British Journal of Pharmacology (1999) 127, 1003–1011

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Ligand-induced differentiation of glucocorticoid receptor (GR) trans-repression and transactivation: preferential targetting of NF-κB and lack of I-κB involvement

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1 Glucocorticoids are highly effective in controlling chronic inflammatory diseases, such as asthma and rheumatoid arthritis, but the exact molecular mechanism of their anti-inflammatory action remains uncertain. They act by binding to a cytosolic receptor (GR) resulting in activation or repression of gene expression. This may occur *via* direct binding of the GR to DNA (transactivation) or by inhibition of the activity of transcription factors such as AP-1 and NF-κB (transrepression).

2 The topically active steroids fluticasone propionate ($EC_{50} = 1.8 \times 10^{-11}$ M) and budesonide ($EC_{50} = 5.0 \times 10^{-11}$ M) were more potent in inhibiting GM-CSF release from A549 cells than tipredane ($EC_{50} = 8.3 \times 10^{-10}$ M), butixicort ($EC_{50} = 3.7 \times 10^{-8}$ M) and dexamethasone ($EC_{50} = 2.2 \times 10^{-9}$ M). The anti-glucocorticoid RU486 also inhibited GM-CSF release in these cells ($IC_{50} = 1.8 \times 10^{-10}$ M).

3 The concentration-dependent ability of fluticasone propionate ($EC_{50} = 9.8 \times 10^{-10}$ M), budesonide ($EC_{50} = 1.1 \times 10^{-9}$ M) and dexamethasone ($EC_{50} = 3.6 \times 10^{-8}$ M) to induce transcription of the β_2-receptor was found to correlate with GR DNA binding and occurred at 10–100 fold higher concentrations than the inhibition of GM-CSF release. No induction of the endogenous inhibitors of NF-κB, IκBα or I-κBβ, was seen at 24 h and the ability of IL-1β to degrade and subsequently induce IκBα was not altered by glucocorticoids.

4 The ability of fluticasone propionate ($IC_{50} = 0.5 \times 10^{-11}$ M), budesonide ($IC_{50} = 2.7 \times 10^{-11}$ M), dexamethasone ($IC_{50} = 0.5 \times 10^{-9}$ M) and RU486 ($IC_{50} = 2.7 \times 10^{-11}$ M) to inhibit a 3×κB was associated with inhibition of GM-CSF release.

5 These data suggest that the anti-inflammatory properties of a range of glucocorticoids relate to their ability to transrepress rather than transactivate genes.

Keywords: Glucocorticoids; inflammation; GM-CSF; NF-κB; cross-coupling; RU486

Abbreviations: AP-I, activator protein-1; Bud, budesonide; Dex, dexamethasone; ECL, enhanced chemiluminescence; FCS, foetal calf serum; FP, fluticasone propionate; GCs, glucocorticoids; GM-CSF, granulocyte-macrophage colony stimulating factor; GR, glucocorticoid receptor; I-κBα, inhibitor of NF-κB; NF-κB, nuclear factor-κB; P/CAF, p300/CBP associated protein; PAO, phenylarsine oxide; PBS, phosphate buffered saline; PCIP, p300/CBP co-integrator protein; SRC-1, steroid-receptor coactivator-1; STAT, signal transduction and activation of transcription; TNFα, tumour necrosis factor-α; TRE, TPA-response element

Introduction

Cytokines, such as IL-1β, tumour necrosis factor-α (TNFα) and granulocyte-macrophage colony stimulating factor (GM-CSF), are released in a co-ordinate network and play an important role in chronic inflammation. As such, the pattern of cytokine expression largely determines the nature and persistence of the inflammatory response (Barnes & Adcock, 1993). Cytokines produce their cellular effects by activation of various transcription factors such as activator protein-1 (AP-I), nuclear factor-κB (NF-κB), and the signal transduction and activation of transcription (STAT) family. Furthermore, the expression of many of these cytokines and their receptors are also upregulated by these transcription factors. The increased expression of some of these factors may be responsible for the prolonged inflammation seen in asthma. AP-1 and NF-κB can also be induce, and be induced by, numerous other mediators such as NO, histamine, and eicosanoids (Barnes & Adcock, 1993).

Glucocorticoids (GCs) are the most effective anti-inflammatory therapy for the treatment of asthma and act by reducing airway hyper-responsiveness and suppressing the airway inflammatory response (Barnes, 1995). However, their exact mechanisms and cellular targets in the lung are uncertain. Glucocorticoid receptors (GR) are predominantly localized to the airway epithelium and endothelium (Adcock *et al*., 1996), therefore, these are likely to be important sites of the anti-inflammatory action of steroids, especially when delivered by the inhaled route. Airway epithelial cells do not act solely as a physical barrier but act as important regulators of the inflammatory reaction, responding to various inflammatory mediators by the production of a wide range of cytokines, chemokines and other inflammatory mediators (Levine, 1995).

Classically GCs act by binding to, and activating, the cytosolic GR. Upon activation the GR dimerizes and translocates to the nucleus. Within the nucleus GR binds to specific DNA elements (GREs) in the promoters of responsive genes resulting in modulation of transcription (Beato *et al*., 1996; Strahle *et al*., 1988). Important genes induced by

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glucocorticoids include the β_2-receptor (Collins *et al*., 1988) and lipocortin-1 (Flower & Rothwell, 1994). Lipocortin inhibits the activation of cytosolic phospholipase A_2 and thus may inhibit the synthesis of leukotrienes and prostanoids, although there is some doubt whether this mechanism is important in inhibiting asthmatic inflammation (Davidson *et al*., 1990).

The mechanisms involved in GR-mediated gene repression are less well understood. Experiments involving overexpression of the subunits of AP-1 and NF-κB along with deletion mutants of specific DNA binding moieties have indicated that the predominant mechanism of glucocorticoid down-regulation of inflammatory genes requires a direct protein-protein interaction with these, or related, transcription factors (Karin, 1998). An additional mechanism of repression by glucocorticoids on NF-κB mediated transcription has been reported in cultured monocytes and T-lymphocytes (Auphan *et al*., 1995; Scheinman *et al*., 1995a). Following steroid treatment a rapid induction of the inhibitor of NF-κB (I-κBα) mRNA and protein synthesis occurs. Newly synthesized I-κBα interacts with NF-κB heterodimers within the cytoplasm, and possibly the nucleus (Zabel *et al*., 1993) thereby inhibiting NF-κB DNA binding and transcriptional activation by cytokines. This mechanism does not appear to occur in all cells examined (Brostjan *et al*., 1996; Heck *et al*., 1997).

