Endocrine (1995) 3, 5-12

Selective effects of 8-Br-cAMP on agonists and antagonists of the glucocorticoid receptor

Shimin Zhang & Mark Danielsen

Department of Biochemistry and Molecular Biology, Georgetown University Medical School, 3900 Reservoir Road, N.W., Washington, DC 20007, USA

RU486 has been reported to be a glucocorticoid receptor (GR) and a progesterone receptor (PR) antagonist. We have analysed RU486 activity on the GR in WCL-2 (CHO) cells and in COS-7 cells transiently transfected with the mouse GR and with the reporter MMTVCAT (MCAT). These cell lines do not contain any active progesterone or androgen receptors. In both cell lines RU486 is a partial agonist of the GR with 10-15% of the activity of dexamethasone. As expected, RU486 is also a partial antagonist of the GR. Treatment of COS-7 cells with 8-Br-cAMP increases the agonist activity of both dexamethasone and RU486. This cAMP induced superactivation is seen with all steroids that have full or partial agonist activity. In contrast, the activities of ZK98.299 and R5020, which are complete antagonists of the GR without any agonist activity, are not affected by 8-Br-cAMP treatment. This effect of 8-Br-cAMP is not seen in WCL2 cells. 8-Br-cAMP, therefore, is not a switch which changes antagonists to agonists but is, rather, a cell specific activator of all agonists whether they have full or only partial agonist activity.

Keywords: glucocorticoid receptor; RU486; cyclic AMP

Introduction

The mouse GR is a member of the steroid, thyroid and retinoic acid receptor super-family (Danielsen *et al.*, 1986; Evans, 1988; Danielsen, 1991). Each of these intracellular receptors has a C-terminal hormone binding domain of approximately 250 amino acids, a central DNA binding domain consisting of two C_2H_2 zinc fingers, and an N-terminal domain of variable length and function. The glucocorticoid, progesterone, androgen and mineralocorticoid receptors form a subgroup of this super-family (Danielsen *et al.*, 1989; Umesono & Evans, 1989). The DNA binding domains are approximately 90% identical and they can all bind to the DNA sequence AGAACAnnnTGTTCT and activate transcription (reviewed in Beato, 1989). The hormone binding domains are also highly homologous (more than 50% identity) and this is reflected in their cross reactivity for endogenous steroids. For instance, both the mineralocorticoid receptor and the GR are activated by hydrocortisone (Arriza *et al.*, 1987), similarly the androgen receptor (AR) is activated by both androgens and progestins (Quarmby *et al.*, 1990).

Within cells the glucocorticoid receptor is found bound to the heat shock proteins HSP90 and HSP56 (reviewed in Danielsen, 1991; Pratt, 1993; Smith & Toft, 1993). It is thought that hormone binding brings about a conformational change in the receptor that causes dissociation of this complex, allows the receptor to bind to DNA, and activate transcription. Partial agonists, such as progesterone, activate the receptor but bring about less transcriptional activation than full agonists such as dexamethasone. For this reason partial agonists are also competitive antagonists of the glucocorticoid response. The defect in the response to partial agonists/antagonists is not understood. However, there is increasing evidence that the major defect occurs after the association of the receptor with DNA and is probably due to altered interaction with the transcriptional machinery (reviewed in Baulieu, 1991; Simons *et al.*, 1992).

RU486 (Agarwal *et al.*, 1987; Ulmann *et al.*, 1990) is an 11β (4-dimethylaminophenyl) substituted steroid that binds tightly to both the GR and PR and has antagonist activity for both receptors *in vivo* and *in vitro* (Chrousos *et al.*, 1988; Laue *et al.*, 1988; Baulieu, 1989). It may also be an antagonist of the AR (Chrousos *et* al., 1988). Although its main clinical use is to terminate pregnancy, it may also be useful for contraception, and for treatment of breast cancer, glaucoma, meningiomas and Cushing's syndrome (Laue *et al.*, 1988; Baulieu, 1989, Schneider *et al.*, 1989). *In vivo*, RU486 has some agonist activity, for instance it suppresses pituitary ACTH secretion (Laue *et al.*, 1988). *In vitro*, RU486 has been reported to be either a complete or a partial antagonist depending on the cell line, receptor type and promoter studied (Bourgeois *et al.*, 1984; Gagne *et al.*, 1986; Webster *et al.*, 1988; Simons *et al.*, 1989; Gruol & Altschmied, 1993). ZK98.299 (Neef *et al.*, 1984) differs from RU486 only in the configuration and the substituents on the D-ring (Figure 1 compares the structure of these steroids). It is thought that ZK98.299 is a full antagonist on both receptors with no agonist activity (Neef *et al.*, 1984; Klein-Hitpass *et al.*, 1991; Sartorius *et al.*, 1993).

We have characterized the response of the GR to various steroids and determined the effect of 8-Br-cAMP on their activity. We find that 8-Br-cAMP increases the activity of all steroids that show some transcriptional activity in the absence of 8-Br-cAMP. These steroids include dexamethasone, progesterone and RU486. Steroids which bind to the GR but which do not activate transcription such as R5020 and ZK98.299 are not affected by 8-Br-cAMP. The superinduction of transcriptional activity by 8-Br-cAMP is seen in COS-7 cells but not in WCL-2 cells even though the hormone binding characteristics of the receptor in the two cell lines are almost identical. We conclude that 8-Br-cAMP does not act as a switch

