphenyl]-17β-hydroxyl-17α-(1-propinyl)-4,9-estradiene-3-one) and ZK98299 (11β-[4-dimethylaminophenyl]-17α-hydroxyl-17β-[3-hydroxypropyl]-13α-methyl-4,9-gonediene-3-one) were provided by Schering AG Pharmaceuticals; and R5020 (promegestone, 17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione) was obtained from DuPont/NEN.

Cell Cultures and Reporter Gene Assay. T47D human breast cancer cells were cultured as described (5, 21) in minimum essential medium supplemented with 5% fetal bovine serum. A cloned derivative (B-11) of T47D, stably transfected with a construct containing the CAT gene linked to the MMTV promoter/enhancer (MMTV-CAT), was used to study PR-mediated target gene expression *in vivo* (21). Cells plated in six-well dishes (Falcon) at a density of 0.5×10^6 cells per well were grown for 3 days at 37°C (5, 21) and then incubated for another 24 hr with medium stripped of steroid hormones by treatment with dextran-coated charcoal (5, 21). This was followed by an additional 24-hr incubation with hormone and other compounds in the same medium. Harvested cells were washed twice in TE_{N150} [40 mM Tris (pH 7.6), 150 mM NaCl, and 1 mM EDTA] and lysed in buffer containing 20 mM potassium phosphate (pH 7.8), 5 mM $MgCl_2$, and 0.5% Triton X-100.

Cell lysates were analyzed for CAT enzyme activity by the radiometric enzymatic/organic extraction method as described (25). Enzyme activity was expressed as cpm of [^{3}H]Ac-CoA converted per μg of protein in the cell extract. In each case, cell culture treatment groups were in duplicate and enzyme activity determinations were performed in duplicate for each lysate. Values for enzyme activity, therefore, are averages from quadruplicate assay determinations.

Gel Mobility Shift Assay. The DNA-binding activity of PR in whole cell extracts of T47D cells was analyzed by gel mobility shift assay as described (15, 21). A 32-bp double-stranded synthetic oligonucleotide corresponding to the progesterone/glucocorticoid response element (PRE/GRE) of MMTV was used as target DNA. The PRE/GRE oligonucleotide was end-labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP (ICN; 3000 Ci/mmol; 1 Ci = 37 GBq) by Klenow polymerase (Pharmacia LKB) fill-in to a specific activity of ≈20,000–30,000 cpm/0.1 ng of DNA. Receptor (25 fmol) and the ^{32}P-labeled oligonucleotide (0.3 ng) were incubated at 4°C for 1 hr in 20 μl of binding buffer and samples were subjected to electrophoresis on nondenaturing 5% acrylamide gels. Gels were dried without fixation under vacuum and autoradiographed.

RESULTS

RU486 Acquires Partial Agonist Activity with Respect to Induction of the MMTV-CAT Target Gene in Cells Treated with 8-Bromoadenosine 3′,5′-Cyclic Monophosphate (8-Br-cAMP). To study hPR trans-activation in the intact cell we have used PR-rich T47D cells (clone B-11) stably transfected with a MMTV-CAT reporter gene (21). As reported in earlier studies, these cells contain very low nonfunctional levels of GR, and MMTV-CAT induction is progestin and PR dependent (21, 26). Fig. 1 shows induction of MMTV-CAT by the synthetic progestin R5020 and indicates that RU486 behaves as a pure

