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Differential effect of glucocorticoid receptor antagonists on glucocorticoid receptor nuclear translocation and DNA binding

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Abstract

The effects of RU486 and S-P, a more selective glucocorticoid receptor antagonist from Schering-Plough, were investigated on glucocorticoid receptor nuclear translocation and DNA binding. In the in vitro study, AtT20 cells were treated with vehicle or with RU486, S-P or corticosterone (3–300 nM) or co-treated with vehicle or glucocorticoid receptor antagonists (3–300 nM) and 30 nM corticosterone. Both glucocorticoid receptor antagonists induced glucocorticoid receptor nuclear translocation but only RU486 induced DNA binding. RU486 potentiated the effect of corticosterone on glucocorticoid receptor nuclear translocation and DNA binding, S-P inhibited corticosterone-induced glucocorticoid receptor nuclear translocation, but not glucocorticoid receptor-DNA binding. In the in vivo study, adrenalectomized rats were treated with vehicle, RU486 (20 mg/kg) and S-P (50 mg/kg) alone or in combination with corticosterone (3 mg/kg). RU486 induced glucocorticoid receptor nuclear translocation in the pituitary, hippocampus and prefrontal cortex and glucocorticoid receptor-DNA binding in the hippocampus, whereas no effect of S-P on glucocorticoid receptor nuclear translocation or DNA binding was observed in any of the areas analysed. These findings reveal differential effects of RU486 and S-P on areas involved in regulation of hypothalamic–pituitary–adrenal axis activity in vivo and they are important in light of the potential use of this class of compounds in the treatment of disorders associated with hyperactivity of the hypothalamic–pituitary–adrenal axis.

Keywords

corticosterone; DNA binding; depression; glucocorticoid receptor; glucocorticoid receptor antagonist; HPA axis; nuclear translocation; RU486

Introduction

Hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis has been found in major depression and other stress related disorders such as anxiety, post-traumatic stress disorders and psychosis. A significant percentage of depressed patients have increased size of both pituitary and adrenal glands associated with increased release of corticotropin (ACTH) from the anterior pituitary and increased cortisol concentration in serum, urine and the cerebrospinal fluid.

Depression associated with HPA-axis hyperactivity is thought to be due to hypersecretion of corticotropin releasing hormone (CRH) and arginine–vasopressin (AVP) (De Kloet et al., 2005; Holsboer, 2000; Nemeroff, 1996), which regulate ACTH secretion. Both peptides are under the negative transcriptional feedback control of glucocorticoids via ligand-activated glucocorticoid receptors (GR) and it has been hypothesized that it is this negative feedback control that may be impaired in depression (Holsboer, 2000; Pariante, 2004; Pariante and Lightman, 2008; Pariante and Miller, 2001). In fact, a great percentage of depressed patients are unable to inhibit endogenous cortisol release following administration of dexamethasone, a synthetic GR agonist (dexamethasone suppression test [DST]) (Gold et al., 1988). Restoration of this HPA dysfunction may have antidepressant potential, and therapy with GR antagonists has been proposed.

The glucocorticoid receptor is a ligand activated transcription factor belonging to the steroid receptor superfamily that also includes the mineralocorticoid receptor (MR) and the progesterone receptor (PR) (Mangelsdorf et al., 1995). In the absence of ligand, the inactive form of GR is associated with heat shock proteins and it is found predominately in the cytoplasm (Pratt and Toft, 2003). When activated by ligand (e.g. corticosterone and cortisol), GR undergoes conformational changes and translocates to the cell nucleus (Picard and Yamamoto, 1987). Within the nucleus, one of the mechanisms of GR-mediated transcriptional regulation is through binding, upon dimerization, to specific sequences of DNA named glucocorticoid response elements (GREs) (trans-activation). Activated GR can also mediate gene regulation via interaction with other transcription factors (trans-repression) with subsequent inhibition of gene expression (reviewed by Heitzer et al., 2007).

RU486 is a GR antagonist with high affinity for both GR and PR and a strong in vitro and in vivo GR antagonist activity (Gagne et al., 1985). The mechanism through which RU486 exerts its antagonist effects remains unclear. Clinical data have shown that RU486 exerts therapeutic effects in the treatment of cognitive dysfunction in bipolar disorder (Young et al., 2004), although its therapeutic potential is still controversial (Nihalani and Schwartz, 2007). The clinical use of RU486 is limited by potential side effects including those related to its anti-PR activity (Gagne et al., 1985).