We have, therefore, investigated the role of these various mechanisms in mediating the ability of the glucocorticoids to inhibit IL-1β-induced release of GM-CSF, an NFκB-inducible gene (Kochetkova & Shannon, 1996), from a human lung epithelial-like cell line (A549). We have also examined the GR-induced inhibition of NFκB and AP-1 activity and the ability of GR to bind to its DNA binding motif and induce the β_2-receptor and IκBα and β proteins. In addition, we have examined the relative abilities of a number of topically acting steroids to activate or repress gene transcription to investigate whether current steroids can differentiate between these two modes of glucocorticoid action.

Methods

Drugs and chemicals

Fluticasone propionate, budesonide, tipredane and butixicort were kindly supplied by Dr M. Johnson (Glaxo-Wellcome, U.K.). RU486 (Mifepristone) was supplied by Dr A. Phillibert (Rousel-Uclaf, France). Enzymes were obtained from Promega (Cambridge, U.K.) and all other reagents, except where stated, were obtained from Sigma (Dorset, U.K.).

Cell culture

A549 cells were grown to confluence in Dulbecco's modified medium containing 10% foetal calf serum (FCS) before incubation for 72 h in serum-free media, as previously described (Newton *et al*., 1996). Cells were then used for the analysis of glucocorticoid action on GM-CSF release, β_2-receptor, I-κB expression and transcription factor activity.

GM-CSF release

A549 cells were cultured for 24 h with 1 ng ml^{-1} IL-1β in the presence or absence of various concentrations of steroids. After this time the culture supernatant was removed and stored at -70°C until samples were analysed. GM-CSF concentrations were measured using a specific ELISA calibrated with human recombinant GM-CSF (0–200 pg ml^{-1}, PharMingen, Lugano, Switzerland). Coating solution (0.1 M $NaHCO_3$, pH 8.2) containing 2 μg ml^{-1} rat anti human GM-CSF monoclonal antibody was used to coat an enhanced protein binding ELISA plate and incubated overnight at 4°C. The plate was washed three times in 0.05% Tween-20 in phosphate buffered saline (PBS) before blocking with 10% FCS in PBS for 2 h at 18°C. Plates were washed again and incubated with samples and standards (diluted in 10% serum in PBS) overnight at 4°C before washing again with 0.05% Tween-20 in PBS. 1 μg ml^{-1} biotinylated rat anti human GM-CSF diluted in 0.05% Tween-20 in PBS was incubated for 45 min at 18°C before extensive washing. The signal was detected following a 30 min incubation at 18°C with a 1:400 dilution of 1 mg ml^{-1} avidin-peroxidase solution (Sigma), extensive washing and final addition of ABTS substrate solution containing 1 μl ml^{-1} H_2O_2. The colour reaction products were read at 405 nm.

Electrophoretic mobility shift assay

Nuclear and cytosolic proteins were extracted from A549 cells as previously described (Adcock *et al*., 1995). Briefly, cells were collected and lysed in 200 μl of Buffer A 10 (mM) HEPES $MgCl_2$ 1.5, KCl 10, DTT 0.5, 0.1% Nonidet P40, and incubated at 4°C for 15 min. After microcentrifugation for 10 s and collection of the cytoplasmic fraction, the nuclear pellet was lysed with 20 μl of Buffer B (mM) HEPES 20, $MgCl_2$ 1.5, NaCl 0.42, DTT 0.5, 25% glycerol, PMS 0.5, EDTA 0.2. The subsequent soluble fraction was mixed with 100 μl of buffer C (mM) HEPES 20, KCl 50, DTT 0.5, PMSF 0.5, EDTA 0.2. 2 μg nuclear protein from each sample was pre-incubated at 4°C for 30 min in binding buffer (mM) Tris HCl 10, pH 7.5 $MgCl_2$ 1, EDTA 0.5, DTT 0.5, NaCl 50, 4% glycerol, 0.1 μg μl^{-1} salmon sperm DNA). Double-stranded oligonucleotides encoding the specific sequence of GRE (5′-TCGACTGTACAGGATGTTCTAGCTACT-) (Promega) were end-labelled with [γ^{-32}P]-ATP and T_4 polynucleotide kinase. Each sample was then incubated with 50,000 c.p.m. of labelled oligonucleotide for 40 min at 4°C. Protein-DNA complexes were separated on a 6% polyacrylamide gel using 0.25 × Tris-Borate-EDTA running buffer. Specificity was determined by the addition of excess unlabelled double stranded oligonucleotides.

Western blotting

Total cellular proteins were extracted from A549 cells by freeze-thawing samples in lysis buffer (mM) HEPES 20, $MgCl_2$ 1.5, NaCl 0.42, DTT 0.5, 25% glycerol, PMSF 0.5, EDTA 0.2 (Adcock *et al*., 1996). 30–50 μg total soluble protein extracts were size fractionated on 10% PAGE gels and transblotted onto Nitrocellulose-ECL membranes (Amersham International, Amersham, U.K.). Membranes were blocked overnight with 2% casein prior to incubation with either 1:1000 rabbit anti-human I-κBα antibody (Santa Cruz Biotechnology Inc., Devizes, U.K.), 1:1000 rabbit anti-human I-κBβ antibody (Santa Cruz Biotechnology Inc) or 1:600 rabbit anti-human human β_2 receptor (kindly donated by Dr J. MacDermot, RPMS, U.K.) at 18°C for 3 h. After washing (3 × 20 min in PBS-Tween), bound antibody was detected using 1:7000 sheep anti-rabbit antibody ($F(ab')_2$ fragment) linked to horseradish peroxidase (Amersham International, U.K.) and bound complexes detected using Enhanced Chemiluminescence (ECL, Amersham International) (Adcock *et al*., 1996).

Immunoprecipitation

Cells were treated for 30 min with 1 μM dexamethasone or fluticasone propionate in the presence of 1 ng ml^{-1} IL-1β. After incubation the cells were washed three times with fresh media before extraction in Buffer A above and two cycles of freeze-thaw. At the end of this time, soluble proteins (100 μg) were incubated for 18 h at 4°C with either an anti-human p65 antibody, an anti-human GR antibody or pre-immune serum (Santa Cruz). Immune complexes were precipitated with protein A-sepharose and loaded onto 10% PAGE gels. Following electrophoretic separation, proteins were electroblotted and transferred to ECL-nitrocellulose membranes and probed for the presence of p65 or GR.

Transient transfection and luciferase assay

Sense and antisense oligonucleotides encoding 6 copies of the consensus DNA binding site for AP-1 (TRE, 5′-CGCTTGATGAGTCAGCCGGAA-) were annealed by allowing to cool slowly to room temperature after incubation at 95°C for 2 min. The double stranded oligonucleotide was then inserted into pGL3-Basic at the *Sma*I site. The number of incorporated TRE sites and the direction of incorporation were confirmed by sequencing.