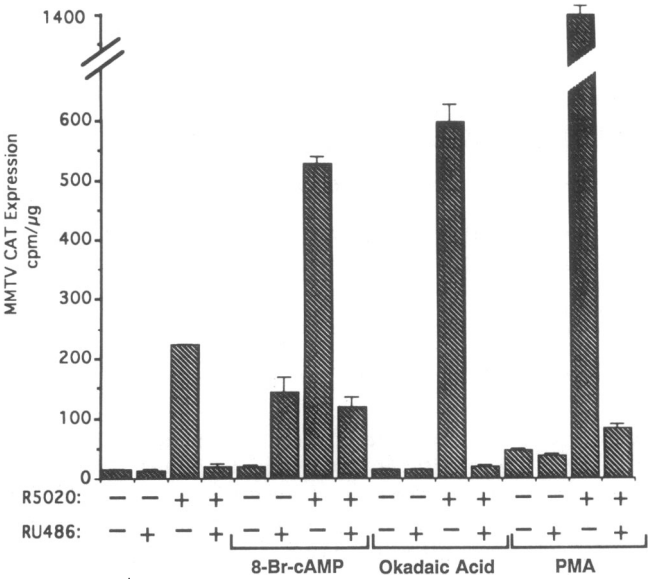

FIG. 1. Effect of cellular modulators of protein kinases/phosphatases on RU486 and R5020 induction of a progesterone-responsive target gene. PR-expressing T47D breast cancer cells (clone B-11) stably transfected with the progesterone-responsive MMTV-CAT reporter gene were incubated for 24 hr with 20 nM R5020, 40 nM RU486, or both steroids. In some experimental groups, cells were treated simultaneously with 8-Br-cAMP (0.5 mM), okadaic acid (OA; 50 nM), or phorbol 12-myristate 13-acetate (PMA; 100 ng/ml). Cells were harvested and lysed and CAT enzyme activity was measured. Data are expressed as cpm of [^{3}H]Ac-CoA converted per μg of total extracted protein and the values are averages from quadruplicate assay determinations. Bars are standard deviations.

antagonist, failing by itself to induce MMTV-CAT expression while completely blocking induction by R5020. However, the combined treatment of 8-Br-cAMP and RU486 results in a substantial induction of MMTV-CAT that approaches the level induced by R5020. The induction level in Fig. 1 is 65% of that stimulated by R5020 alone and in five replicate experiments ranged from 20% to 85% (data not shown). It should be noted that 8-Br-cAMP alone has no effect on MMTV-CAT expression; thus cAMP potentiates the ability of RU486 to exhibit agonist activity. Combined treatment with 8-Br-cAMP also compromises the ability of RU486 to antagonize R5020 action since substantial levels of MMTV-CAT expression remain when cells are treated simultaneously with 8-Br-cAMP, R5020, and a high concentration of RU486 (Fig. 1).

Fig. 1 also shows the effect of 8-Br-cAMP on potentiating R5020 induction of the MMTV-CAT reporter gene that we reported in an earlier study (5). Combined treatment with R5020 and 8-Br-cAMP produces a severalfold (3- to 4-fold on average) higher induction of MMTV-CAT expression than that of R5020 alone. To investigate whether other modulators of protein phosphorylation could similarly alter the biological activity of RU486, we tested OA, an inhibitor of protein phosphatases 1 and 2A, and PMA, a stimulator of protein kinase C. As shown in Fig. 1, the combination of OA or PMA and R5020 resulted in a severalfold higher level of MMTV-CAT expression than that induced by R5020 alone. PMA had the largest effect (7- to 10-fold higher than R5020 alone) and also produced a small but reproducible induction in the absence of hormone. However, neither OA nor PMA was able to confer partial agonist activity to RU486. RU486 continued to behave as a pure antagonist in cells treated with OA or PMA.

Type II Antagonists (RU486 and ZK112993) Acquire Agonist Activity in Cells Treated with 8-Br-cAMP, Whereas a Type I Antagonist (ZK98289) Does Not. Working with a series of RU486-related 11β-aryl-substituted compounds, Klein-Hit-

pass *et al.* (24) have shown that antiprogestins fall into two types based on their mechanism of action: those that prevent receptor binding to DNA (type I) and those that permit DNA binding (type II). The hormone dependency of hPR binding to a PRE oligonucleotide and the effects of the type I antagonist ZK98299 and type II antagonist RU486 are shown by gel mobility shift assay in Fig. 2. No binding of hPR to the PRE occurs in the absence of ligand or after addition of ZK98299 (Fig. 2). Receptor binding to the PRE requires addition of R5020 and is also inducible by RU486 (Fig. 2). We have previously shown by supershifts with receptor-specific antibodies that the three closely spaced DNA complexes detected in Fig. 2 contain receptors corresponding to the different dimers that form between the A (94 kDa) and B (120 kDa) isoforms of PR: AA, AB, and BB dimers (15, 21). Fig. 2 also shows that PR-RU486 bound to DNA exhibits a slightly faster electrophoretic mobility than PR-R5020, suggesting there are structural differences between PR–agonist and PR–antagonist complexes when bound to DNA (15, 21, 23, 24). Interestingly, treatment of cells with 8-Br-cAMP did not effect this faster mobility, suggesting that cAMP does not restore the conformation to that of PR bound initially to hormone agonist.