Correspondence: Mark Danielsen
Received 21 March 1994; accepted 2 August 1994

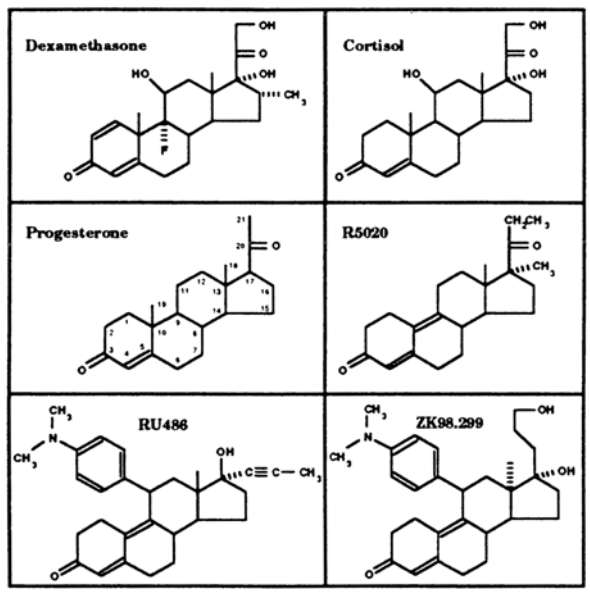

Figure 1 Structure of selected glucocorticoid agonists and antagonists

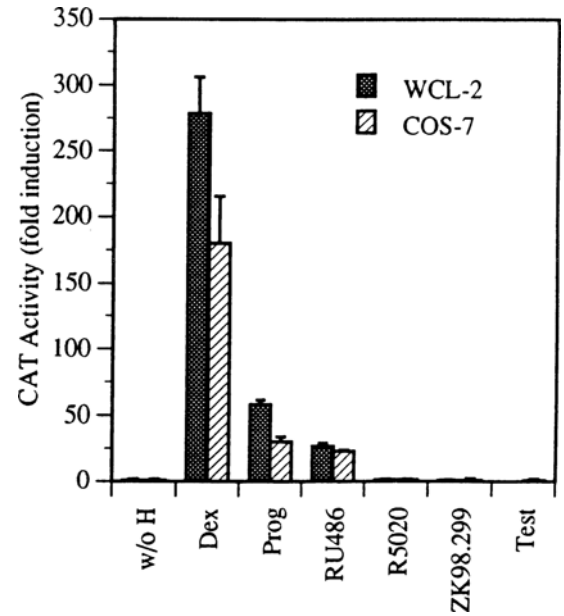

Figure 2 Agonist activity of RU486 and progesterone on MCAT in WCL-2 and COS-7 cells. Cells were transfected with pMCAT (10.5 μg/10 cm dish) and, in the case of COS-7 cells, with 150 ng pmGR. Two days later, the cells were induced with concentrations of hormone ranging form 10^{-11} to 10^{-5} M as shown in Figure 3. CAT activity was determined 40 h later. The fold inductions for WCL-2 (shaded) and COS-7 (striped) cells were calculated using the maximum value obtained from the dose response curve. The basal level of CAT activity in these assays was 0.68 fmole [^{3}H]acetyl chloramphenicol produced per minute per mg protein. w/o H, no hormone; Dex, dexamethasone; Prog, progesterone; Test, testosterone.

changing antagonists to agonists, rather it brings about a cell specific increase in the activity of all steroids that show some inherent agonist activity.

Results

RU486 and progesterone are partial agonists of the GR

The CHO cell line WCL-2 is an excellent cell line to study GR activation since it expresses high levels of GR (Hirst *et al.*, 1990) but no detectable levels of PR or AR (see below). To measure GR transcriptional activity in these cells, we transiently transfected them with the well characterized reporter plasmid pMCAT and treated with steroid for 2 days (Danielsen *et al.*, 1986, 1989). To ensure that direct comparisons could be made between each steroid treatment, each set of experiments was performed on aliquots of the same transfection. At saturating levels of steroid, RU486 induced CAT activity 23-fold to levels of approximately 13% those obtained with dexamethasone (Figure 2). The agonist activity of RU486 is seen even more clearly in Figure 3 where dose response curves of RU486, dexamethasone and progesterone are compared. It should be noted that the data in Figure 3 have been normalized to the maximum value obtained for each steroid (Figure 2) so that EC_{50} values can be compared easily. RU486 induces CAT activity with an EC_{50} of 11.8 nM which is only 2.5-fold higher than the EC_{50} of the full agonist dexamethasone (Figure 3). Progesterone also induces CAT activity but requires approximately 300-fold more hormone than RU486. It should be noted that the curves are computer fit using the assumption that the CAT activity obtained is proportional to the amount of occupied receptor as defined by the law of mass action (Materials and methods). The EC_{50} values obtained are in close agreement with the Kd/Ki values obtained from hormone binding experiments (Figure 4). This indicates that the response of these cells reflects the amount of occupied receptor, and by extension that the GR is not in excess in these cells [discussed in (Simons *et al.*, 1992)].

Since pmCAT can be induced by both PR and AR in some cells (Cato *et al.*, 1986; Brinkmann *et al.*, 1989) it was important to ensure that RU486 and progesterone were activating transcription through the GR and not through these other receptors. This is demonstrated in Figures 2 and 3 where neither testosterone, an AR agonist, nor R5020, a specific PR agonist, induced CAT activity.

We have also examined the agonist activity of RU486 in COS- 7 cells. These cells are often used to study the transcriptional activity of steroid receptors because they are easily transfected and have no measurable levels of any active steroid receptor (Evans, 1988). We cotransfected these cells with the mouse GR expression plasmid pmGR and with pMCAT and then measured the response to steroids as shown in Figures 2 and 3b. R5020 was again inactive, confirming the absence of progesterone receptors. RU486 and progesterone were again partial agonists with EC_{50}'s that are similar to those obtained in the WCL-2 cells (4.3 and 428 nM respectively. In the experiment shown in Figure 3, RU486 and progesterone gave 13% and 17% of the activity obtained with dexamethasone.

RU486 and progesterone are partial antagonists, while ZK98.299 and R5020 are complete antagonists of the GR

We used transfected COS-7 cells to examine the antagonist activity of RU486 and other steroids. Cells