Cells were grown to confluence and then treated for 2 days in serum-free media. pGM-CSF(−123)-Luc (containing the −123 to +1 sequence of the human GM-CSF promoter), pGL-3κBLuc or pGL-6 × TRE-Luc were incubated with 2.5 μl Tfx−50 reagent (Promega) μg^{-1} DNA ml^{-1} of serum free media for 15 min at room temperature. Cells were transfected by the addition of 1 ml of media containing DNA-Tfx−50 (5 μl Tfx−50 μg−1 DNA) for 1.5 h before washing in fresh media and incubation in 1 ml serum free media. All cells were transfected with 1 μg pSV-β-gal vector (Promega) to control for transfection efficiency. After 18 h the media was changed and cells stimulated with varying concentrations of glucocorticoids in the presence or absence of 1 ng ml^{-1} of IL-1β. Cells were harvested by scraping and resuspended in 1 × reporter Lysis Buffer (Promega). After incubation at room temperature for 15 min lysates were vortexed for 10 s and subjected to one freeze-thaw cycle. Cellular debris was pelleted and total protein was measured. Luciferase assays were performed using 20 μl of extract and 50 μl Luciferase Assay Reagent (Promega) and luminescence measured with a TD 20/20 Luminometer (Turner Designs, Hemel Hempstead, U.K.). Relative luminescence readings were normalized to β-galactosidase expression and expressed as a percentage of activation relative to control or IL-1β-stimulated release.

Results

Effects of glucocorticoids on IL-1β-stimulated GM-CSF release

IL-1β caused a concentration-dependent increase in GM-CSF release after 24 h in A549 cells ($EC_{50} = 0.3$ ng ml^{-1}). IL-1β (1 ng ml^{-1}) stimulated the production of approximately 80% maximal levels of GM-CSF (22.5 ± 4.5 ng ml^{-1}). No detectable GM-CSF was found in the supernatant of control untreated cells. Co-incubation with fluticasone propionate, budesonide or dexamethasone all produced a concentration-dependent inhibition of IL-1β-stimulated GM-CSF release over this time period. Fluticasone propionate ($EC_{50} = 1.8 \times 10^{-11}$ M) and budesonide ($EC_{50} = 5.0 \times 10^{-11}$ M) produced a 122 fold and a 44 fold greater inhibition of GM-CSF release respectively than dexamethasone ($EC_{50} = 2.2 \times 10^{-9}$ M) (Figure 1a and Table 1). The anti-glucocorticoid RU486 also caused a concentration-dependent inhibition of IL-1β-stimulated GM-CSF release from A549 cells with an $EC_{50} = 1.8 \times 10^{-10}$ M. At concentrations greater than 10^{-7} M RU486 was less effective at inhibiting IL-1β-induced GM-CSF release (Figure 1b). Furthermore, the ability of 10^{-10} M fluticasone propionate, budesonide or dexamethasone to inhibit IL-1β-stimulated GM-CSF release was not inhibited by 10^{-8} M RU486 (Figure 1c). Both tipredane ($EC_{50} = 8.3 \times 10^{-10}$ M) and butixicort ($EC_{50} = 3.7 \times 10^{-8}$ M) also caused a concentration-dependent inhibition of IL-1β-stimulated GM-CSF release. The ability of IL-1β to stimulate GM-CSF release was markedly attenuated by the NF-κB inhibitor phenylarsine oxide (PAO) in a concentration-dependent manner with a maximal effect at 10 μM (Figure 1d). The proteosome inhibitor CBZ-leucine-leucine-leucinal (LLLal) caused a concentration-dependent inhibition of IL-1β (1 ng ml^{-1})-stimulated GM-CSF release ($EC_{50} = 30$ μM).

Effect of glucocorticoids on DNA binding activity

The ability of fluticasone propionate and dexamethasone to stimulate DNA binding was assessed by EMSA. Fluticasone propionate and dexamethasone produced a concentration-dependent increase in DNA binding. Fluticasone propionate ($EC_{50} = 5.2 \times 10^{-9}$ M) and budesonide ($EC_{50} = 6.4 \times 10^{-9}$ M) caused a marked increase in DNA binding with an approximately 10 fold higher potency than that seen for dexamethasone ($EC_{50} = 4.6 \times 10^{-8}$ M) (Table 1). Moreover the maximal level of DNA binding induced by fluticasone propionate was greater (30 fold stimulation) than that seen with dexamethasone (5 fold stimulation). At higher concentrations of fluticasone propionate (10^{-6} M) the inducibility of DNA binding was much reduced possibly reflecting the relative partial agonist activity of this drug. RU486 gave no consistent increase in DNA binding (Figure 2). Confirmation that the correct band was detected was obtained by using an excess of unlabelled oligonucleotide and also by the use of a specific GR antibody that supershifted the retarded band.

Effects of glucocorticoids on the induction of the β_2-adrenoceptor

In order to ensure that the glucocorticoids were able to induce gene expression in these cells the ability of these glucocorticoids to induce the expression of the β_2-adrenoceptor after 24 h was investigated by Western blotting (Figure 3). The concentration-dependent ability of fluticasone propionate ($EC_{50} = 1.0 \times 10^{-9}$ M), budesonide ($EC_{50} = 1.1 \times 10^{-9}$ M) and dexamethasone ($EC_{50} = 3.6 \times 10^{-8}$ M) to induce a 2–3 fold increase in the expression of the β_2-receptor at 24 h correlated with the induction of GR DNA binding. This occurred at 10–100 fold higher concentrations than that which repressed IL-1β-stimulated GM-CSF release (Figure 3). Fluticasone propionate caused a greater increase in β_2-receptor expression than either budesonide or dexamethasone. RU486 had no effect on β_2-adrenoceptor expression at any time or at any concentration tested (Table 1).

Effects of glucocorticoids on I-κBα and β expression

The ability of glucocorticoids to affect the induction of the cytoplasmic inhibitor of NF-κB, I-κBα, was also investi-