To test whether other antiprogestins have the capacity to exhibit agonist activity in combination with 8-Br-cAMP, we have analyzed the type I antiprogestin ZK98299 and one other type II antiprogestin, ZK112993. As shown in Fig. 3*A*, ZK98299 behaves as a pure antagonist in the absence and presence of 8-Br-cAMP. Under both conditions, ZK98299 fails to induce MMTV-CAT and it completely blocks induction by R5020. In contrast, ZK112993 combined with 8-Br-cAMP exhibits considerable agonist activity (Fig. 3*B*).

Partial Agonist Activity of RU486 Exhibited in the Presence of 8-Br-cAMP Appears To Be Receptor Associated. Experiments were conducted to determine whether the agonist activity exhibited by RU486 in cells treated with 8-Br-cAMP is the result of authentic nonmetabolized RU486 acting through hPR. Earlier studies from Horwitz's group showed that RU486 is stable in breast cancer cells in culture (27). To rule out that RU486 might be converted to a metabolite with

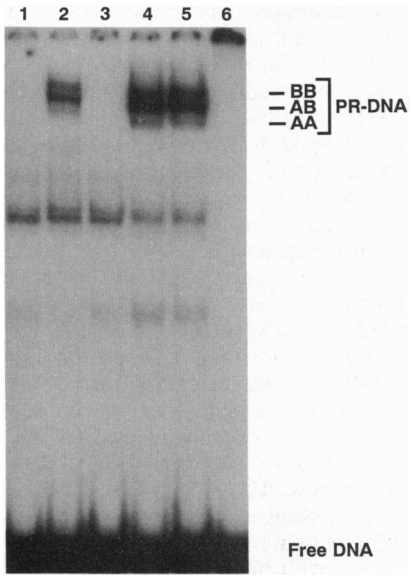

FIG. 2. Progestin and antiprogestin effects on hPR binding to a PRE. T47D breast cancer cells were left untreated (lane 1) or treated for 2 hr at 37°C with R5020 (lane 2), ZK98299 (lane 3), RU486 (lane 4), or RU486 and 8-Br-cAMP (0.5 mM) (lane 5). Lane 6, no protein. Aliquots of whole cell extracts containing 25 fmol of PR were bound to 0.3 ng of a ^{32}P-labeled PRE and analyzed by gel mobility shift assay as described in the text.