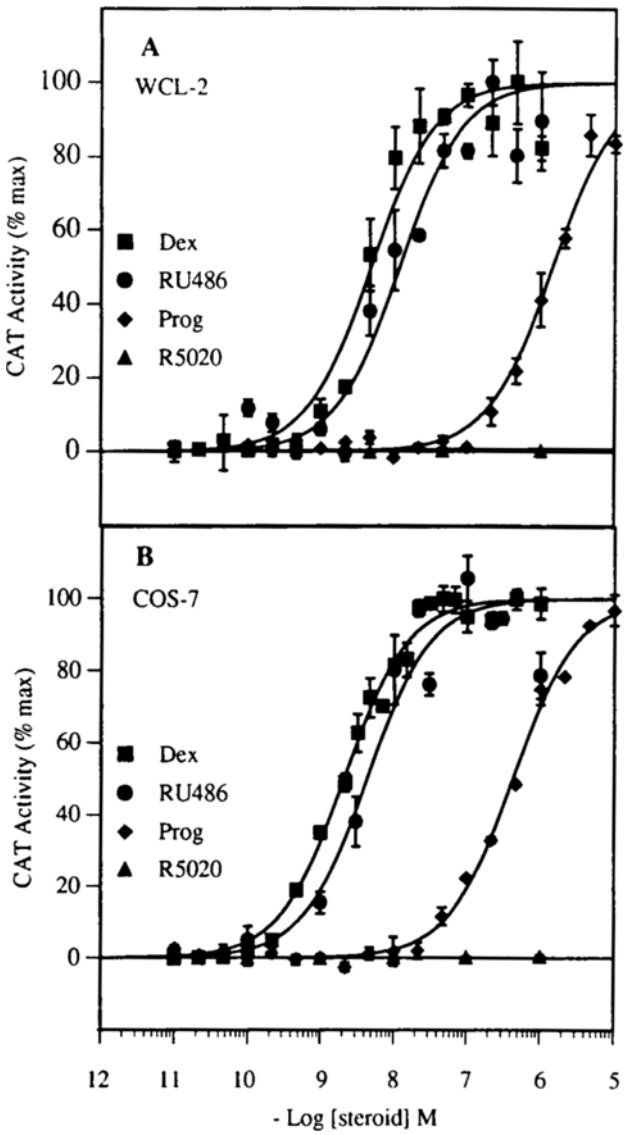

Figure 3 Dose response of GR to dexamethasone, RU486 and progesterone in WCL-2 and COS-7 cells. Cells were transfected with pMCAT (10.5 μg/10 cm dish) and, in the case of COS-7 cells, with 150 ng pmGR. Two days later, the cells were induced with the indicated concentrations of hormone and CAT activity was determined 40 h later. The dose response curves to dex (■), RU486 (●), Progesterone (◆), and R5020 (▲) were computer fit based on the Michaelis-Menton equation and assuming that the amount of transcriptional activity is proportional to hormone bound receptor (Materials and methods). The computer fits shown all have r^2 values of above 0.94. All data points were performed in at least triplicate (average ±SE shown) (**A**) WCL-2 cells (**B**) COS-7 cells

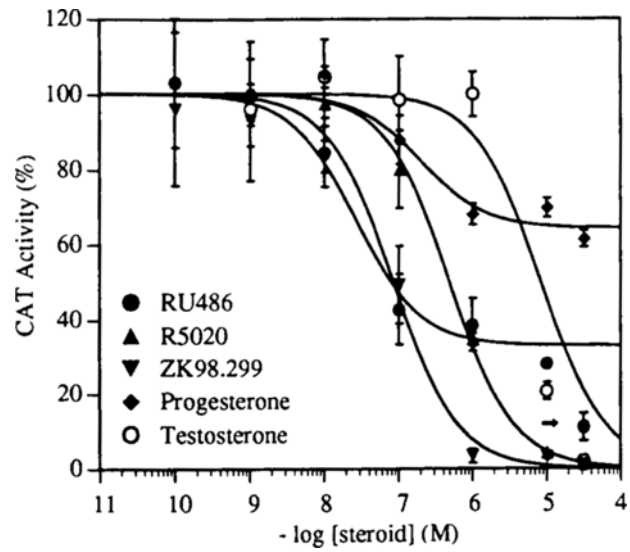

Figure 4 Inhibition of dexamethasone induction of CAT activity by various steroids. COS-7 cells were transfected with pmGR and pMCAT as described in Materials and methods. The cells were incubated with a mixture of 2 mM dex and various concentrations of RU486 (●), R5020 (▲), ZK98.299 (▼), progesterone (◆), testosterone (○), and CAT activity was determined 40 h later. The results are expressed as a percentage of CAT activity obtained with 2 nM dexamethasone alone. The data is computer fit (Materials and methods) based on the Michaelis-Menton equation assuming that the amount of transcriptional activity is proportional to agonist bound receptor and that antagonist bound receptor is inactive. Inhibition curves for RU486 and progesterone do not reach 0% due to the intrinsic agonist activity of these compounds (see text). The arrow indicates a data point for RU486 inhibition at 3×10^{-4} M that does not lie on the computer drawn fit (see text). The computer fits shown all have r^2 values of above 0.95. All data points were determined in triplicate (average ± SD shown). The data shown is from one experiment which is representative of three similar, independent experiments

were transfected with pmGR and pMCAT and 2 days later cells were induced with a mixture of 2 nM dexamethasone and increasing concentrations of other steroids (Figure 4). ZK98.299, R5020, and testosterone act as pure antagonists of the GR with calculated Ki values of 51 nM, 291 nm and 5010 nM respectively. It should be noted that the curves shown in Figure 4 are computer fit and reflect competitive binding to a single site on the GR (Materials and methods). RU486 and progesterone, on the other hand, give complex inhibition curves due to their partial agonist activity. The computer generated curve fitting ($r^2 = 0.95$ for RU486, $r^2 = 0.96$ for progesterone) takes into account the antagonist properties of these steroids due to their displacement of dexamethasone and also their intrinsic agonist activity (Materials and methods). The increased inhibition of CAT activity seen with 0.3 mM RU486 (shown by an arrow in Figure 4) is probably due to non-specific 'squelching' that is seen occasionally with all agonists at very high concentrations. The amount of agonist activity shown by progesterone (25%) and RU486 (13%) in this experiment is similar to the partial agonist activity of these compounds when used individually.

