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Expression and function of nuclear receptor coregulators in brain: understanding the cell-specific effects of glucocorticoids

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CHAPTER IV

TIMING IS CRITICAL FOR EFFECTIVE GLUCOCORTICOID
RECEPTOR MEDIATED REPRESSION OF THE cAMP-INDUCED
CORTICOTROPIN-RELEASING HORMONE GENE

S van der Laan, ER de Kloet and OC Meijer

Abstract

Glucocorticoid negative feedback of the hypothalamus-pituitary-adrenal axis is mediated in part by direct repression of gene transcription in glucocorticoid receptor (GR) expressing cells. We have investigated the cross talk between the two main signalling pathways involved in activation and repression of corticotrophin releasing hormone (CRH) mRNA expression: cyclic AMP (cAMP) and GR. We report that in the At-T20 cell-line the glucocorticoid-mediated repression of the cAMP-induced human CRH proximal promoter activity depends on the relative timing of activation of both signalling pathways. Activation of the GR prior to or in conjunction with cAMP signalling results in an effective repression of the cAMP-induced transcription of the CRH gene. In contrast, activation of the GR 10 minutes after onset of cAMP treatment, results in a reduced ability to repress gene expression. In addition, translocation of ligand-activated GR to the nucleus was found as early as 10 minutes after glucocorticoid treatment. Interestingly, while both signalling cascades counteract on the CRH proximal promoter, they synergize on a synthetic promoter containing 'positive' response elements. Since the order of activation of both signalling pathways may vary considerably *in vivo*, we conclude that a critical time-window exists for effective repression of the CRH gene by glucocorticoids.

1. Introduction

Cross-talk of intracellular signalling pathways is central to many neuroendocrine control systems (1;2). The expression and/or secretion of the two main neuroendocrine secretagogues of the hypothalamus-pituitary-adrenal axis (HPA axis) are both stimulated by cAMP and suppressed by glucocorticoids, the end-product of the HPA axis: adrenocorticotrophic hormone (ACTH) from anterior pituitary corticotrophs and corticotrophin releasing hormone (CRH) from the hypothalamus (3-6). At the molecular level, these signals are represented by protein kinase A (PKA), the transcription factor cAMP element-binding protein (CREB), and the glucocorticoid receptor (GR), respectively.

The proximal promoter of the human corticotrophin releasing hormone (hCRH) gene contains a canonical, functional cAMP response element (CRE) and a negative glucocorticoid receptor response element (nGRE). Induction of the hCRH gene expression by cAMP-dependent activation of the protein kinase A (PKA) pathway is mediated by phosphorylation of the CRE-binding protein (CREB) at serine residue 133 (7;8). *In vivo*, Wölfel *et al.* showed that binding of CREB to the canonical CRE located at the nucleotide position -224 (upstream exon 1) was specifically induced after activation of the PKA pathway with forskolin (9). Additionally, Kovacs *et al.* demonstrated that in the hypothalamic parvocellular neurons of rodents subjected to ether stress, CREB phosphorylation was induced in a time course that parallels the increase of CRH heteronuclear RNA levels (10).

The At-T20 cell-line is a well-established *in vitro* model system for studying glucocorticoid-induced repression of the hCRH proximal promoter. Nested deletions and site-specific point mutations of the CRE located at nucleotide -224 resulted in a significant loss of induction by cAMP, demonstrating that CREB binding is necessary for the stimulation of the gene (11). In parallel, electrophoretic mobility shift assays (EMSA) identified a GR-binding site at position nt -249 that was indispensable for GR-mediated repression of the cAMP-induced promoter. Internal deletion of the entire nGRE and specific point mutations resulted in a loss of repression by the ligand-activated GR, indicating that DNA binding is essential for the glucocorticoid-induced repression (12).

The nGRE in the hCRH promoter is separated by as few as 25 bp with the canonical CRE, a distance that clearly permits functional interactions at the promoter (13). Since, *in vivo* the order of activation of the cAMP and glucocorticoid signalling pathways may vary considerably, and this is known to affect responses at the level of neuroendocrine secretion (14), we tested the hypothesis that effective repression of the cAMP-induced hCRH proximal promoter depends on the relative timing of GR activation in the At-T20 cell-line.