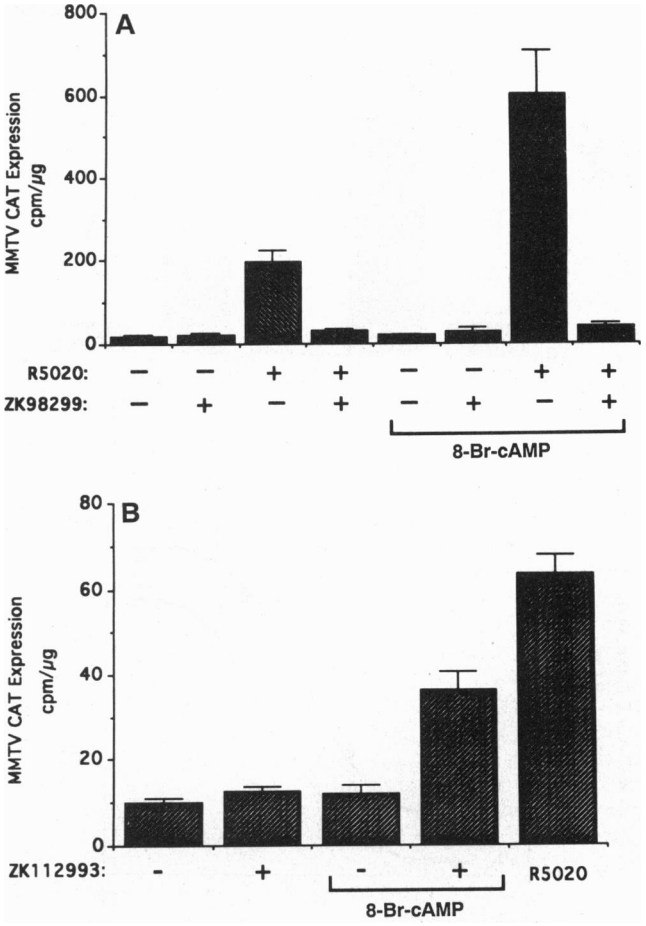

FIG. 3. Effect of 8-Br-cAMP on the biological activity of the progesterone antagonists ZK98299 and ZK112993. (*A*) B-11 cells were incubated for 24 hr without hormone or with 10 nM R5020, 200 nM ZK98299, or both steroids in the presence or absence of 8-Br-cAMP (1 mM). (*B*) B-11 cells were incubated for 24 hr without or with 10 nM ZK112993 in the presence or absence of 8-Br-cAMP (1 mM). As an internal control for the level of stimulation by hormone agonist, cells were also incubated with R5020 alone. Cells were lysed and CAT activity was assayed; values are expressed as in Fig. 1. In Figs. 3 and 4, R5020 concentration was lower than in Fig. 1 (10 nM instead of 20 nM). This is a near-saturating dose of R5020 and was used to ensure efficient competition with ZK98299, which has a lower binding affinity for hPR than other progestin antagonists.

weak agonist activity in response to elevated intracellular cAMP, we determined by HPLC analysis that [^{3}H]RU486 added to T47D cell cultures for 24 hr is virtually unmetabolized in the absence and presence of added 8-Br-cAMP (not shown). Fig. 4*A* shows that the type I antiprogestin ZK98299, which does not exhibit agonist activity (Fig. 3*A*) when combined with cAMP, is capable of inhibiting the MMTV-CAT expression (Fig. 4*A*) induced by the combined treatment of RU486 and 8-Br-cAMP. ZK98299 is also effective in inhibiting in total the augmented induction achieved by the combination of 8-Br-cAMP and R5020, indicating that the potentiating effect of 8-Br-cAMP on R5020 action is also receptor associated (Fig. 3*A*). In further support of a receptor-dependent mechanism, RU486 in cells cotreated with 8-Br-cAMP stimulates MMTV-CAT expression in a dose-dependent and saturable manner that is virtually superimposable upon the dose–response curve obtained with the agonist R5020 alone (Fig. 4*B*). It should be noted that the dose–response curves in Fig. 4*B* are plotted on different scales; the magnitude of RU486 stimulation in cells treated with 8-Br-cAMP is less than that of R5020 alone. We have

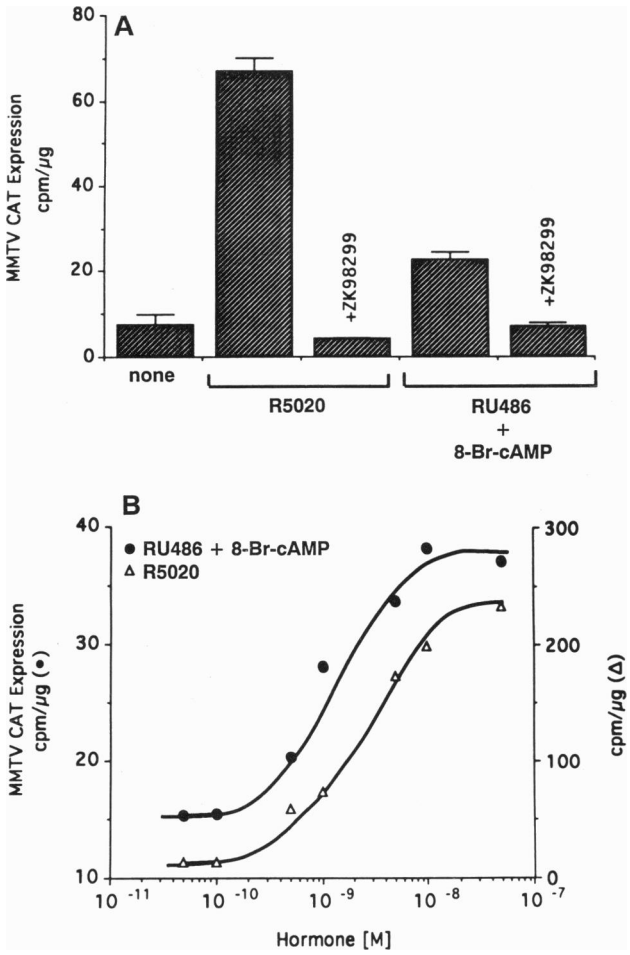

FIG. 4. Evidence that agonist activity exhibited by RU486 in cells treated with 8-Br-cAMP is receptor associated. (*A*) Stably transfected B-11 cells were incubated for 24 hr without or with hormone in the absence or presence of 8-Br-cAMP, 10 nM R5020, 10 nM R5020 plus 100 nM ZK98299, 10 nM RU486 plus 1 nM 8-Br-cAMP, or 10 nM RU486 plus 100 nM ZK98299 plus 1 mM 8-Br-cAMP. (*B*) B-11 cells were incubated for 24 hr with increasing concentrations of R5020 alone or with the same concentrations of RU486 in the presence of a constant amount of 8-Br-cAMP (1 mM). Cells were harvested and lysed and CAT activity was measured as in Fig. 1.