More selective glucocorticoid receptor antagonists have been developed by Schering-Plough (Peeters et al., 2004). Those compounds are structurally related to RU486 and lack appreciable affinity for MR, oestrogen and androgen receptors. In this report the effect of RU486 and its methylenedioxy phenyl analogue (called S-P) on GR activity is described.

The pharmacokinetic characteristics of both RU486 and S-P have been previously described (Peeters et al., 2004). In vitro nuclear receptor binding experiments have shown that compared with RU486, S-P has a two-fold lower affinity for the human glucocorticoid receptor. Furthermore, in vitro cytoplasmic receptor binding experiments have shown that S-P, compared with RU486, is 5.3-fold less potent as a PR antagonist, indicating greater GR selectivity than RU486. Furthermore, RU486 and S-P inhibited cortisol-induced GR activity with similar potency in vitro (Fitzsimons et al., 2009; Thomson et al., 2004). Both GR antagonists are equally able to cross the blood–brain barrier (Bachmann et al., 2003; Peeters et al., 2004). It has been recently reported that S-P is unable to induce GR nuclear translocation in AtT20 cells although it is able to reduce corticosterone-induced GR nuclear translocation (Peeters et al., 2008). However, the effect of S-P on GR activity in vivo has not been investigated. To further characterize the mechanism of effect of these compounds, we have investigated the effects of RU486 and S-P alone or in combination with corticosterone on GR nuclear translocation and DNA binding in vitro in AtT20 cells and in vivo in adrenalectomized rats.

Methods

Drugs

The following GR antagonists were used: RU486 (mifepristone) was purchased from Sigma (Cat. No. M 8046, Sigma-Aldrich, St. Louis, MO), and S-P was provided by Schering-Plough (Schering-Plough Corporation, Newhouse, UK). Corticosterone (CORT) was also purchased from Sigma in the form of a preformed water-soluble complex of corticosterone and 2-hydroxypropyl- β -cyclodextrin (Cort-HBC, Cat. No. C-174; Sigma-Aldrich, St. Louis, MO).

In vitro experiments

AtT20 cells—Adherent AtT-20 cells (European collection of cell cultures, ECACC), a murine corticotropic tumour cell line, were cultured in 60mm tissue culture plates (Iwaki, Japan) at 5% CO₂ atmosphere and 37°C with Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine and 4500 mg/l glucose (Gibco, UK) supplemented with 10% foetal calf serum (FCS) (Gibco, UK). The culture media was replaced with D-MEM/F-12 (1 : 1) liquid with L-glutamine, 15mM HEPES, without phenol red (Gibco, UK), supplemented 100 μ g/ml bovine serum albumin fraction V (BSA) (Gibco, UK) and 10 μ g/ml transferrin (Sigma, UK) prior to the administration of steroids.

Drugs and experimental design—RU486, S-P and CORT were dissolved in DMSO to reach the stock concentration of 100 mM. Drugs were then further diluted in DMEM to reach a concentration of 100 μ M. For each drug, serial dilutions were done in DMEM containing 0.1% DMSO (0.1% DMSO/DMEM) to final concentrations as shown in Figures 1–4. To investigate the dose-dependent effect of RU486 and S-P on GR nuclear translocation and DNA binding, and to compare the effect to those induced by CORT, AtT20 cells were treated with either vehicle or with each compound at 1–300 nM concentration (Experiment 1). To investigate whether RU486 and S-P would reduce CORT-induced GR nuclear translocation and DNA binding, AtT20 cells were incubated with vehicle, RU486 and S-P

(1–300 nM) in combination with CORT (30 nM) (Experiment 2). After one hour of treatment, medium was removed, plates were washed twice in cold 0.05 M PBS and cells were harvested and processed for nuclear extract preparation.

In vivo experiments

Animals and surgery—All experiments were conducted in male Sprague Dawley adult rats (Harlan-Olac, Bicester, UK) weighing 180–200 g upon arrival. Rats were housed in groups of four animals per cage under standard environmental conditions ($21 \pm 1^\circ\text{C}$) under a 14 h light, 10 h dark schedule (lights on at 05 : 15) and food and water (or saline when specified) were provided ad libitum throughout the experiment. Before surgery, animals were allowed to acclimatize to the animal facility for one week. All animal procedures were approved by the University of Bristol Ethical Review Group and were conducted in accordance with Home Office guidelines and the UK Animals (Scientific Procedures) Act, 1986. All efforts were made to minimize the number of animals used and their suffering.