2. Material & methods

2.1 Cell culture and transient transfections

AtT-20/D-16V mouse tumor cells (kindly provided by dr. J. van der Hoek, Erasmus Medical Centre, Rotterdam, The Netherlands) were grown and maintained in DMEM containing 4.5 g/l glucose supplemented with 0.5 % penicillin/streptomycin, 10% horse serum and 10% fetal bovine serum (Gibco, United Kingdom) in a humidified atmosphere of 5% CO₂ at 37°C. A day prior to transfection 0.1 × 10⁶ cells per well were plated in 24 wells plate (Greiner). For each well, the cells were transfected using 1.6 μl Lipofectamine 2000 (Invitrogen, United Kingdom) per 0.8 μg plasmid according to the manufacturer's instructions. To induce the CRH-promoter the cells were treated with 10 μM forskolin (Calbiochem, Darmstadt, Germany) which leads to an increase of intracellular cyclicAMP (cAMP). Subsequent protein kinase A (PKA) activation results in CREB phosphorylation (15). Repression of the forskolin-induced

CRH promoter was performed with the synthetic glucocorticoid dexamethasone (DEX) co-treatment. The cells were harvested and assayed according to the luciferase kits instructions (Promega, Madison, USA) using a luminometer (LUMAT LB 9507, Berthold, Bad Wildbad, Germany). The total amount of DNA for each transfection was kept constant using empty vector.

First, GR-mediated repression was measured when both treatments were given simultaneously. Then, to address the hypothesis that the relative timing of activation is of importance we assessed whether GR activation prior to or after the FSK treatment differentially affected the promoter activity of CRH. In these experiments (fig 1B), all groups were treated 3 hours with FSK but the time of onset of DEX treatment relative to FSK varied. Of note, DEX treatment was added at the time mentioned in the figure (relative to start of FSK treatment which was set at $t=0$). Finally, to test promoter-specificity we used the synthetic TAT3-Luc GRE-containing reporter (fig 3). In the experiments using the GRE-containing promoter, all groups were treated for 4 hours with DEX and the time of onset of FSK treatment varied. If there is no crosstalk between the cAMP/CREB pathway and GR, then the expectation is that all groups have similar promoter activity since the time of DEX treatment is identical.

2.2 Immunocytochemistry

A day prior stimulation, 30×10^3 cells were grown in chamber slides. Following stimulation, cells were fixed in 4% paraformaldehyde, permeabilized with Triton X-100 and blocked with 5% normal goat serum. Cells were incubated with a GR-specific antibody (M20; dilution 1:500; Santa Cruz biotechnologies) during 60 minutes, washed and subsequently incubated for 60 minutes with a secondary goat anti-rabbit Alexa Fluor 488 antibody (dilution 1:750; Invitrogen, Breda, The Netherlands). After incubation, cells were washed and counterstained for 10 min with Hoechst 33528. All sections were mounted with polyaquamount (Polysciences, Inc.) and visualized with an immunofluorescence microscope (Leica DM6000). Control cells were incubated with equal amounts of non-immune rabbit serum (Santa Cruz), which was used as substitute for the primary antibodies. Guided by the Hoechst staining, nuclear immunoreactivity of at least 20 cells was measured using ImageJ 1.32j software (NIH, USA). Nuclear immunoreactivity expressed as relative optical density in Igg-stained controls was used for determination of background signal.

2.3 Data analysis

First, we determined the repression induced by simultaneous DEX/forskolin cotreatment (Figure 1A). Since the aim of the study was to compare the DEX-induced repression on the CRH promoter, we compared all groups to the repression measured in the three hours cotreatment group (set at 100%). In the next experiment (figure 1B) only the onset time of DEX treatment varied between the groups whereas all groups were stimulated for three hours with FSK. The DEX-induced repression is compared.

2.4 Statistics

The values are expressed as the average of 4 paralleled transfections within one experiment and the error bars represent the standard deviation. All transfection experiments were performed at least three times, yielding similar results. Overall statistical analysis was performed using one way analysis of variance (ANOVA) and statistical significance was determined with Tukey's multiple comparison tests with $p < 0.05$.