also shown in previous studies that RU486 alone over the same concentration range as that used in Fig. 4*B* gives no stimulation of MMTV-CAT (21) and that 8-Br-cAMP alone over a concentration range of 1–1000 μM also has no inductive effects (5). These results taken together suggest that RU486 when combined with 8-Br-cAMP is acting as a partial agonist through the PR.

DISCUSSION

There are several reports that cAMP and steroid hormones can synergize positively in the regulation of transcription of various target genes (5, 7, 8, 12, 28, 29) and in the case of glucocorticoids in the induction of cell lysis (30). Although the precise molecular mechanism underlying cooperative interactions between cAMP signaling pathways and steroid hormones is not known, some studies have suggested that cAMP can alter the efficacy of the steroid receptor as a trans-activator of gene expression (5, 7, 12). In a previous report, we showed that cAMP can exert a strong positive synergistic effect on progesterone-dependent PR-mediated regulation of the MMTV-CAT reporter gene (5).

The major finding of the present study is that the potent progesterone antagonist RU486 has the potential to acquire substantial agonist activity in response to stimulation of cAMP signaling pathways. Moreover, agonist activity appears to be the result of authentic RU486 activity through the PR. The other potentially important finding of this study is that the phenomenon of acquiring agonist activity in cells treated with cAMP extends to one other antiprogestin, ZK112993, but not to the antiprogestin ZK98299. Because RU486 and ZK112993 are both type II antagonists capable of promoting hPR-DNA binding (24) and ZK98299 is a type I antagonist that does not permit DNA binding (Fig. 2), these results suggest that detection of cross-talk between cAMP signaling pathways and antiprogestins may require binding of the receptor–antagonist complex to DNA. It is of interest that other agents such as OA and PMA, which can also potentiate progesterone-dependent increases in target gene expression, fail to confer agonist activity to RU486 (Fig. 1). Thus, functional synergism with RU486 may be fairly selective for cAMP-enhancing agents.

It is well known that steroid antagonists can display varying amounts of partial agonist activity depending on the end-point response, the cell or tissue type, or the growth state of the cell (see review, ref. 31). With RU486 this can also depend on whether the antagonist is bound to GR, hPR, or the A and B isoforms of hPR (23, 31). The variation in agonist activity exhibited by RU486 in the present study appears to be due to quite a different mechanism than that described previously, since RU486 dramatically converts from a pure antagonist to a substantial partial agonist in the same cells (and same growth stage) and with the same target gene. The combined effect of RU486 and 8-Br-cAMP can also confer significant agonist activity with respect to induction of another target gene in T47D cells (32), the endogenous progesterone/glucocorticoid-responsive human metallothionein II-A (M.L.M., D.P.E., and S.K.N., unpublished). These results taken together with the evidence that RU486 is acting as a partial agonist through hPR suggest that functional properties of the receptor–antagonistic complex can become activated by cooperative interactions with cAMP signaling pathways.

Recent studies from Simons' groups (31) have described an analogous variation in the percent agonist activity for antiglucocorticoids in the same cell with the same target gene. They identified a functional cis-acting element, distinct from classical GREs, termed glucocorticoid modulatory element (GME), that appears to mediate variation in the percent agonist activity of glucocorticoid antagonists. This does not appear to be related to the present results, at least initially, because this GME appears to require binding of a trans-acting protein that is functionally dependent on cell growth stage. Also, the GME has been described only with the TAT gene and does not appear to be operative on some other glucocorticoid-responsive genes (31).