Rats were anaesthetized with isoflurane (Merial Animal Health Ltd, UK) and bilateral adrenalectomy was performed to deplete endogenous corticosteroids. After surgery, adrenalectomized (ADX) rats were returned to their home cage, and allowed to recover for five days prior to the experiment with 0.9% NaCl in drinking water provided ad libitum.

Drugs and experimental design—GR antagonists were dissolved in 5% DMSO/ 5% Cremophor in 5% mulgofen/saline (vehicle, 2 ml/kg), CORT (3 mg/kg, i.p.) was dissolved in saline (1 ml/kg). The dose of CORT used in this study has been previously described to produce a plasma CORT profile similar to that of an acute stress response (Conway-Campbell et al., 2007; Kitchener et al., 2004). Rats were injected with (1) S-P (50 mg/kg, s.c.), RU486 (20 mg/kg, s.c.) or vehicle (VEH, 2 ml/kg, s.c.) (Experiment 3) or (2) vehicle or GR antagonists followed, 30 min later, by CORT or saline (vehicle group only) (Experiment 4). Thirty minutes after CORT administration, rats were anaesthetized with isoflurane and killed by decapitation. The doses of RU486 and S-P used in this study are respectively four- and 2.5-fold higher than the threshold dose able to induce an anti-GR effect in rat (Peeters et al., 2004). Furthermore, the dose of S-P is five-fold higher than the threshold dose able to bind both pituitary and central GR in ADX rats (Bachmann et al., 2003) and it has been previously shown to reverse dexamethasone suppression of stress-induced corticosterone release in mice (Thomson et al., 2004).

Tissue and blood collection—After decapitation, the brain was quickly removed from the skull for dissection of hippocampus, hypothalamus, prefrontal cortex and amygdala, and the pituitary was also collected. All tissue was rapidly frozen in liquid nitrogen and stored at -80°C until nuclear extracts were prepared. Trunk blood was collected into heparinized tubes and the plasma obtained by centrifugation was stored at -20°C until measurement of CORT.

Corticosterone measurement—CORT plasma levels were measured by radioimmunoassay (RIA) as previously described (Spiga et al., 2007). Briefly, 5 μl of each plasma sample was diluted in 500 μl of a citrate buffer and processed in triplicate. Samples

were incubated overnight at 4°C with 50 µl of 125I corticosterone tracer (Oxford BioInnovation DSL Ltd, Oxford, UK) and 50 µl of rabbit anti-rat corticosterone primary antibody (kindly donated by G. Makara, Hungary). On day 2, a charcoal/dextran solution was added to the samples, which were then centrifuged (15 min, 4000 rpm, 3120g, 4°C) and aspirated before being loaded onto a gamma counter. Intra- and inter-assay coefficients of variation of the CORT assay were 16.65% and 13.30% respectively.

Nuclear extract preparation—Nuclear extracts were prepared according to the method of Vallone et al. (1997). All procedures were performed at 4°C and on ice. For nuclear extract preparation from AtT20, cultured cells were harvested by scraping in cold PBS (0.1 M) and then centrifuged (2200g, 5 min). The pellet was resuspended in S1 buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA [pH 8], supplemented with 0.5 mM dithiothreitol, 0.2 mM Na orthovanadate, 2 mM NaF, and Complete Protease Inhibitor [Roche Diagnostics Ltd., Burgess Hill, UK]). After centrifugation (2200g, 5 min) the supernatant was removed and kept as the cytoplasmic fraction, and the pellet was incubated in 1% Igepal Ca-630 (Cat. No. I3021, Sigma-Aldrich) in S1 buffer for 10 min. After centrifugation the pellet was washed in S1 and then resuspended in S2 buffer (10 mM HEPES, pH 7.9, 400 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EGTA, pH 7, and 5% glycerol) and kept under agitation for 1 h, before centrifugation at 22,000g for 30 min. The supernatant was kept as the nuclear fraction and stored at –80°C until use.

Nuclear extract from tissue samples was obtained by a similar protocol as previously described (Conway-Campbell et al., 2007). Protein concentrations were determined by bicinchoninic acid assay (BCA) (Pierce, Rockford, IL, US).