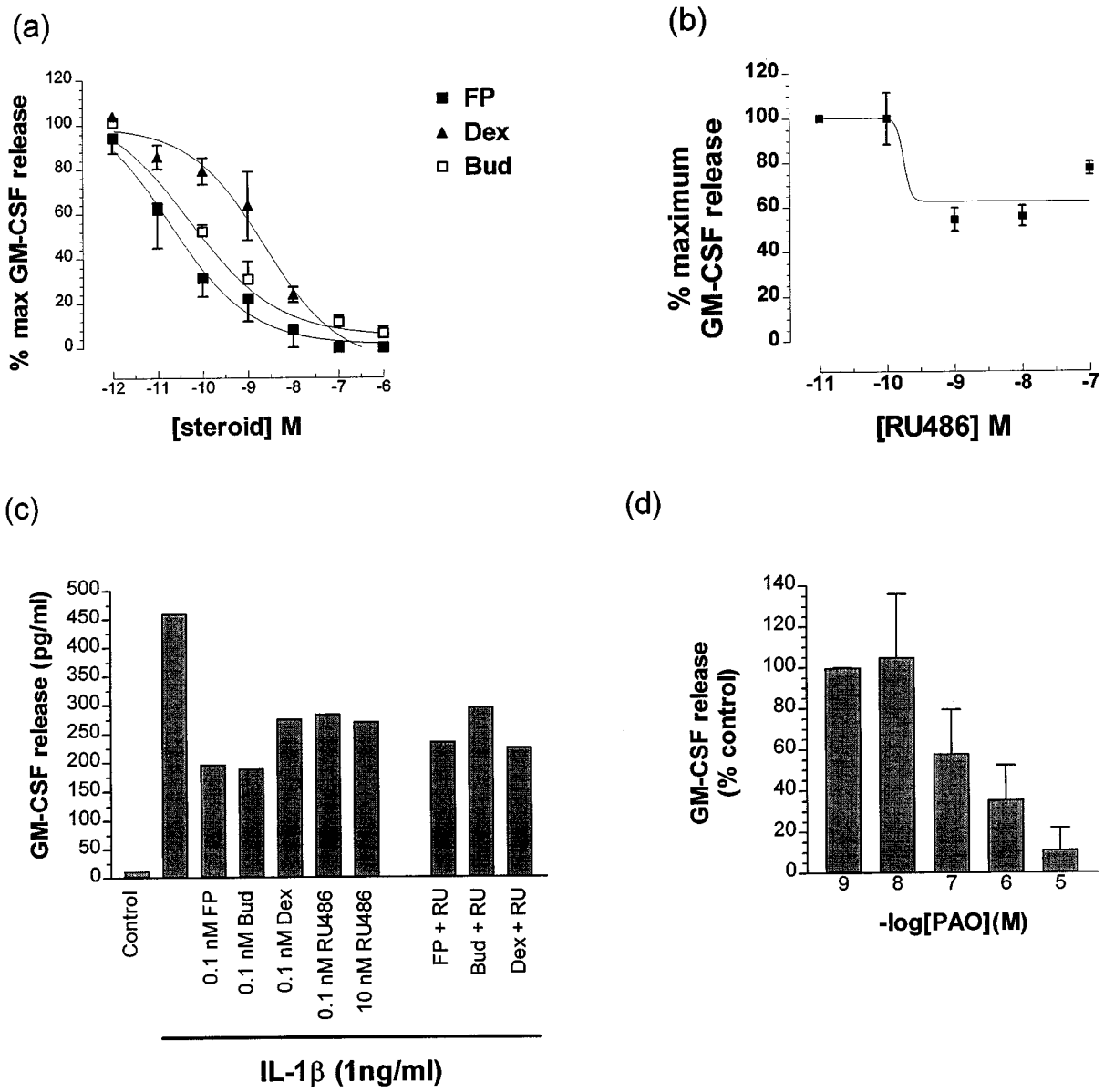

Figure 1 (a) Concentration-dependent inhibition of interleukin (IL)-1β (1 ng ml^{-1})-stimulated granulocyte-macrophage colony stimulating factor (GM-CSF) release into the media from A549 cells at 24 h following fluticasone propionate (FP), budesonide (Bud) and dexamethasone (Dex) treatment. (b) Concentration-dependent inhibition of IL-1β (1 ng ml^{-1})-stimulated GM-CSF release from A549 cells at 24 h following treatment with the anti-glucocorticoid RU486. (c) The effect of low concentration (10^{-9} M) RU486 (RU) treatment on the inhibition of IL-1β-stimulated GM-CSF release by 10^{-10} M FP, Bud and Dex. (d) The effects of increasing concentrations of phenylarsine oxide (PAO) on IL-1β (1 ng ml^{-1})-stimulated induction of GM-CSF release into culture medium at 24 h. Results are plotted as the means$\pm$s.e.means of the percentage of maximal IL-1β-stimulated GM-CSF release in the absence any drug. $n=4-7$ for each data point except in (c) where results are the mean of two independent experiments.

Table 1 Glucocorticoid effects on transactivation and transrepression in A549 cells

	FP IC_{50}	*Bud IC_{50}*	*Dex IC_{50}*	*RU486 IC_{50}*
GM-CSF release*	1.8×10^{-11} M	5.0×10^{-11} M	2.2×10^{-9} M	1.8×10^{-10} M
β_2 Receptor	1.0×10^{-9} M	1.1×10^{-9} M	3.2×10^{-8} M	No induction
GRE Binding	5.0×10^{-9} M	2.4×10^{-9} M	4.6×10^{-8} M	No induction
I-κBα	No induction	No induction	No induction	No induction
I-κBα degradation	No effect	No effect	No effect	No effect
I-κBβ	No induction	No induction	No induction	No induction
I-κBβ degradation	No effect	No effect	No effect	No effect
κB activity (*stim)	1.8×10^{-11} M	2.7×10^{-11} M	0.8×10^{-9} M	8.0×10^{-11} M
κB activity (basal)	0.5×10^{-11} M	2.7×10^{-11} M	0.5×10^{-9} M	2.5×10^{-11} M
TRE activity (*stim)	1.7×10^{-10} M	ND	0.9×10^{-9} M	ND
TRE activity (basal)	1.1×10^{-10} M	1.0×10^{-10} M	0.3×10^{-9} M	7.1×10^{-11} M
GM-CSF promotor*	0.6×10^{-11} M	ND	1.3×10^{-9} M	ND

FP, fluticasone propionate; Bud, budesonide; Dex, dexamethasone; GM-CSF, granulocyte macrophage colony stimulating factor; GRE binding, glucocorticoid receptor DNA binding. *After stimulation with interleukin (IL)-1β (1 ng ml^{-1}). ND, experiment not performed.