3. Results and discussion

Three hours forskolin (FSK) treatment strongly induced the CRH-promoter activity. In line with previous reports (12;16), simultaneous DEX cotreatment strongly suppressed the FSK-induced stimulation of the hCRH-promoter activity. DEX cotreatment resulted in 75% repression of the FSK-induced promoter activity (fig. 1A). However, DEX treatment alone (0.1 μ M) did not significantly suppress the basal activity of the CRH-promoter (data not shown). To test our hypothesis that the order of activation of both signalling cascades is important for the level of GR-mediated repression, we initiated the DEX treatment at different time points prior or during the 3-hours FSK treatment (fig. 1B). We compared the resulting GR-mediated repression to the simultaneous cotreatment group (to be able to adequately compare repression, the cotreatment group (75%) was set to a 100% reference value). Two hours of DEX pre-treatment resulted in a significant increased repression compared to the simultaneous cotreatment (data not shown). However, activation of the GR up to one hour prior to FSK treatment resulted in similar levels of GR-mediated repression as in the simultaneous cotreatment group (fig. 1B). The relatively slow onset of the additional repression suggests that *de novo* protein synthesis is involved in this effect.

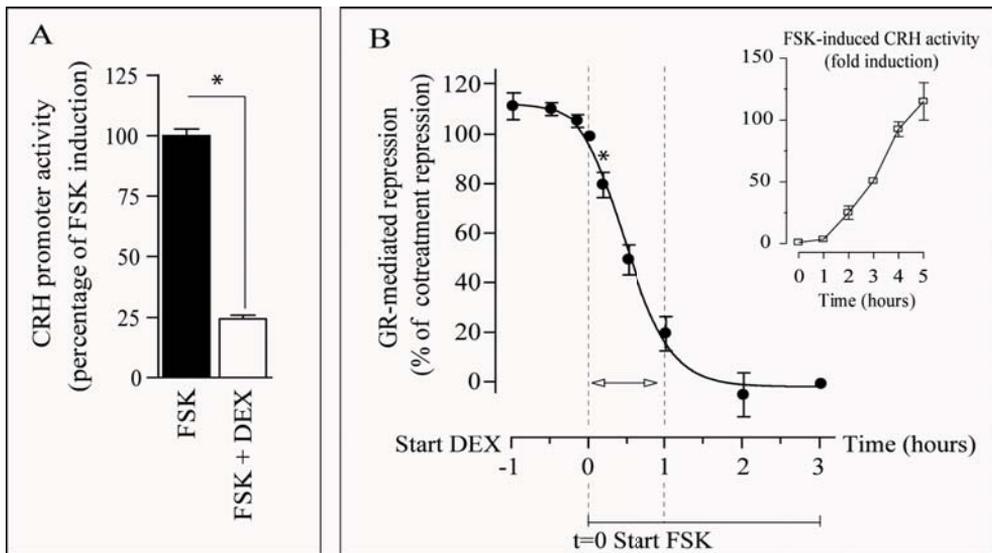


Fig. 1: Luciferase reporter assay in AtT-20 mouse tumour cells. 0.1×10^6 cells were transiently transfected in 24-wells plate using Lipofectamine 2000 (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. The day after transfection, the cells were treated with 10 μ M forskolin (Calbiochem, Darmstadt, Germany) and/or 0.1 μ M of the synthetic glucocorticoid dexamethasone (DEX) and assayed for luciferase activity. (1A) CRH-promoter activity expressed as percentage of maximal induction after 3 hours forskolin (FSK) treatment (filled bar). Simultaneous co-treatment with DEX (open bar) resulted in a strong repression of the CRH-promoter activity. (1B) The repression induced by DEX in the cotreatment group was set at 100%. All groups were treated for three hours with FSK. Different time of onset of the DEX treatment relative to the FSK treatment results in a significant loss of repression when DEX treatment is started 10 minutes after FSK treatment (*). FSK treatment leads to a progressive increase in CRH-luc promoter activity over a period of at least 5 hours (inset).

When DEX treatment is started after forskolin stimulation of the CRH promoter, the time-window separating both treatments was of great consequence for the level of repression (fig. 1B). A 10 minutes delay in DEX treatment resulted in a 20% loss of repression compared to the simultaneous cotreatment group. Strikingly, a 30 minutes delay (a reduction of approximately

15% of the DEX treatment time compared to simultaneous cotreatment) resulted in a 50% loss of GR-mediated repression, indicating the importance of the relative time of onset of treatments. Clearly, the reduced time of DEX exposure is not proportional to the loss of GR-mediated repression, pointing to a ‘GR resistance’ at the promoter. Because FSK treatment induces a progressive increase of the CRH-luc promoter activity over a period of at least 5 hours (inset fig. 1B), we assume that FSK induces binding of CREB to the promoter over that period. However, activation of the GR in the first hour following FSK treatment is critical for effective repression.