Western immunoblotting—Western blot was performed according to the original method of Laemmli (1970). Aliquots of each sample (20 µg for hippocampus, hypothalamus, prefrontal cortex and amygdala, 10 µg for pituitary and AtT20 cells) were processed as previously described (Spiga and Lightman, 2009). Data were expressed as fold induction relative to vehicle (Experiments 1–3) or vehicle + saline (Experiment 4).

GR-DNA binding assay—A commercially available ELISA-based transcription factor binding assay kit (TransAM GR, Cat. No. 45496, Active Motif, Carlsbad, CA, USA) was used to measure GRE-binding activity for each nuclear sample as previously described (Spiga and Lightman, 2009). Data were expressed as fold induction relative to vehicle (Experiments 1–3) or vehicle + saline (Experiment 4).

Statistical analysis—All data are expressed as mean ± SEM. One-way ANOVA followed, when appropriate, by Fisher's least significant difference (LSD) post-hoc test was used to analyse the effect of treatment. A *p*-value of *p* < 0.05 was considered significant.

Results

In vitro experiments

Experiment 1: Effect of CORT, RU486 and S-P on GR nuclear translocation and DNA binding in AtT20—The effect of CORT, RU486 and S-P on GR nuclear

translocation is shown in Figure 1(A) and (B). There was a significant effect of CORT ($F(6,20) = 23.911$, $p < 0.00001$), RU486 ($F(6,20) = 14.335$, $p < 0.0001$) and S-P ($F(6,20) = 4.353$, $p = 0.011$) on GR nuclear translocation in AtT20 cells and this effect was significant at concentrations 30–300 nM. No difference between RU486 and CORT on GR nuclear translocation was observed for any of the concentrations used. However, the effect of S-P on GR nuclear translocation was lower compared with both CORT and RU486 ($p < 0.05$).

The effect of CORT, RU486 and S-P on GR-DNA binding is shown in Figure 1(C). In accordance with the GR nuclear translocation data, there was a significant effect of both CORT ($F(6,20) = 3.828$, $p = 0.018$) and RU486 ($F(6,20) = 10.463$, $p = 0.00017$) and this effect was observed at 100 and 300 nM (CORT) and at 10–300 nM (RU486). In contrast, only a trend of effect for S-P was observed ($F(6,20) = 2.333$, $p = 0.0898$).

Experiment 2: Effect of RU486 and S-P on CORT-induced GR nuclear translocation and DNA binding in AtT20

The effect of RU486 and S-P on GR nuclear translocation induced by 30 nM CORT is shown in Figure 2(A) and (B). There was a significant effect of treatment for both RU486 ($F(7,23) = 6.003$; $p = 0.0015$) and S-P ($F(7,23) = 11.777$; $p < 0.0001$) on GR nuclear translocation. As expected 30 nM CORT induced GR nuclear translocation in cells treated with VEH ($p < 0.05$). S-P was able to reduce this effect (1–300 nM, $p < 0.0005$ compared with VEH + CORT) and the effect of CORT was completely blocked with 100 and 300 nM concentrations ($p > 0.05$ compared with VEH). In contrast, RU486 did not reduce the CORT effect but at 30–300 nM concentration it was able to potentiate the effect of CORT ($p < 0.05$ compared with VEH + CORT).

The effect of RU486 and S-P on GR-DNA binding induced by 30 nM CORT is shown in Figure 2(C). There was a significant effect of treatment for both RU486 ($F(7,23) = 5.736$; $p = 0.0018$) and S-P ($F(7,23) = 3.311$; $p = 0.0224$) on GR nuclear translocation. As seen for the nuclear translocation experiment, there was a significant effect of CORT in cells treated with VEH ($p < 0.05$). In contrast with the GR nuclear translocation data, no effect of S-P was observed on CORT-induced DNA binding at any of the concentrations tested ($p > 0.05$ compared with VEH + CORT). However, in accordance with the nuclear translocation data, RU486 (30–300 nM) was able to potentiate the effect of CORT ($p < 0.05$ compared with VEH + CORT).

In vivo experiments

Experiment 3: Effect of RU486 and S-P on GR nuclear translocation and DNA binding in brain areas and pituitary

Plasma CORT levels: As expected, very low levels of plasma CORT were found in ADX rats treated with either VEH (6.16 ± 3.11 ng/ml, $n = 8$), RU486 (3.02 ± 3.02 ng/ml, $n = 6$) or S-P (0 ± 0 ng/ml, $n = 6$) and no significant difference was found between the three groups.