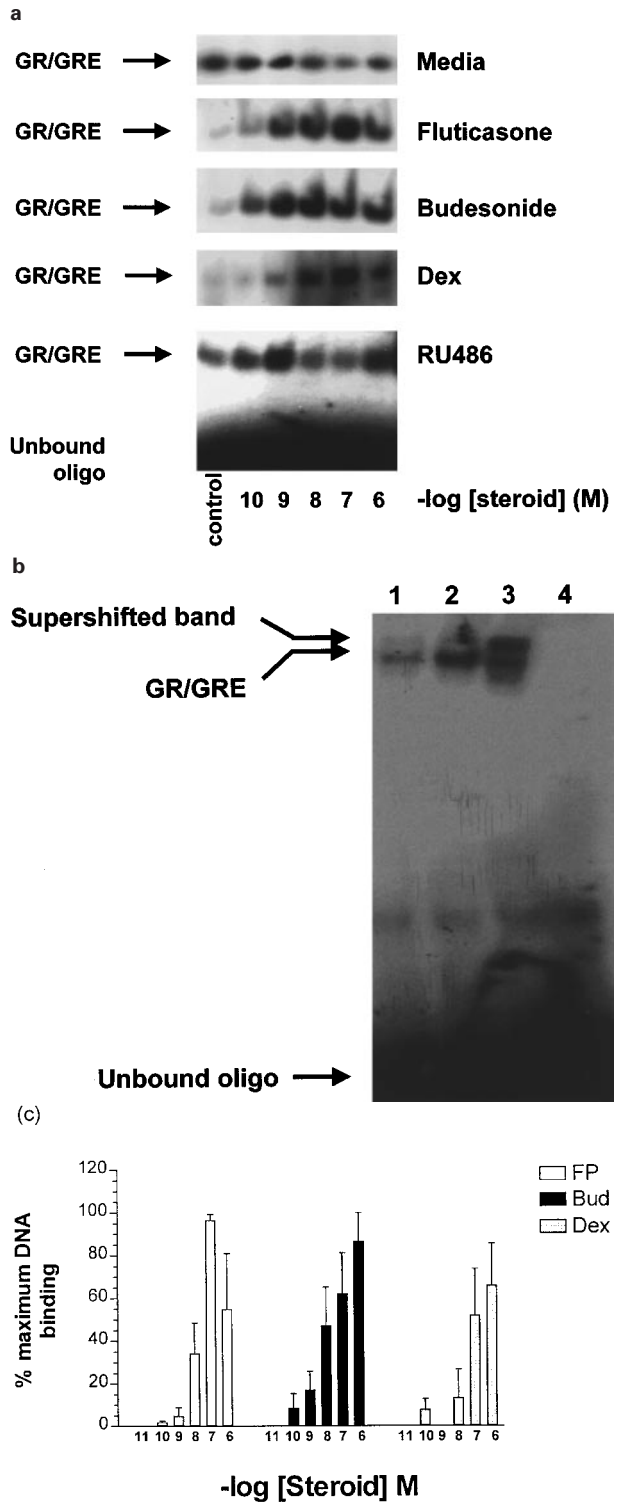

Figure 2 (a) Representative electrophoretic mobility shift assay showing the concentration-dependent effect of fluticasone propionate (FP), Budesonide (Bud) and dexamethasone (Dex) on glucocorticoid receptor (GR)-induced activation as represented by increased DNA binding (GRE binding) (arrowed) within the nucleus after 2 h incubation. (b) Supershift assay of dexamethasone (10^{-6} M)-stimulated GR DNA binding. Increased DNA binding is seen following dexamethasone treatment (lane 2). Pre-incubation of retarded complexes with an anti-GR antibody (lane 3) shows specific enhanced retardation of GR/GRE band. Specificity of binding was indicated by the addition of 100 fold excess unlabelled oligonucleotide (lane 4). Unbound oligonucleotide is indicated by an arrow at the bottom of the gel. (c) Densitometric analysis of the retarded bands in (a) and corrected for maximal band intensity showing the concentration-dependent increase in GRE binding following 2 h incubation with FP, Bud and Dex within the nucleus as a percentage of the maximal increase observed.

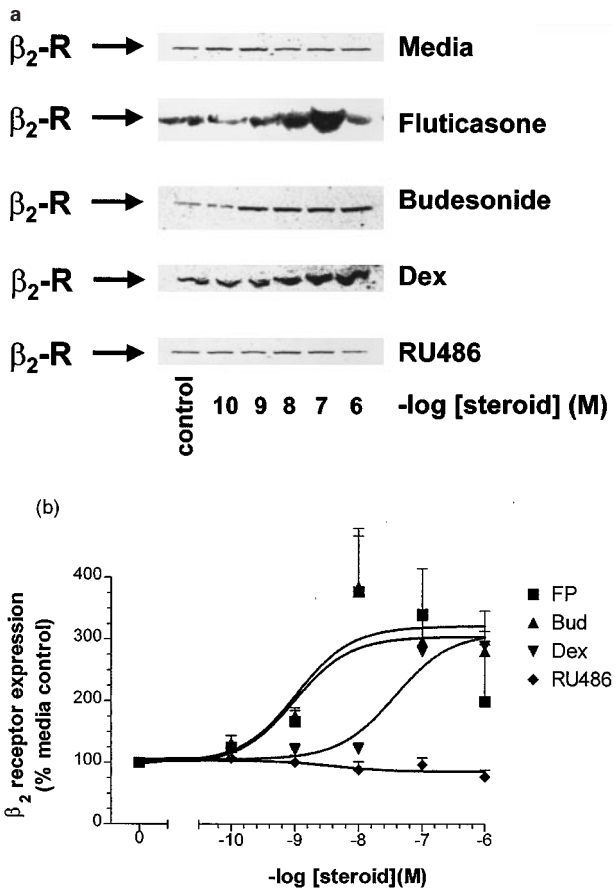

Figure 3 (a) Western blot analysis of β_2-receptor (β_2R) expression at 24 h following increasing concentrations of fluticasone propionate (FP), budesonide (Bud), dexamethasone (dex) or RU486. The single 47 kD band representing the β_2-receptor is indicated by the arrow. Incubation with control media does not affect β_2-receptor expression. (b) Graphical representation of the results shown in (a). Results are shown as the percentage change in β_2-receptor band density compared to control untreated cells and are representative of four individual experiments and are reported as the means ± s.e.means.

gated in these cells by Western blot analysis. Cells were incubated with glucocorticoids (10^{-12}–10^{-6} M) for various time periods of up to 24 h. None of the glucocorticoids investigated (fluticasone propionate, budesonide, dexamethasone or RU486) had any effect on the expression of I-κBα protein at any concentration tested in these cells (Figure 4a). IL-1β (1 ng ml^{-1}) stimulation caused a rapid phosphorylation and subsequent degradation of I-κBα which was inhibited by the proteosome inhibitor CBZ-leucine-leucine-leucinal (LLLal, 50 μM) (Figure 4b). This was followed at 60–90 min by the induction of *de novo* synthesized I-κBα (Figure 4c). We further examined the effects of these glucocorticoids on this degradation and reappearance of I-κBα within the cytoplasm of these cells. IL-1β caused a total loss of I-κBα protein from the cytoplasm within 2–5 min. The expression of I-κBα protein returned to control levels between 90 and 120 min. None of the drugs tested had any effect on the time course of I-κBα degradation or synthesis (Figure 4c). Glucocorticoids may affect NF-κB activation by altering the level of the I-κB associated with longer term induction of NF-κB, I-κBβ in these cells. None of the glucocorticoids tested had any effect on the induction of I-κBβ at 24 h. In contrast to I-κBα, IL-1β did not cause a rapid degradation of I-κBβ but

caused a decrease in I-κBβ levels at 4–6 h before returning to control levels which was not affected by glucocorticoids (Figure 4d and Table 1).