Antagonist activity is due to inhibition of hormone binding

In order to confirm that the antagonist activity seen in the CAT assay was indeed due to competition for binding to the GR, we performed a competition binding experiment. A hormone binding assay was set up in which a fixed concentration of [^{3}H]triamcinolone acetonide (10 nM) was mixed with increasing concentrations of various cold steroids (Figure 5). The curve fits shown are based on the assumption of competitive binding for the GR and, except for testosterone, have r^2 values above 0.95. The calculated Ki values are TA, 2.6 nM; dex, 3.3 nM; RU486, 5 nM; ZK98.299, 38 nM; R5020, 79 nM; progesterone, 140 nM; testosterone, 12μM. Although testosterone shows competition with a

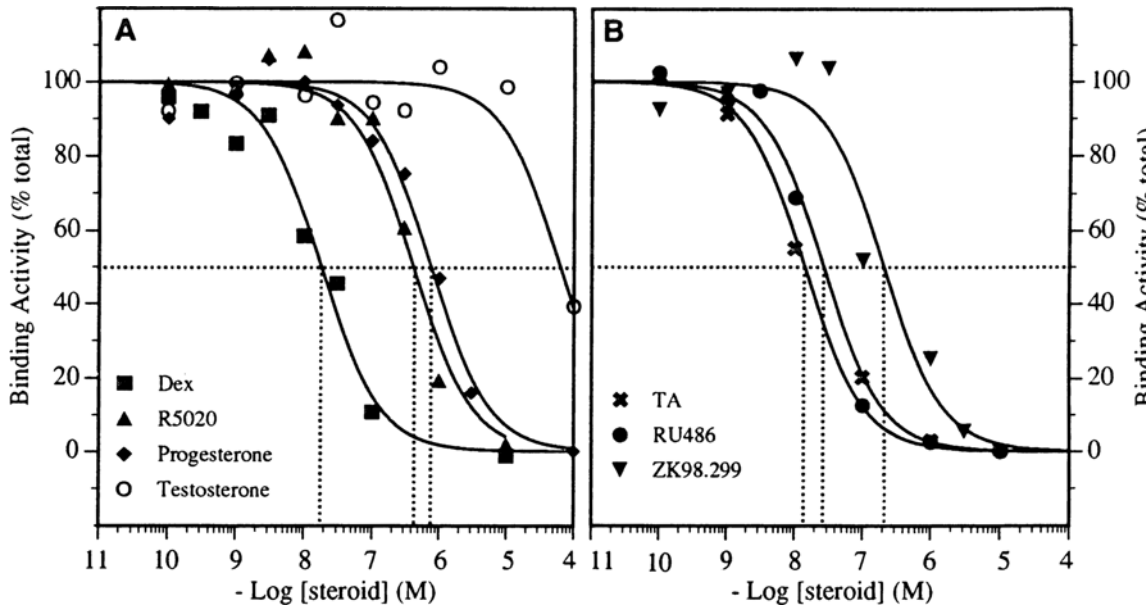

Figure 5 Binding of triamcinolone acetonide to mGR and competition with other steroids. COS-7 cells were transfected with pmGR and cytosol prepared 4 days later. The cytosol was equilibrated with 10 nM [^{3}H]TA in the presence of various concentrations of cold hormones on ice overnight. Binding was determined using the charcoal adsorption method as described in Materials and methods. Error bars have been omitted for clarity. (**A**) and (**B**) are different experiments. Dexamethasone (■), ZK98.299 (▼), RU486 (●), R5020 (▲), progesterone (◆), testosterone (○). All points were performed in triplicate (average shown). The experiment was performed three times with similar results. The curves are computer fit assuming competitive equilibrium binding (Materials and methods) and the curves have r^2 values above 0.95 except for testosterone ($r^2 = 0.83$)

calculated Ki of 12μM, this value is only approximate, since the maximum competing concentration of testosterone used was 100μM which did not yield complete inhibition. These data indicate that each of these steroids binds to the same site on the GR, and that antagonism is due to competition for receptor occupancy.

8-Br-cAMP increases the activity of both full and partial agonists but has no effect on complete antagonists in COS-7 cells but not in WCL-2 cells

While this work was in progress (Beck *et al.*, 1993b) showed that intracellular 8-Br-cAMP levels could convert RU486 from being an antagonist to a partial agonist of the PR. This is not the case with the GR since, at least in our system, RU486 is always a partial agonist. To determine if 8-Br-cAMP has any effect on the transcriptional activity of the GR, we measured the response of transfected COS-7 cells to RU486 and dexamethasone in the presence or absence of 1 mM 8-Br-cAMP. In these experiments cells were first transfected and then pooled and redistributed to multiwell dishes to ensure that direct comparisons could be made between the various hormone treatments.

1 mM 8-Br-cAMP has no effect on the basal level of transcription obtained from MCAT but it does increase the transcriptional activity of GR induced by dexamethasone, progesterone and by RU486 (Figure 6). This super-induction by 8-Br-cAMP occurs when the cells are treated with RU486 alone, or when they are treated with a mixture of RU486 and dexamethasone (Figure 7). The increase in activity of RU486 activation is similar to that with dexamethasone, so that the agonist activity as measured as a percentage of the dexamethasone response remains constant. On the other hand, 8-Br-cAMP has no effect on the full antagonists ZK98.299, and R5020. 8-Br-cAMP does not change the EC_{50} of the response to either agonists or partial agonists (not shown). The superinduction is also not due to increased GR protein levels since no increase in GR protein can be detected by Western blot analysis (not shown). These effects of 8-Br-cAMP on GR transcriptional activation are not universal, however, since 8-Br-cAMP has no stimulatory effect on GR activity in WCL-2 cells (Figure 6).

Discussion

It has been suggested recently that 8-Br-cAMP acts as a switch which converts RU486 from being an antagonist of the GR to a partial agonist (Gruol & Altschmied, 1993; Nordeen *et al.*, 1993). We have found that this is not the case in either CHO cells, or in GR transfected COS-7 cells, since in both cell lines RU486 induces significant transcriptional activity from MCAT even in the absence of 8-Br-cAMP. Treatment of COS-7 cells with 8-Br-cAMP cells with 8-Br-cAMP results in increased agonist activity of RU486. However, this activation by 8-Br-cAMP is not unique to RU486, but rather, also occurs with other partial agonists such as progesterone and with full agonists such as dexamethasone. Two GR antagonists, R5020 and ZK98.299, were found to bind to the GR but were unable to activate transcription even in the presence of 8-Br-cAMP. This suggests that 8-Br-cAMP can only increase the activity of either partial or full agonists but has no effect on complete antagonists. The effects