GR nuclear translocation: The effect of acute treatment with RU486 and S-P on GR nuclear translocation in brain areas and pituitary is shown in Figure 3. A significant effect of treatment was observed in the hippocampus ($F(2,18) = 8711$; $p = 0.0027$), in the prefrontal

cortex ($F(2,19) = 16.390$; $p = 0.0001$) and in the pituitary ($F(2,19) = 7.972$; $p = 0.0036$) but not in the hypothalamus and in the amygdala. Specifically, RU486 induced GR nuclear translocation in hippocampus ($p = 0.0008$), prefrontal cortex ($p < 0.0001$) and pituitary ($p = 0.0011$). In contrast, no effect of S-P was observed in any of the areas analysed.

GR-DNA binding: The effect of acute treatment with RU486 and S-P on GR-DNA binding in brain regions is shown in Figure 4. Consistent with the GR nuclear translocation results, there was a significant effect of treatment in the hippocampus ($F(2,14) = 21.924$; $p < 0.0001$). However, no effect of treatment was observed on GR-DNA binding in any other areas. RU486 induced GR-DNA binding in the hippocampus ($p < 0.0001$) and, consistent with the nuclear translocation data, no effect of S-P was observed.

Experiment 4: Effect of RU486 and S-P on CORT-induced GR nuclear translocation and DNA in brain areas and pituitary

Plasma CORT levels: Plasma CORT levels of ADX rats treated with VEH, CORT or RU486 + CORT and S-P + CORT are shown in Figure 5. As expected, very low levels of plasma CORT were found in ADX rats treated with vehicle followed by saline. However, acute administration of CORT produced significantly high levels of circulating CORT in ADX rats injected with either vehicle, RU486 or S-P (treatment effect: $F(3,25) = 54.670$; $p < 0.00001$). No significant difference was found between rats injected with vehicle or GR antagonists followed by CORT.

GR nuclear translocation: The effect of acute treatment with RU486 and S-P on GR nuclear translocation induced by i.p. injection of CORT in rat brain areas and pituitary is shown in Figure 6. There was a significant effect of the treatment in all the areas analysed (hippocampus: $F(3,24) = 12.320$, $p < 0.0001$; hypothalamus: $F(3,25) = 7.466$, $p = 0.0013$; prefrontal cortex: $F(3,21) = 3.503$, $p = 0.0368$; amygdala: $F(3,23) = 3.211$, $p = 0.0449$; pituitary: $F(3,22) = 8.802$, $p = 0.0007$). Specifically, in all areas, CORT induced GR nuclear translocation in rats treated with either vehicle or GR antagonists and no differences was found between RU486 + CORT or S-P + CORT compared with VEH + CORT in any of the areas analysed. However, trend of effect of RU486 toward increased CORT-induced GR nuclear translocation was found in the hippocampus ($p = 0.0645$).

GR-DNA binding: The effect of acute treatment with RU486 and S-P on GR-DNA binding induced by i.p. injection of CORT in brain areas is shown in Figure 7. Consistent with the GR nuclear translocation data, there was a significant effect of the treatment in all the areas analysed (hippocampus: $F(3,19) = 17.011$, $p < 0.0001$; hypothalamus: $F(3,19) = 20.776$, $p < 0.0001$; prefrontal cortex: $F(3,18) = 3.347$, $p = 0.047$; amygdala: $F(3,19) = 3.524$, $p = 0.039$). In all areas analysed, CORT increased GR nuclear translocation in rats treated with either vehicle or GR antagonists, and no differences between VEH + CORT, RU486 + CORT or S-P + CORT was found.

Discussion

We have used complementary in vitro and in vivo approaches to investigate the effect of two GR antagonists, RU486 and the more selective S-P on GR nuclear translocation and DNA

binding, as well as the effect of those compounds on CORT-induced GR nuclear translocation and DNA binding. Both RU486 and S-P induced nuclear translocation in AtT20 cells, whereas only RU486 had a significant effect in vivo. Furthermore, whereas S-P was able to reduce CORT-induced GR nuclear translocation in AtT20 cells, the same effect was not seen in vivo.