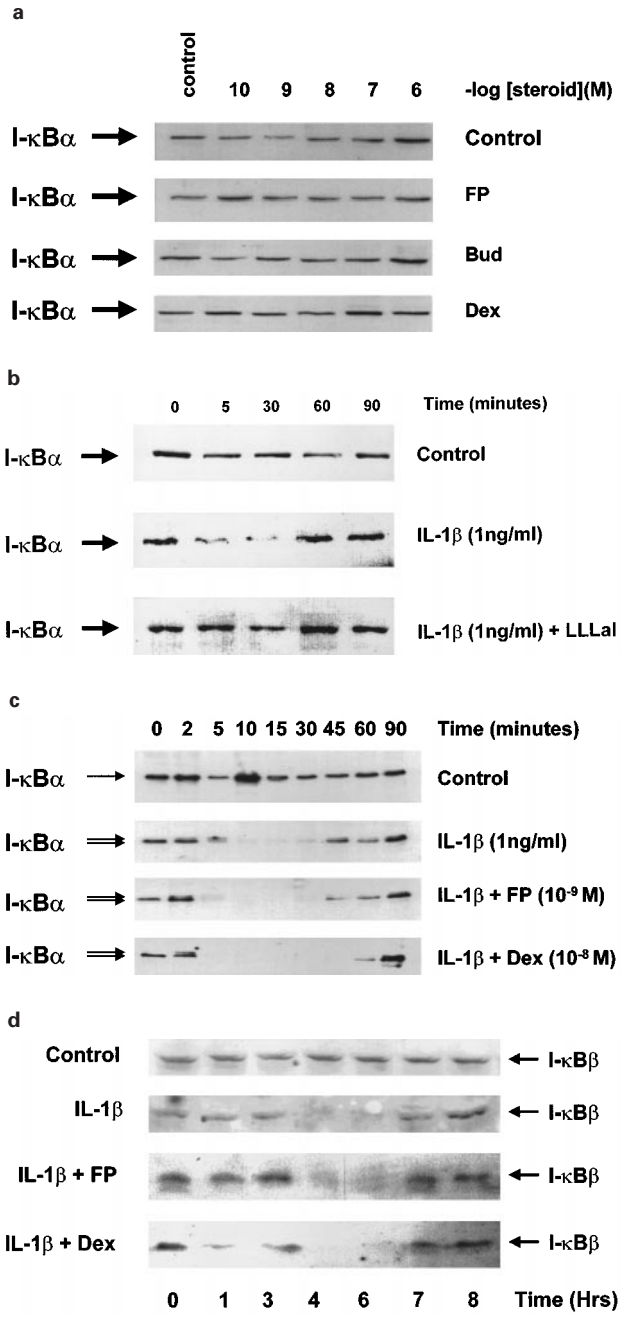

Figure 4 Western blot analysis of the time course of I-κBα expression following 24 h treatment with various concentrations of fluticasone propionate (FP), budesonide (Bud) and dexamethasone (Dex). Concentrations are reported as −log of Molar concentrations. (b) Western blot analysis of the time course of I-κBα expression following up to 90 min treatment with IL-1β (1 ng ml^{-1}) in the presence and absence of the proteosome inhibitor CBZ-leucine-leucine-leucinal (LLLal) (50 μM) I-κBα is indicated by the arrow. (c) Western blot analysis of the time course of I-κBα expression following up to 90 min treatment with IL-1β (1 ng ml^{-1}). The effects of fluticasone propionate (FP, 10^{-10} M), budesonide (Bud, 10^{-10} M) and dexamethasone (Dex, 10^{-9} M) on IL-1β-stimulated I-κBα phosphorylation, degradation and subsequent induction are shown. I-κBα and the slower migrating phosphorylated form of I-κBα are indicated by the arrows. (d) The lack of effect of fluticasone propionate (FP, 10^{-10} M) budesonide (Bud, 10^{-10} M) and dexamethasone (Dex, 10^{-9} M) on IL-1β-stimulated I-κBβ phosphorylation, degradation and subsequent induction over 0–8 h are shown. Results are representative of four individual experiments.

GR/p65 immune complex

Immunoprecipitation studies showed that the p65 subunit of NF-κB was associated with GR, either directly or within a complex, within A549 cells. Western blot analysis of the immunoprecipitated p65 complex showed the presence of a specific GR band. In the reverse experiment the immunoprecipitated GR complex showed the presence of p65 (Figure 5). In contrast, immunoprecipitation with pre-immune serum showed no binding of GR or p65.

NF-κB- and TRE-driven reporter gene constructs

IL-1β (1 ng ml^{-1}) stimulation produced a significant 3 fold induction in luciferase activity with both the pGM-CSF(−123)-Luc and the pGL-3κBLuc vectors. IL-1β-stimulated 3×κB-activated luciferase activity was inhibited in a concentration-dependent manner by fluticasone propionate (FP, $IC_{50} = 1.8 \times 10^{-11}$ M), budesonide (Bud, $IC_{50} = 2.7 \times 10^{-11}$ M), dexamethasone (Dex, $IC_{50} = 0.8 \times 10^{-9}$ M) and RU486 ($IC_{50} = 8 \times 10^{-11}$ M) (Figure 6a). The basal expression of pGL-3κB-Luc was also modulated by glucocorticoids. Fluticasone propionate ($IC_{50} = 0.5 \times 10^{-11}$ M), budesonide ($IC_{50} = 2.7 \times 10^{-11}$ M) and dexamethasone ($IC_{50} = 0.5 \times 10^{-9}$ M) caused a 50–70% decrease in luciferase activity compared to those seen in control unstimulated cells. RU486 also caused a concentration-dependent inhibition of κB-driven luciferase activity ($IC_{50} = 2.5 \times 10^{-11}$ M) but the maximal reduction seen was 50% of basal levels. IL-1β-stimulated GM-CSF-promoter driven luciferase activity was inhibited in a concentration-dependent manner by fluticasone propionate ($IC_{50} = 0.6 \times 10^{-11}$ M) and dexamethasone ($IC_{50} = 1.3 \times 10^{-9}$ M) (see Table 1).

IL-1β (1 ng ml^{-1}) caused a 2.4 fold increase in a 6×TRE promoter-driven luciferase activity after 18 h of stimulation. IL-1b-stimulated TRE-promoter driven luciferase activity was inhibited in a concentration-dependent manner by fluticasone propionate ($IC_{50} = 1.7 \times 10^{-11}$ M) and dexamethasone ($IC_{50} = 0.9 \times 10^{-9}$ M) (Figure 6b). These glucocorticoids also reduced basal expression of TRE activity, fluticasone propionate ($IC_{50} = 1.1 \times 10^{-10}$ M), budesonide ($IC_{50} = 1.0 \times 10^{-10}$ M) and dexamethasone ($IC_{50} = 0.3 \times 10^{-9}$ M) all reducing levels to less than 50% of those seen in control unstimulated cells. RU486 also caused a concentration-dependent inhibition of TRE activity ($IC_{50} = 7.1 \times 10^{-11}$ M) but the maximal reduction seen was 50% of basal levels. Inhibition of TRE activity occurred at approximately 10 fold lower concentrations than that which caused inhibition of κB-driven luciferase activity and GM-CSF release (see Table 1).