To gain insight in the dynamics of GR translocation to the nucleus, we performed immunocytochemistry on DEX treated cells. Translocation data show that DEX treatment induces maximal nuclear GR-immunoreactivity (GR-ir) as early as 10 minutes after treatment (figure 2). No difference in nuclear GR-ir was observed between the 10 and 30 minutes treatment groups (fig. 2A). As expected, FSK treatment did not influence translocation dynamics of the GR although it is known that PKA activation can modulate the steroid sensitivity by enhancing DNA binding properties of GR (17). These data indicate that GR is capable of mediating its genomic effects as early as 10 minutes after treatment.

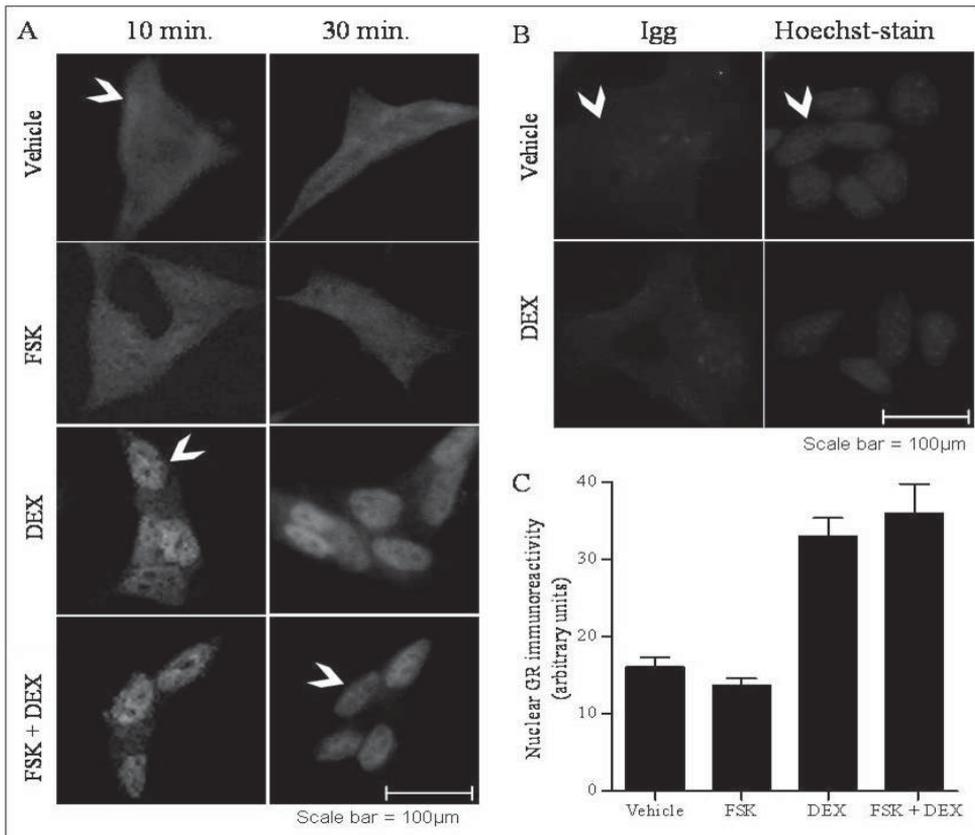


Fig. 2: Immunofluorescent staining of the GR in AtT-20 cells. (2A) Time course of GR-ir in different treatment groups. DEX alone and FSK + DEX cotreatment, show nuclear GR staining after 10 minutes treatment. (2B) Control IgG staining show specificity of the GR-specific antibody. (2C) Nuclear quantification of GR-ir after 10 minutes treatment (The average values \pm SEM are shown) (see colour page 126).