In vitro study

Both RU486 and S-P induced GR nuclear translocation in AtT20 cells. However, whereas the effects of RU486 were similar to those induced by CORT, GR nuclear translocation induced by S-P was significantly lower compared with both RU486 and CORT. Furthermore, there was an effect of RU486 on GR-DNA binding in AtT20 cells, whereas no effect of S-P was found. The high potency of RU486 to induce GR nuclear translocation in AtT20 was also confirmed by the finding that RU486 was able to potentiate the effects of CORT on GR nuclear translocation. In contrast, S-P actually reduced CORT-induced GR nuclear translocation, although no effect was observed on CORT-induced GR-DNA binding. The discrepancy on the effects of S-P alone or in combination with CORT between GR nuclear translocation and DNA binding could be due to the temporal difference between the two processes. As only one time point (1 h) was analysed, it is possible that analysis of a later time point would be necessary to observe an effect of S-P on DNA binding. On the other hand, it is also possible that in the presence of S-P, nuclear GR is in a conformation that is not able to bind to DNA. Our results on the effects of RU486 are consistent with previous reports describing the effects of RU486 on GR nuclear translocation (Jewell et al., 1995; Pariante et al., 2001; Qi et al., 1990) and GR binding to DNA (Pandit et al., 2002; Schmidt, 1986) in vitro. However, with regards to S-P, a recent report has shown no effect of this compound on GR nuclear translocation in AtT20, although the same compound, in agreement with our results, was able to reduce CORT-induced GR nuclear translocation (Peeters et al., 2008).

In vivo study

To further characterize the mechanism of effect of those two GR antagonists, we investigated the effect of RU486 and S-P alone or in combination with CORT on GR nuclear translocation and GR binding to DNA in vivo in ADX rats.

Consistent with a previous report where the ability of RU486 to increase nuclear GR immunostaining in rat hippocampus was described (van Eekelen et al., 1987) we found that acute systemic administration of RU486 induced GR nuclear translocation in pituitary, hippocampus and prefrontal cortex. The effect of RU486 on GR nuclear translocation in the hippocampus was associated with increased GR-DNA binding. However, no effect of RU486 on GR-DNA binding was found in the prefrontal cortex. Interestingly, no effect of RU486 on either GR nuclear translocation or GR-DNA binding was found in the hypothalamus or in the amygdala, two key areas involved in the modulation of the neuroendocrine response to stress.

Our data suggest that the effect of RU486 on GR nuclear translocation is region specific. The lack of effect of RU486 on GR nuclear translocation in the hypothalamus and in the

amygdala could possibly be due to differential bioavailability of these compounds in these brain areas. There is evidence, in rats, for regional differences in cerebral blood flow (Lin et al., 1999) and the hypothalamus is one of the most richly vascularized areas in the brain (Sposito and Gross, 1987). However, as we only measured the effect of RU486 on GR activity at one time point (60 min), we cannot exclude the possibility of an effect of RU486 on hypothalamic and amygdala GR within a different timeframe. Further time course studies would be necessary to clarify this. It is also possible that there is a differential potency of RU486 to occupy GR in vivo in different brain areas, but this seems unlikely as there is no evidence for differential affinities for GR in different brain regions.

Consistent with the nuclear translocation data, we also found that RU486 is able to significantly increase GR-DNA binding in hippocampus, but not in hypothalamus and amygdala. Interestingly, despite our evidence that RU486 induces GR nuclear translocation in the prefrontal cortex, no effect was observed on GR-DNA binding. This discrepancy may be the result of different kinetic processes in this area and that RU486-induced GR-DNA binding could have occurred later than the one measured time point at 60 min. Certainly, differential temporal kinetics of CORT induced GR-DNA binding between brain regions has previously been described. Notably, the duration of GR-DNA binding is more prolonged in hippocampus relative to prefrontal cortex, as measured by electrophoretic mobility shift assay (EMSA) (Kitchener et al., 2004). Furthermore, we have recently shown that, when both GR nuclear translocation and DNA binding were investigated, the hippocampus appears to be more responsive to CORT than other brain areas (Spiga and Lightman, 2009). The final possibility is that RU486 activated GR could be acting more on trans-repression mechanisms binding to others transcription factors such as AP-1 and NF- κ B or activating alternate pathways (Revest et al., 2005).

From the current data it is not possible to determine whether the effects of RU486 on GR nuclear translocation and DNA represent agonist or antagonist activities. Indeed, several in vitro studies have shown that RU486 exerts its antagonist effect acting downstream from the GR-DNA binding step required for the trans-activation process (Beck et al., 1993; Mikuni et al., 2007; Pandit et al., 2002).