Discussion

Fluticasone propionate and budesonide were more potent as inhibitors of GM-CSF release and NF-κB activity than dexamethasone. Although all these ligands were acting through the same receptor, fluticasone propionate and budesonide were approximately five times more potent at these targets than would be predicted purely by ligand binding affinity. In contrast, the ability of these drugs to modulate AP-1 reporter gene activity was more closely related to their GR ligand binding affinity (see Brattsand & Linden (1996)). This suggests that altered conformational changes in the GR monomer may alter the ability to repress gene expression in a ligand dependent manner. This was further suggested by the ability of low concentration, but not high concentration, RU486 to suppress IL-1β-stimulated GM-CSF release and κB activity.

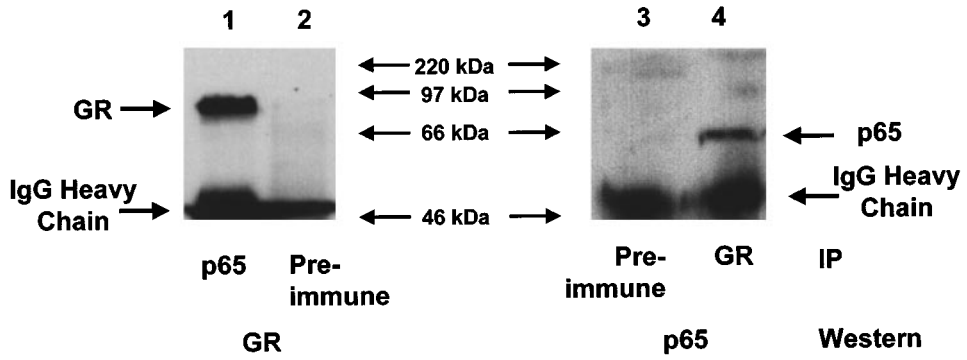

Figure 5 Western blot analysis of immunoprecipitated p65 and GR complexes. Cells were treated for 30 min with a combination of IL-1β (1 ng ml^{-1}) and dexamethasone (10^{-6} M) before cell were lysis. Total cell extracts were immunoprecipitated with an anti-human p65 antibody (lane 1), an anti-human GR antibody (lane 4) or with pre-immune serum (lanes 2 and 3) before separation by 10% PAGE and detection of bands by either anti-human GR antibody (lanes 1 and 2) or anti-human p65 antibody (lanes 3 and 4). The specific GR or p65 bands are indicated arrows. The 50 kDa IgG heavy chain is detected in all samples and is also arrowed. Molecular weight markers are as indicated. The results are representative of three independent experiments.

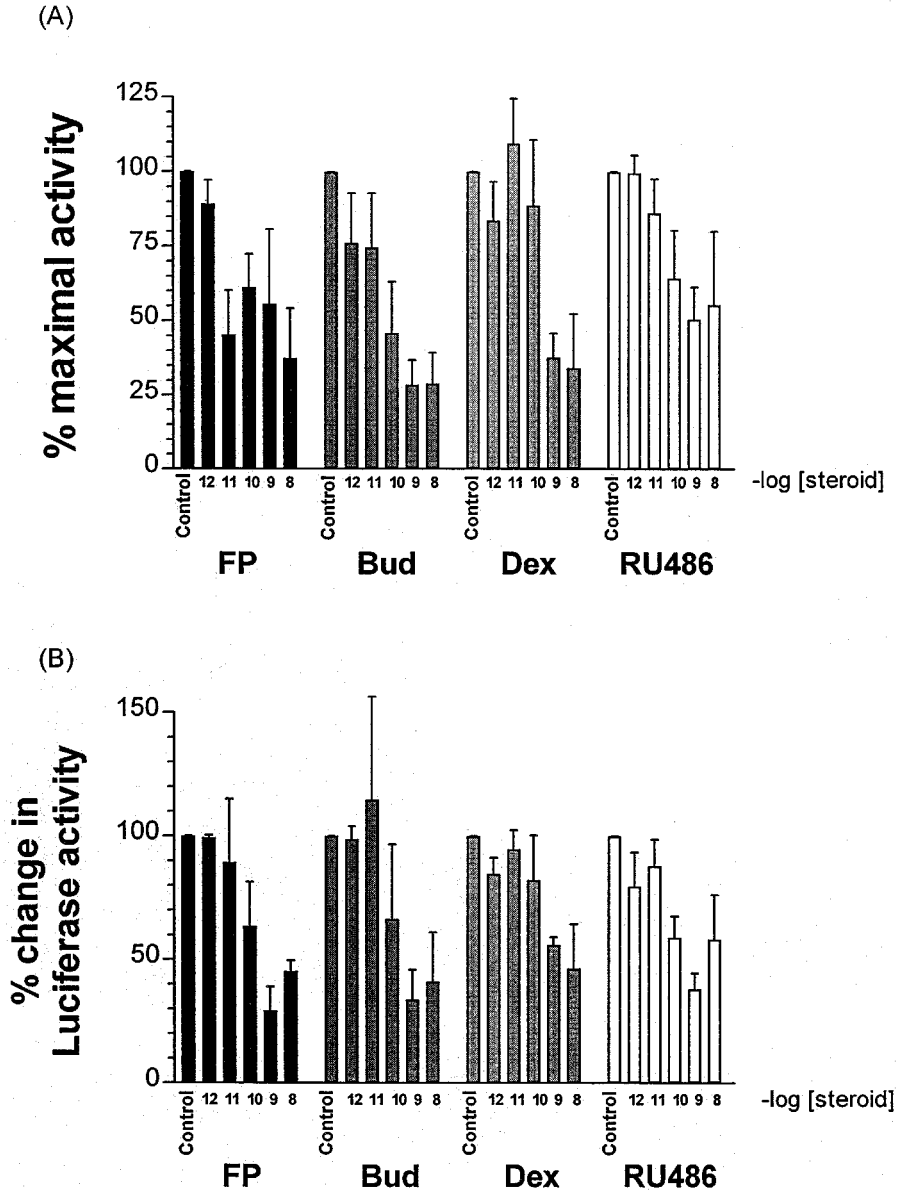

Figure 6 (a) Inhibition of an IL-1β (1 ng ml^{-1})-stimulated 3 × κB-driven luciferase reporter gene by fluticasone propionate (FP), budesonide (Bud), dexamethasone (Dex) and RU486. Results are expressed as relative light units/unit β-galactsidase activity (means ± s.e.mean) and represent the results of at least four independent experiments. (b) Inhibition of IL-1β (1 ng ml^{-1})-stimulated 6 × TRE-Luc reporter gene by fluticasone propionate (FP) and dexamethasone (Dex). Results are expressed as relative light units/unit β-galactsidase activity (means ± s.e.mean) and represent the results of at least four independent experiments.