Posttranslational modification such as phosphorylation is known to affect DNA binding properties, transcriptional activation and stability of numerous nuclear receptors including GR (18). Although translocation to the nucleus was not affected by FSK treatment, we tested whether FSK influenced the transcriptional activity of the GR in these cells. We measured the effect of FSK and DEX cotreatment on a positively regulated promoter (a synthetic GRE-containing promoter; TAT3-luc (19)). FSK cotreatment synergistically induced transcription on an exclusively GRE-containing promoter compared to DEX treatment alone (fig. 3). FSK treatment prior DEX treatment resulted in an increased transcriptional activity of the GR. Likewise, the longer the time of FSK cotreatment the higher the transcriptional activity of the GR.

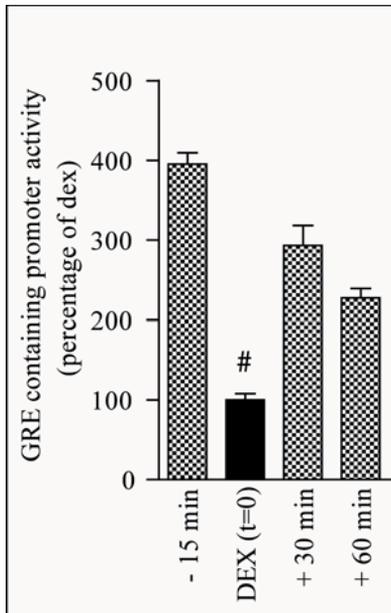


Fig. 3: TAT3-luc (GRE-containing promoter) activity expressed as percentage of maximal induction after 4 hours DEX treatment (filled bar; $t=0$). All groups (hatched bars) were treated for 4 hours with DEX and only the time of onset of FSK treatment was different. Forskolin treatment strongly enhanced the transcriptional rate of GR at all time points (# indicates significantly different from DEX group with $p < 0.05$). Pre-treatment with FSK resulted in the highest potentiation of the GR transcriptional rate.

The current data demonstrate that time-dependent interactions between GR and cAMP/CREB can occur at the level of the CRH gene, where these factors seem to functionally compete for the same promoter. We suggest that the observed 'primacy' effect for transcription factor action at this promoter is due to the close proximity of the two response elements involved. The spacing of the elements is such that it is likely that both GR and CREB may bind simultaneously (13). Possibly, sterical hindrance at the promoter due to the formation of larger protein complexes is responsible for the importance of timing of stimuli. Alternatively, CREB-mediated chromatin remodeling events that disfavor GR-binding may account for the apparent 'GR resistance'. The latter option is attractive because sterical hindrance neglects the dynamic nature and short residence time of transcription factors on the DNA (20).

While CREB-driven transcription is repressed by glucocorticoids on a composite promoter such as hCRH, it is unaffected on a 5xCRE-containing promoter (21). On the other hand, glucocorticoid signalling is modulated by FSK-induced PKA activation on both the composite

hCRH and the exclusively 3xGRE-containing promoters. Therefore, PKA activation can determine the transcriptional outcome at glucocorticoid target genes, independent of the presence of CREs in the promoter. We postulate that there is no cross-talk between the GR and CREB off the DNA but that PKA activation modulates GR-mediated transcription through phosphorylation of the receptor. Chromatin immunoprecipitation assays on the human CRH promoter would give additional information on the actual binding of these transcription factors to the genomic DNA *in vivo* or in a stably transfected cell-line. These ChIP-assays would allow more molecular insights related to the sequence of binding at the promoter.

It is well known that acute exogenous steroid treatment effectively suppresses stress-induced expression of CRH mRNA in rats (22). However, the current study using a model system shows that repression is markedly attenuated if GR activation is initiated with as little as a 10 minutes delay. Comparable observations were found at the level of ACTH secretion using an *in vivo* perfusion system. Glucocorticoid inhibition of cAMP stimulated ACTH secretion from rat pituitary tissue was shown to be impaired by cAMP treatment prior to glucocorticoid treatment. Both systems show that cAMP activation prior to glucocorticoid treatment results in an impaired action of glucocorticoids at the level of the pituitary and the hypothalamus.

The critical time-window for effective repression by glucocorticoids may have interesting implications in the control of CRH expression *in vivo*. The order of activation of both signalling pathways is variable, and depends on the history of stress and glucocorticoid exposure, as well as the circadian and ultradian pulsatility of glucocorticoid levels (23;24). Therefore, it is likely that effective GR-mediated repression of the stress-induced CRH mRNA expression will only occur in specific situations. We conclude that the differences in timing of stimulatory and repression signals are of consequence for adaptation of the organism to stress.

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