The in vivo antagonist effect of RU486 is well established (Gagne et al., 1985; Mayer et al., 2006; Oitzl et al., 1998; van Haarst et al., 1997). However, RU486 has been shown to act as a GR agonist in vitro to induce apoptosis, an activity mediated by activation of a cyclic 5'3'; adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) (Gruol and Altschmied, 1993). This finding suggests that a tissue-specific activation of this pathway could be responsible for the selective effect of RU486 in different brain areas; however, the functional significance of this pathway within distinct brain regions is unknown.

Interestingly agonist-like effects of RU486 on HPA axis activity have been described in rats following acute (Havel et al., 1996) and chronic (Bachmann et al., 2003) administration. Havel and colleagues (Havel et al., 1996), suggest that RU486 could display agonist effects in situations where glucocorticoid levels are low. This situation would certainly apply to our study, as we used ADX rats which had no CORT replacement during the 6 days between the

surgery and the experiment. Whether RU486 would induce GR nuclear translocation and DNA binding in vivo in intact animals with normal CORT levels, is not known.

It has been proposed that the antagonistic effect of RU486 could be due to formation of unproductive DNA-GR interactions which are unable to initiate transcriptional enhancement of genes regulated by glucocorticoids (Segnitz and Gehring, 1990). Therefore, further studies investigating the effect of RU486 on GR-mediated gene regulation would be necessary to elucidate the anti-GR mechanism of this compound.

As the effect of RU486 on both GR nuclear translocation and DNA binding was found only in the hippocampus, our results suggest that this area is preferentially involved the central effect of RU486.

In contrast with the in vitro data, we found no effect of acute administration of S-P on GR nuclear translocation in any of the areas analysed. Furthermore, we found no effect of this compound on GR-DNA binding.

Our in vivo results are consistent with our data on AtT20 cells where no effect of S-P was observed on GR-DNA binding, and suggests that the two GR antagonists exert their effect at different steps of the GR-mediated molecular mechanism of glucocorticoid hormone action. However, our data are not able to elucidate the mechanism of anti-GR activity of S-P.

With respect to the effects of the two GR antagonists on CORT-induced GR nuclear translocation and DNA binding in vivo, we found neither RU486 nor S-P were able to reduce CORT-induced GR activity in any of the areas analysed. The dose of CORT used in this study in vivo has been previously described to produce a plasma CORT profile similar to that of an acute stress response (Conway-Campbell et al., 2007; Kitchener et al., 2004). Similar effects of RU486 and S-P were found when a lower dose of CORT was used (Spiga et al., 2008).

In summary, we have found that RU486 induces GR nuclear translocation and GR-DNA binding in the hypothalamus, an area involved in HPA axis modulation. Our data confirm a previous observation from van Eekelen and colleagues regarding an in vivo effect of RU486 on GR nuclear translocation in rat hippocampus (van Eekelen et al., 1987). Our results also provide the first evidence for GR-DNA binding induced by RU486 in vivo. These effects of RU486 appear to be area specific, and may be a significant factor to consider in the design of further studies using glucocorticoid antagonists to investigate HPA feedback or as potential therapeutic compounds.

We have also found that the GR antagonists RU486 and S-P exert their anti-GR activity through different mechanisms both in vitro and in vivo. In light of current interest in the use of this class of compound for treatment of depression and other stress-related disorders, it will be important to gain a better understanding of their mechanism of action.

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Figure 1.

Effect of corticosterone (CORT), RU486 and S-P on glucocorticoid receptor (GR) nuclear translocation and DNA binding in AtT20 cells. AtT20 cells were treated for 1 h with vehicle (VEH), CORT (1–300 nM), RU486 (1–300 nM) or S-P (1–300 nM). Nuclear translocation was evaluated as GR content in the nuclear fraction. In (A) is shown a representative western immunoblot of GR. Densitometry quantitation of GR bands were normalized to H1 (B). GR-DNA binding was evaluated as induction of the complex GR-GRE quantified using an ELISA-based method and normalized to induction of the nuclear factor NF- κ B (C). The

results are shown as the mean $\pm$ SEM of three independent experiments and are expressed in logarithmic scale as fold induction relative to VEH-treated cells. * $p < 0.05$ significant difference compared with VEH; # $p < 0.05$ significant difference compared with the same concentration of RU486.

Figure 2.