In order to examine the potential mechanisms for this repression of GM-CSF release we examined the ability of these drugs to increase the expression of the β_2-adrenoceptor and the inhibitor of NF-κB-driven transcription, I-κB. The ability of these glucocorticoids to induce the β_2-adrenoceptor was found to correlate with GR/GRE binding and occurred at 10–100 fold higher concentrations than the inhibition of GM-CSF release (see Table 1). These results are similar to those for fluticasone and dexamethasone suppression of TNFα-induced E-selectin expression (Ray *et al*., 1997). No induction of I-κBα, was seen in these cells by any steroid at concentrations up to 1 μM for time periods up to 24 h. Furthermore, the ability of IL-1β to cause I-κBα or I-κBβ degradation and subsequent induction was not affected by steroids. This confirms results obtained in several other cell types where no effect of dexamethasone was observed (Heck *et al*., 1997; Brostjan *et al*., 1996; Ray *et al*., 1997) and suggest that induction of gene transcription by the activated glucocorticoid receptor was not required for inhibition of GM-CSF release.

The possibility of a direct interaction between activated GR and NF-κB in the suppression of NF-κB activated gene transcription was indicated by the ability of these glucocorticoids to inhibit a reporter gene construct containing three κB sites alone and was confirmed by immunoprecipitation experiments. This interaction is likely to occur through a leucine charged domain in the p65 subunit (Heery *et al*., 1997). The inhibition of luciferase activity correlated with inhibition of GM-CSF release suggesting that this is indeed an important mechanism in regulating GR actions in these cells. However only a 50–60% inhibition of luciferase activity was seen at concentrations of steroid at which GM-CSF release was completely inhibited. This suggests that although repression of gene transcription plays a major role in the suppression of GM-CSF release in these cells other post-transcriptional events may also be important in glucocorticoid-repression of GM-CSF release.

The data presented here suggests that the anti-inflammatory properties of a range of glucocorticoids relate to their ability to transrepress rather than transactivate genes. Furthermore, the results seen with RU486 suggests that repression of NF-κB and AP-1 activity by GR does not require the transactivation function of GR. The results also suggest that transrepression occurs at approximately 10 fold lower concentrations than that required for transactivation of genes such as the β_2-receptor. The ability of the more modern inhaled glucocorticoids fluticasone propionate and budesonide, but not dexamethasone, to inhibit NF-κB activity appears to correlate more closely with GM-CSF release than the ability to inhibit AP-1 activity. This suggests that NF-κB may be a more important target for glucocorticoid actions, at least in the regulation of inflammatory genes in A549 cells, than AP-1. However, in the context of other diseases and other cells AP-1 may be a more important target. The importance of inhibiting both NF-κB and AP-1 within these cells may be relevant in the control of inflammatory responses since these transcription factors are important for the expression of many genes and often act in concert with each other (Stein *et al*., 1993).

Similar results have been demonstrated for the interaction between GR and AP-1 (Heck *et al*., 1994). In these studies using overexpression vectors, a variety of GR mutants and a choice of glucocorticoid ligands, it has been possible to dissociate the transrepressive and transactivation functions of GR on AP-1 mediated reporter gene activities. Moreover, DNA binding and activation of glucocorticoid-regulated promoters require GR dimerisation, whereas AP-1, and probably NF-κB, repression may be mediated by GR monomers (Heck *et al*., 1994). Increased levels of either AP-1 or NF-κB, raised by overexpression of cDNAs, also have profound effects on the ability of GR to inhibit transcription driven by these genes. Expression of GR at levels that cause marked inhibition of either AP-1 or NF-κB alone fails to repress either factor when both are activated together (Scheinman *et al*., 1995b).

Recent results from several laboratories have provided similar but slightly different results regarding the exact mechanisms of glucocorticoid suppression of gene transcription. These differences may be due to the level of expression of GR in each system since some depend upon over-expression of GR, some occur in the presence of serum and others in the absence of serum. We have recently found that the removal of serum (or the use of charcoal-stripped serum) results in markedly up-regulated expression of GR at 48 h (Adcock *et al*., unpublished observations).

Studies of transcription factor interactions may have therapeutic potential in the control of inflammatory disease. Glucocorticoids exert their anti-inflammatory effects largely by interference with the ability of transcription factors that have been activated by inflammatory cytokines to induce transcription of inflammatory genes. This interaction between GR and transcription factors may be either direct or through an integrator molecule such as CBP or associated co-activators such as p300/CBP associated protein (P/CAF), steroid-receptor coactivator-1 (SRC-1) or p300/CBP co-integrator protein (pCIP) (Janknecht & Hunter, 1996). Interaction of these factors with CBP allows interaction, modulation of histone acetylation and subsequent activation of the basal transcription initiation complex. Although not shown here for NF-κB, it is possible that this is the mechanism by which glucocorticoids interfere with NF-κB-driven gene transcription (Perkins *et al*., 1997; Sheppard *et al*., 1998). These interactions may affect transactivation by the pro-inflammatory transcription factor due to effects on DNA-binding, association with the integrator molecules or activation of RNA polymerase II to varying degrees. The exact contribution of each mechanism may vary between cell types and depend upon the cell stimulus. The differences in activity of different glucocorticoids did not correlate solely with ligand binding but may reflect differential interaction of the same receptor with DNA or proteins according to the activating ligand. Similar ligand-induced differentiation of transrepression and transactivation activities has been reported previously for the interaction between retinoic acid and its receptors (RARα) (Yang Yen *et al*., 1991). Thus, there is potential for the development of novel glucocorticoids with enhanced transrepressive and reduced transactivation actions. Other drugs that regulate the activity of specific transcription factors may also be developed in the future.

In conclusion, both trans-repression and trans-activation by GR may be affected by the ligand in a manner that reflects not only ligand affinity but also the differential abilities of ligands to influence GR interaction with DNA, other transcription factors, integrator molecules or the basal transcription apparatus. The differentiation of these two activities (trans-repression and trans-activation) leads to the possibility of the development of more specific glucocorticoids. Indeed, these results suggest that current drugs used in asthma therapy may already have been selected for an enhanced ability to transrepress rather than activate gene transcription.

This work was funded by the Medical Research Council (U.K.), The National Asthma Campaign, The European Union Biomed II programme and Glaxo-Wellcome.

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(Received January 25, 1999
Revised March 18, 1999
Accepted March 23, 1999)