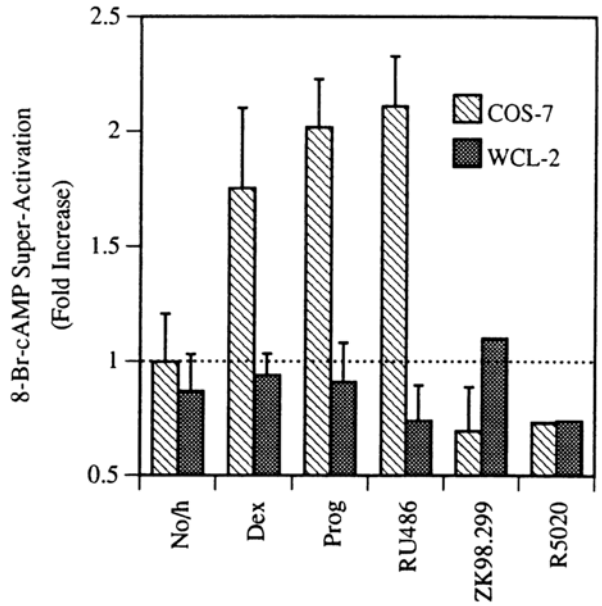

Figure 6 8-Br-cAMP increases the agonist activity of dexamethasone, RU486 and progesterone in COS-7 cells but not WCL-2 cells. COS-7 cells were transfected with pmGR and pMCAT, and WCL-2 cells were transfected with pMCAT alone as detailed in Materials and methods. After transfection the cells were pooled and redistributed into multiwell dishes. Two days later cells were induced with 10 nM dexamethasone (dex), 1 μM progesterone (Prog), 10 nM RU486, 10 nM ZK98.299, 100 nM R5020, either with or without 1 mM 8-Br-cAMP. CAT activity was determined 40 h later. The graph shows the fold stimulation of CAT activity with 8-Br-cAMP. The values shown are mean values with standard errors from 1–8 independent experiments in which CAT activity was determined in triplicate for each

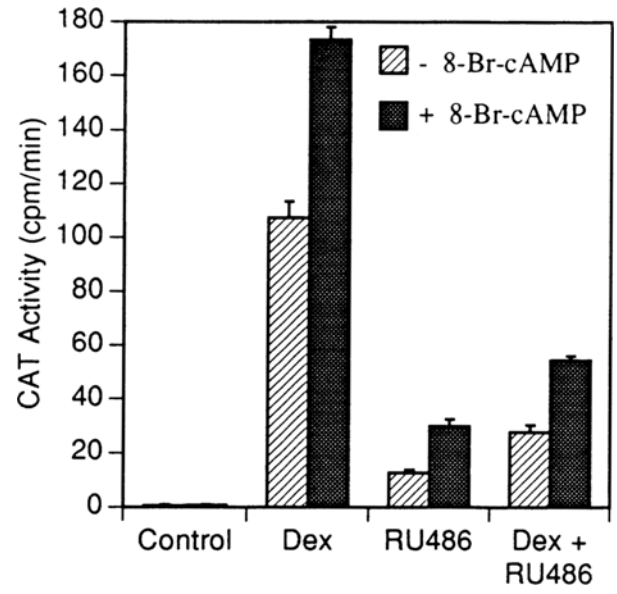

Figure 7 RU486 is still an antagonist in the presence of 8-Br-cAMP COS-7 cells were treated as detailed in Figure 6, legend; except that cells were induced with 10 nM dex, 1 μM RU486, or both as indicated

of 8-Br-cAMP are cell specific since 8-Br-cAMP does not increase the activity of glucocorticoid agonists in WCL-2 cells.

Partial GR agonists/antagonists, such as RU486 and progesterone, bring about transcriptional responses that are only a fraction of the transcriptional response to full agonists such as dexamethasone. The reason for this partial transcriptional activity is not understood, although we do know that it is not related to the affinity for the receptor (RU486 binds to the GR with about the same affinity as dexamethasone and with higher affinity than cortisol). The defect in the RU486 response could be due to a number of mechanisms including inefficient release of HSP90, formation of abortive receptor complexes, decreased nuclear localization, altered or decreased DNA binding, or inefficient coupling with the transcriptional machinery. It is clear that RU486 is less efficient than dexamethasone in releasing receptor from HSP90 and bringing about nuclear localization (reviewed in Baulieu, 1991; Mao *et al.*, 1992; Beck *et al.*, 1993a). For instance in the mouse lymphoma cell line W7-MG1, RU486 is 30% as efficient as triamcinolone acetonide in promoting tight nuclear binding (Gruol & Altschmied, 1993). It seems unlikely, however, that this rather modest effect on nuclear localization could account for the dramatic effect seen on transcription. Defective nuclear translocation also does not easily explain why the degree of agonist activity shown by partial agonists is at least partly determined by the cell type, growth state of the cells (Oshima & Simons, 1992), and by the expression levels of the receptor (S. Zhang and M. Danielsen, unpublished). Taken together, we believe that the data support a model in which RU486 (and other partial agonists) brings about a conformational change in the receptor that is different from the conformational change induced by a full agonist. This results in inefficient release of HSP90 and accounts for the poor nuclear localization seen in some cells. In addition, the altered conformation of the receptor brings about inefficient coupling with the transcriptional machinery. This could be in the form of weak homodimerization on the GRE, weak heterodimerization (i.e. interaction) with other transcription factors or steric hindrance associated with a malfolded hormone binding domain.