Effect of RU486 and S-P on corticosterone (CORT)-induced glucocorticoid receptor (GR) nuclear translocation and DNA binding in AtT20 cells. AtT20 cells were treated for 1 h with vehicle (VEH) or co-treated with 30 nM CORT and VEH, RU486 (1–300 nM), S-P (1–300 nM). Nuclear translocation was evaluated as GR content in the nuclear fraction. In (A) is shown a representative western immunoblot of GR. Densitometry quantitation of GR bands were normalized to H1 (B). GR-DNA binding was evaluated as induction of the complex GR-GRE quantified using an ELISA-based method and normalized to induction of the

nuclear factor NF- κ B (C). The results are shown as the mean $\pm$ SEM of three independent experiments and are expressed in logarithmic scale as fold induction relative to VEH-treated cells. * p < 0.05 significant difference compared with VEH; # p < 0.05 significant difference compared with VEH + CORT.

Figure 3.

Effect of RU486 and S-P on glucocorticoid receptor (GR) nuclear translocation in rat brain and pituitary. (A) Western immunoblot of GR in the nuclear fraction produced from hippocampus (HC), hypothalamus (HYP), prefrontal cortex (PFCx), amygdala (AMY) and pituitary (PIT) of ADX rats treated with VEH, RU486 (20 mg/kg, s.c.) or S-P (50 mg/kg, s.c.). (B) Densitometry quantitation of GR bands normalized to H1 (not shown). GR nuclear translocation was evaluated as GR content in the nuclear fraction. The results are shown as the mean $\pm$ SEM ($n=5-6$ /group) and are expressed as fold induction relative to rats treated

with VEH. * $p < 0.05$ significant difference compared with VEH; # $p < 0.05$ significant difference compared RU486.

Figure 4.

Effect of RU486 and S-P on glucocorticoid receptor GR-DNA binding in rat brain. GR-DNA binding was evaluated in the nuclear fraction produced from hippocampus (HC), hypothalamus (HYP), prefrontal cortex (PFCx) and amygdala (AMY) of ADX rats treated with vehicle (VEH), RU486 (20 mg/kg, s.c.) or S-P (50 mg/kg, s.c.). Induction of the complex GR-GRE was quantified using an ELISA-based method and normalized to induction of the nuclear factor NF-YA. The results are shown as the mean $\pm$ SEM ($n = 5$ /group) and are expressed as fold induction relative to VEH. * $p < 0.05$ significant difference compared with VEH.

Figure 5.

Circulating corticosterone (CORT) levels. Plasma CORT levels were measured in ADX rats treated with vehicle (VEH) followed by saline (SAL), VEH followed by CORT (3 mg/kg, i.p.), S-P (50 mg/kg, s.c.) followed by CORT or RU486 (20 mg/kg, s.c.) followed by CORT.

* $p < 0.05$ significant difference compared with VEH + SAL.

Figure 6.

Effect of RU486 and S-P on corticosterone (CORT)-induced glucocorticoid receptor (GR) nuclear translocation in rat brain and pituitary. (A) Western immunoblot of GR in the nuclear fraction produced from hippocampus (HC), hypothalamus (HYP), prefrontal cortex (PFCx), amygdala (AMY) and pituitary (PIT) of ADX rats treated with vehicle (VEH) followed by saline (SAL), VEH followed by CORT (3 mg/kg, i.p.), S-P (50 mg/kg, s.c.) followed by CORT or RU486 (20 mg/kg, s.c.) followed by CORT. (B) Densitometry quantitation of GR bands normalized to H1 (not shown). GR nuclear translocation was

evaluated as GR content in the nuclear fraction. The results are shown as the mean $\pm$ SEM ($n = 5-6$ /group) and are expressed as fold induction relative to rats treated with VEH. * $p < 0.05$ significant difference compared with VEH; # $p < 0.05$ significant difference compared RU486.

Figure 7.

Effect of glucocorticoid receptor (GR) antagonists in corticosterone (CORT)-induced GR-DNA binding in rat brain. GR-DNA binding was evaluated in the nuclear fraction produced from hippocampus (HC), hypothalamus (HYP), prefrontal cortex (PFCx) and amygdala (AMY) of ADX rats treated with vehicle (VEH) followed by saline (SAL), VEH followed by CORT (3 mg/kg, i.p.), S-P (50 mg/kg, s.c.) followed by CORT or RU486 (20 mg/kg, s.c.) followed by CORT. Induction of the complex GR-GRE was quantified using an ELISA-based method and normalized to induction of the nuclear factor NF- κ B. The results are

shown as the mean $\pm$ SEM ($n = 5/\text{group}$) and are expressed as fold induction relative to VEH. $*p < 0.05$ significant difference compared with VEH + SAL.