The underlying molecular mechanisms responsible for positive cooperativity between cAMP and the receptor–RU486 complex are not known. Two conceptual models are presented in Fig. 5. A basic assumption is that cAMP selectively enhances trans-activation function of PR and not other functional properties. This is supported by the fact that 8-Br-cAMP does not affect cellular levels of PR, steroid, or DNA-binding activities (ref. 5 and Fig. 2). It is generally believed that the hPR–RU486 complex binds efficiently to specific DNA but assumes a distinct conformation that does not permit efficient communication (through protein–protein interaction) of PR with the transcriptional machinery at the core promoter (Fig. 5 *Upper*) (21–24). Thus, trans-activation is either very weak or does not ensue. We propose that cross-talk with cAMP signaling pathways, through changes in protein phosphorylation, increases the efficiency of PR interaction with the transcriptional machinery and, thus, partially overcomes the antagonistic effect of RU486. This could occur by direct modification of receptor itself (Fig. 5 *Lower*

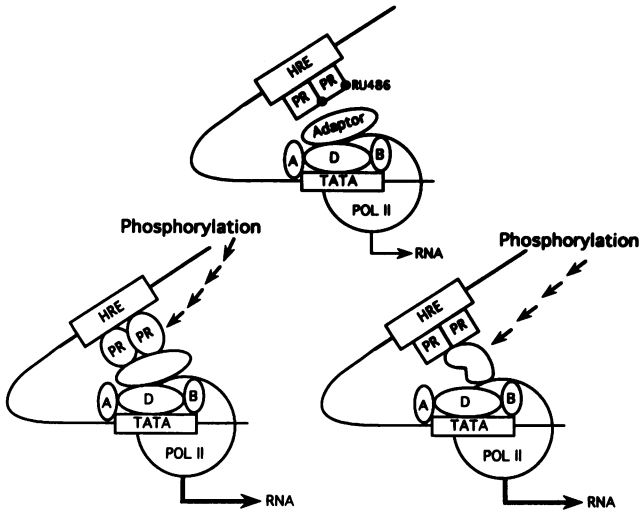

FIG. 5. Proposed mechanism for acquired agonist activity of RU486 in cells treated with 8-Br-cAMP. The PR–RU486 complex bound to target DNA fails to interact efficiently with the transcriptional machinery because the receptor is in the wrong conformation. Cross-talk between second messenger signal transduction pathways involving protein kinases and nuclear steroid receptors stimulates the PR–RU486 complex to interact more efficiently with the transcriptional apparatus. This could result by a direct modification of PR (*Lower Left*) or indirect modification of an adaptor protein (*Lower Right*). HRE, hormone response element; POL II, RNA polymerase II.

Left) or indirectly through modification of another component or adaptor protein involved in PR-mediated processes (Fig. 5 *Lower Right*). We tend to favor the indirect hypothesis for several reasons. First, 8-Br-cAMP does not convert the mobility of the PR–RU486 complex on gel shift assays to that of PR–R5020, suggesting that the conformation of PR itself has not been affected (Fig. 2). Second, we reported in an earlier study that 8-Br-cAMP has no obvious effect on the net phosphorylation of intact hPR (5). However, because hPR is phosphorylated on as many as six or seven serine residues (33), it is possible that cAMP could affect a single key phosphorylation site without a detectable change in total phosphorylation. In this regard, it is of interest that Somers and DeFranco (13) showed that functional synergism between OA and trans-activation of GR did not correlate with changes in site-specific phosphorylation as determined by tryptic phosphopeptide mapping of GR.

The most widely used hormonal agent for treatment of hormone-dependent breast cancer is the antiestrogen tamoxifen, which acts as a competitive antagonist of estrogen action at the ER level (34). A frequent problem is the eventual development of resistance to tamoxifen upon prolonged administration, despite the fact that resistant tumors often retain expression of ER. Studies with experimental breast cancer have suggested several mechanisms for antiestrogen resistance, including mutations in the steroid receptor, constitutive overproduction of paracrine growth factors, and changes in pharmacology of the antisteroid (34, 35). We propose an alternate mechanism that involves cross-talk between second messenger and steroid receptor signal transduction pathways of the nature observed in the present study with breast cancer cells in culture. Cross-talk could be initiated by changes in growth factor or protooncogene production during the course of tumor progression. By this mechanism, tumors would not become resistant to antisteroids *per se*; rather, the receptor–antagonist complex would acquire agonist activity. Although the present study is with RU486, this may be a paradigm for antiestrogens. Tamoxifen acts by a general mode similar to that of RU486, permitting ER to bind to specific DNA (36). We speculate that tamoxifen may also acquire agonist activity through cooperative interactions between two signal transduction pathways. The present results also suggest that antisteroids that do not permit receptor binding to DNA may be less prone to this mode of resistance.

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