The effects of 8-Br-cAMP are probably due to activation of protein kinase A (PKA), since other investigators have found similar or related transcriptional effects in response to forskolin (Nordeen *et al.*, 1993) or PKA overexpression in cells (Rangarajan *et al.*, 1992). PKA activation increases transcription due to both full and partial agonists indicating that it affects a common rate limiting step in the pathway of GR transcriptional activation. It is particularly interesting that the effect of 8- Br-cAMP is the same with RU486 activated receptors as it is with dexamethasone activated receptors, implying that it affects a process which is limiting with both agonists and partial agonists. One possibility is that 8-Br-cAMP increases the level of phosphorylation of the receptor resulting in improved dissociation from HSP90 or increased transcriptional activity. Increased phosphorylation of the receptor appears not to be involved, however, since we have found that in COS-7 cells transfected with a GR in which the major sites of phosphorylation have been removed by site directed mutagenesis (Mason & Housley, 1993); MCAT is induced normally by both

dexamethasone and RU486 and is super-induced by 8-Br-cAMP (S. Zhang, S.A. Mason, P. Housley and M. Danielsen, in preparation). A similar conclusion was also reached by Moyer *et al.* (1993) who directly examined GR phosphorylation after cAMP treatment. Our data also indicate that the basal level of phosphorylation of the GR is not required for the cAMP response. Another possibility is that 8-Br-cAMP brings about phosphorylation or an increase in the level of some transcription factor that can interact with the GR. The interaction with this factor would be rate limiting for both GR activated by dexamethasone and for GR activated by a partial agonist. Phosphorylation or an increase in the amount of the factor would then lead to an increase in the rate of transcription. Interestingly, in cells deficient in PKA, the GR response is almost completely abrogated (Gruol & Altschmied, 1993) suggesting that this hypothetical factor may be essential for GR activation.

We were surprised to find that 8-Br-cAMP did not super-induce GR transcriptional activity in WCL-2 cells. We have repeated these experiments with forskolin and isobutylmethylxanthine to increase cAMP levels and again we did not detect any superactivation (S. Zhang and M. Danielsen, unpublished). It is possible that cAMP levels are already high in these cells and that this precludes any effects of additional cAMP. This seems unlikely, however, since we have found that 8-Br-cAMP decreases the response of mouse L-Cells to glucocorticoids (S. Zhang and M. Danielsen, unpublished) which suggests that it is the transcription factor complement of a cell which determines the response. The ability of a cell to super-activate the GR in response to 8-Br-cAMP is not directly related to the amount of agonist activity shown by partial agonists since all three cell lines respond to RU486 and progesterone in a similar manner.

We have found that ZK98.299 binds tightly to the GR but has no transcriptional activity even in the presence of cAMP or in cells expressing very high levels of receptor. ZK98.299 differs from RU486 only in the substituents and configuration of the D-ring (Figure 1). This indicates that the bulky 4-dimethylaminophenyl group at the 11β position of both RU486 and ZK98.299 is not the most critical factor in determining antagonistic activity in these steroids. R5020 also lacks any agonist activity on the GR. A comparison of the structures of progesterone, RU486 and R5020 must lead to the conclusion that it is either the 17α methyl group, or the additional carbon in the side chain at the 17β position which leads to pure antagonist activity (Figure 1). In the case of the PR, ZK98.299 is unable to activate the receptor to a DNA binding form (Klein-Hitpass *et al.*, 1991; Sartorius *et al.*, 1993). It would be of interest to determine if ZK98.299 or R5020 can bring about DNA binding of the GR in these cells or whether some other mechanism of antagonist activity occurs with the GR. This would be of particular interest for R5020 since it obviously activates the PR to a DNA binding form. Our data also suggests that care should be used in evaluating the effects of high concentrations of R5020 since appreciable binding to the GR will occur if this receptor is also present in the cells under study.

Materials and methods

Plasmids

pmGR is a derivative of pSV2Wrec which contains the mouse GR under the transcriptional control of the SV40 promoter (Danielsen *et al.*, 1986). pmGR was constructed by replacing the pBR322 origin of replication in pSV2Wrec with the origin of replication from *pBluescript* which improves plasmid yields. pMMTVCAT (pMCAT) was made by inserting the MMTV LTR into the HindIII site of pSVOCAT (Danielsen *et al.*, 1986). pBAG is a β- galactosidase expression vector under the control of the Moloney murine leukemia virus long terminal repeat (Price *et al.*, 1987). All plasmids were prepared by cesium chloride density centrifugation. RNA and proteins were removed by digestion with RNAase A, phenol/chloroform extraction, and ethanol precipitation.

Growth and transfection of tissue culture cells

The WCL-2 cell line is derived from CHO dHFR-cells which were co-transformed with pSV2Wrec and pSV2dHFR (Hirst *et al.*, 1990). These cells express high levels of mGR under the control of the constitutive SV40 early promoter. WCL-2 cells were cultured in high glucose Dulbecco's modified Eagle medium (DMEM) containing 10% defined/supplemented charcoal stripped bovine calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 10^{-5} M methotrexate. COS-7 cells were cultured in the same growth medium but without the methotrexate.

Transfection of WCL-2 and COS-7 cells was performed by the low CO^2 calcium phosphate precipitation method (Chen & Okayama, 1987) with slight modifications. Briefly, exponentially growing stock cells were seeded ($1.27 = 10^4$ cells/cm^2) into fresh 10 cm tissue culture plates 16 to 24 h before transfection, and refed with growth medium 4 h before transfection. A transfection mixture was prepared by mixing stock 1 M $CaCl_2$ 2 × BBS [59 mM *N*-, *N*-Bis(2 hydroxy-ethyl)-2 amino-ethane-sulfonic acid (BES), 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95], plasmid DNA, carrier (salmon sperm) DNA and sterilized distilled water to give a final concentration of 1 × BBS and 125 mM $CaCl_2$. The amount of plasmid DNA used varied according to the purpose of the experiments. For CAT assays, 150 ng of pmGR (COS-7 cells only), 10.5 μg pMMTVCAT and 300 ng pBAG was used. For hormone binding and competition assays 1–3 μg of pmGR was used with an equal amount of pBAG. In all transfections carrier DNA was used to bring the amount of total DNA up to 30 μg. The mixture was incubated for 20 min at room temperature or at 37°C, and then 1.5 ml was added drop wise to each dish of cells. The dishes were gently swirled several times and incubated for 15 to 20 h at 37°C under 3% CO_2. The medium was removed and the cells were washed with 37°C prewarmed PBS, refed and incubated at 37°C under 8–10% CO_2 overnight.

Hormone induction and determination of CAT activity

The transfected cells were detached with 0.05% trypsin and 0.02% EDTA, pooled with cells from other plates if more than one plate of cells was used for the transfection, mixed with growth medium and then distributed into 12-or 24-well plates. Cells from three 10 cm dishes were distributed into four 12-well plates or four 24-well plates. After 4 h culture at 37°C, steroid hormones were added into each well and cells cultured for a further 40–48 h.

CAT assays were based on the phase separation method (Neuman et al., 1987). The transfected cells (in multi-well dishes) were washed twice with phosphate buffered saline. 400 μl (12-well plates) or 200–250 μl (24-well plates) of

0.25 M Tris-HCl, pH 7.8 was added to each well. To prepare cell lysates, the plates underwent three cycles of freeze-thaw at −70°C and room temperature. To determine CAT activity 200 µl (from 12-well plates) or 100 µl (from 24-well plates) of cell lysate from each well was mixed with 150 µl reaction solution (0.25 M Tris-HCl, pH 7.8 containing 16.67 nM [^{3}H]acetyl CoA and 0.267 mg/ml of chloramphenicol) in scintillation vials. To each vial, 2 ml non-aqueous scintillation fluid (Econofluor-2, Dupont) was added and mixed with the reaction by shaking or vortexing. Only the product of the reaction, [^{3}H]chloramphenicol acetate is soluble in the organic scintillation fluid. The rate of the reaction was determined by counting the vials at regular intervals over the course of 1–4 h. Counting efficiency was 42–47% depending on the scintillation counter used.

For β-galactosidase lysate assays, 100 µl of cell lysate was mixed with 900 µl of 80 mM sodium phosphate, pH 7.3 containing 9 mM $MgCl_2$, 102 µM 2-mercaptoethanol and 8 mM of the substrates o-nitrophenyl-β-D-galactopyranoside (ONPG) or chlorophenol-red-β-D-galacto-γ-pyranoside (CPRG) and incubated for 2 h (CPRG) or overnight (ONPG) in a 37°C water bath. The absorbance at 570 nm (for CPRG) or at 414 nm (for ONPG) was measured on a spectrophotometer. Blank controls were the same as experiments except for using lysates of cells transfected without the pBAG vector.

For β-galactosidase whole cell assays, transfected cells were rinsed twice with PBS, fixed with cold fixing solution (0.2% glutaraldehyde, 5.4% formaldehyde) for 5 min, followed by three washes with PBS. Cells were stained with X-Gal solution (1 mg/ml X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 0.01% sodium deoxycholate). Blue stained cells were counted after overnight incubation at 37°C.

Curve fitting

Curve fitting was performed using the Michaelis-Menton equation modified for ligand receptor interactions.

For activation by any agonist (full or partial):

$$\text{activity} = \frac{[\text{agonist}] \times \max}{[\text{agonist}] + EC_{50}}$$

Where max = maximum possible activity

For competition curves of pure antagonists in the presence of full agonists:

$$\text{activity} = \frac{[\text{agonist}] \times \max}{[\text{agonist}] + EC_{50}\left(1 + \frac{[\text{antagonist}]}{K_i}\right)}$$

For competition curves of partial antagonists (PA) in the presence of full agonists (A):

$$\text{activity} = \frac{[A] \times \max}{[A] + EC_{50}\left(1 + \frac{[PA]}{K_i}\right)} + \frac{[PA] \times \text{maxpa}}{[PA] + EC_{pa50}\left(1 + \frac{[A]}{EC_{50}}\right)}$$

Where EC_{50} and max relate to the full agonist, and EC_{50pa}, maxpa and Ki relate to the partial agonist/antagonist. Curve fitting was performed with the computer program DeltaGraph Pro 3.0 for the Macintosh.

Hormone binding and binding competition assay

Cells were transfected as described above except that 1–3 µg pmGR was used without the addition of pMCAT. The transfected cells continued to be cultured in dishes for another two days. The cells were harvested by scraping from dishes using a rubber policeman and then washed three times with phosphate buffered saline by centrifugation for 5 min at 1000 r.p.m. The following manipulations were carried out at 0 to 4°C unless indicated otherwise. The cell pellets were suspended in 3 vol. of binding buffer (20 mM HEPES, pH 7.3, 2.0 mM molybdate, 5 mM EDTA) and placed on ice for 5 min and ruptured by 13 strokes with the A pestle of a Dounce homogenizer. The homogenate was centrifuged at 15 000 g for 30 min. The resulting supernatant was re-centrifuged at 110 000 g for 1 h. The clear supernatant was defined as cytosol which was used for hormone binding assays and Western blots.

Hormone binding assays and bidding competition assays were performed by the charcoal absorption method (Bell & Munck, 1973). For the binding assay, cytosol and steroids were incubated in an ice water bath overnight in a total volume of 100 µl containing 0.6 to 1.2 mg protein. [^{3}H]triamcinolone acetonide was diluted to 10^{-6} M prior to use. Nonspecific binding was determined by incubating the mixture with 1000–2000-fold molar excess of non-radioactive dexamethasone. After the incubation for 18 h on ice, 300 µl of 2.5% dextran coated charcoal was added to each tube, followed by vortexing the tubes for 10 s. The tubes were placed at room temperature for 10 min and then microfuged for 8 min. [^{3}H]triamcinolone acetonide present in the supernatant was determined using a scintillation counter. Specific hormone binding was obtained by subtracting non-specific binding from total binding. Binding competition assays were carried out the same way as the hormone binding assays except that different concentrations of cold steroids were used instead of cold dexamethasone. Steroid stock solutions were made in 95% ethanol at concentrations from 4.4 mM for RU486 to 10 mM for testosterone.

Materials

Cell culture products were from Biofluids Inc. Rockville, MD except that serum was from Hyclone, Logan, Utah, and tissue culture plates were from Corning, Corning, N.Y. [^{3}H]acetyl CoA (57.7 GBq/mmole), [^{3}H]triamcinolone acetonide (42.5 Ci/mmole) and R5020 were from New England Nuclear, Boston, MA. RU486 and ZK98.299 were obtained from Dr David Henderson, Schering AG. 2.5% dextran coated charcoal was from Wein Laboratories, Inc., Succasunna, N.J. Bradford protein assay solution was from Bio-Rad, Richmond, CA. General chemicals were from Sigma, St Louis, MO, or J.T. Baker, Phillipsburg, N.J.

Acknowledgements

The authors wish to thank H. Nakai and members of the Danielsen laboratory for the helpful suggestions concerning this work. This work was supported by USPHS grants R01 DK-42552 and KO4 DK-02105 (to M.D.) from the NIDDK. Postdoctoral support for S.Z. was provided by the Nation's Capital Affiliate of the American Heart Association.

References

Agarwal, M.K., Hainque, B., Moustaid, N. & Lazer, G. (1987). *Febs. Lett.*, **217**, 221–226.

Arriza, J.L., Weinberger, C., Cerelli, G., Glaser, T.M., Handelin, B.L., Housman, D.E. & Evans, R.M. (1987). *Science*, **237**, 268–275.

Baulieu, E.-E. (1991). *Endocrinol. Metabol. Clin. North Am.*, **20**, 873–891.

Baulieu, E.E. (1989). *Science*, **245**, 1351–1357.

Beato, M. (1989). *Cell*, **56**, 335–344.

Beck, C.A., Estes, P.A., Bona, B.J., Muro, C.C., Nordeen, S.K. & Edwards, D.P. (1993a). *Endocrinology*, **133,** 728–740.

Beck, C.A., Weigel, N.L., Moyer, M.L., Nordeen, S.K. & Edwards, D.P. (1993b). *Proc. Natl. Acad. Sci. USA*, **90,** 4441–4445.

Bell, P.A. & Munck, A. (1973). *Biochem. J.*, **136,** 97–107.

Bourgeois, S., Pfahl, M. & Baulieu, E.-E. (1984). *EMBO J.*, **3,** 751–755.

Brinkmann, A.O., Faber, P.W., van Rooij H.C., Kuiper, G.G., Ris, C., Klaassen, P., van der Korput, J.A., Voorhorst, M.M., van Laar, J.H., Mulder, E. & Trapman, J. (1989). *J. Steroid Biochem.*, **34,** 307–310.

Cato, A.C., Miksicek, R., Schütz, G., Arnemann, J. & Beato, M. (1986). *EMBO J.*, **5,** 2237–2240.

Chen, C. & Okayama, H. (1987). *Mol. Cell. Biol.*, **7,** 2745–2752.

Chrousos, G.P., Laue, L., Nieman, L.K., Kawai, S., Udelsman, R.U., Brandon, D.D. & Loreiaux, D.L. (1988). *Kidney Int.*, **34,** S18–S23.

Danielsen, M. (1991). *Nuclear Hormone Receptors, Molecular Mechanisms, Cellular Functions, Clinical Abnormalities*, Parker, M. (eds.). Academic Press: London. pp. 39–78.

Danielsen, M., Hinck, L. & Ringold, G.M. (1989). *Cell*, **57,** 1131–1138.

Danielsen, M., Northrop, J.P. & Ringold, G.M. (1986). *EMBO J.*, **5,** 2513–2522.

Evans, R.M. (1988). *Science*, **240,** 889–895.

Gagne, D., Pons, M. & De-Paulet, A.C. (1986). *J. Steroid Biochem.*, **25,** 315–322.

Gruol, D.J. & Altschmied, J. (1993). *Mol. Endocrinol.*, **7,** 104–113.

Hirst, M.A., Northrop, J.P., Danielsen, M. & Ringold, G.M. (1990). *Mol. Endocrinol.*, **4,** 162–170.

Klein-Hitpass, L., Cato, A.C., Henderson, D. & Ryffel, G.U. (1991). *Nucleic Acids Res.*, **19,** 1227–1234.

Laue, L., Gallucci, W., Loriaux, D.L., Udelsman, R. & Chrousos, G.P. (1988). *J. Clin. Endocrinol. Metab.*, **67,** 602–606.

Mao, M., Regelson, W. & Kalimi, M. (1992). *Molec. Cell. Biochem.*, **109,** 1–8.

Mason, S.A. & Housley, P.R. (1993). *J. Biol. Chem.*, **268,** 21501–21504.

Moyer, M.L., Borror, K.C., Bona, B.J., DeFranco, D.B., Nordeen, S.K. (1993). *J. Biol. Chem.*, **268,** 22933–22940 (1993).

Neef, G., Beier, S., Elger, W., Henderson, D. & Wiechert, R. (1984). *Steroids*, **44,** 349–372.

Neuman, J.R., Morency, C.A. & Russian, K.O. (1987). *Biotechniques*, **5,** 444–447.

Nordeen, S.K., Bona, B.J. & Moyer, M.L. (1993). *Mol. Endocrinol.*, **7,** 731–742.

Oshima, H. & Simons Jr, S. (1992). *Endocrinology*, **130,** 2106–2112.

Pratt, W.B. (1993). *J. Biol. Chem.*, **268,** 21455–21458.

Price J., Turner, D. & Cepko, C. (1987). *Proc. Natl. Acad. Sci. USA*, **84,** 156–160.

Quarmby, V.E., Kemppainen, J.A., Sar, M., Lubahn, D.B., French, F.S. & Wilson, E.M. (1990). *Mol. Endocrinol.*, **4,** 1399–1407.

Rangarajan, P.N., Umesono, K. & Evans, R.M. (1992). *A. Mol. Endocrinol.*, **6,** 1451–1457.

Sartorius, C.A., Tung, L., Takimoto, G.S. & Horwitz, K.B. (1993). *J. Biol. Chem.*, **268,** 9262–9266.

Schneider, M.R., Michna, H., Nishino, Y. & E-Etreby, M.F. (1989). *Eur. J. Cancer Clin. Oncol.*, **25,** 691–701.

Simons Jr, S., Mercier, L., Miller, N.R., Miller, P.A., Oshima, H., Sistare, F.D., Thompson, E.B., Wasner, G. & Yen, P.M. (1989). *Cancer Res.*, **49,** 2244s–2252s.

Simons Jr, S., Oshima, H. & Szapary, D. (1992). *Mol. Endocrinol.*, **6,** 995–1002.

Smith, D.F. & Toft, D.O. (1993). *Mol. Endocrinol.*, **7,** 4–11.

Ulmann, A., Teutsch, G. & Philibert, D. (1990). *Sci. Am.*, **262,** 42–48.

Umesono, K. & Evans, R.M. (1989). *Cell*, **57,** 1139–1146.

Webster, N.J., Green, S., Jin, J.R. & Chambon, P. (1988). *Cell*, **54,** 199–207.