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Agonist-specific modulation of glucocorticoid receptor-mediated transcription by immunosuppressants[1]

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Abstract

Although the immunosuppressive drugs FK506, rapamycin and cyclosporin A have been reported to potentiate transcriptional activation mediated by a non-saturating concentration of the glucocorticoid receptor agonist dexamethasone, the precise mechanism(s) underlying these responses remains unclear. The murine L-929-derived LMCAT cell line stably transfected with the mouse mammary tumor virus promoter-chloramphenicol acetyl transferase reporter gene construct was utilized in the present study to further investigate the mechanism(s) underlying this dexamethasone potentiation as well as the possible agonist specificity of this potentiation.. The present data demonstrate that pretreatment (2 h) of LMCAT cells with 10 μM FK506, rapamycin or cyclosporin A results in the potentiation of reporter gene transcription mediated not only by dexamethasone (~ 12-fold), but also by hydrocortisone (~ 6-fold) and triamcinolone acetonide (~ 2.5-fold). In sharp contrast, the data show for the first time that pretreatment with any one of these immunosuppressive drugs suppresses (~ 2–8-fold) the transcriptional responses mediated by corticosterone, deoxycorticosterone, and cortexolone. Pretreatment of intact LMCAT cells with FK506 increases the subsequent whole cell specific binding of [^{3}H]dexamethasone, but does not increase specific cytoplasmic binding when the tritiated agonist is added directly to cytosolic extracts prepared from the pretreated cells. These data suggest that the FK506-mediated potentiation of the transcriptional responses induced by some agonists, like dexamethasone, may be related to the ability of this immunosuppressant to inhibit the membrane-associated multidrug resistance (MDR) P-glycoprotein, which actively extrudes some steroids from cells. Identical pretreatment with FK506 has no detectable effect on the subsequent whole cell specific binding of [^{3}H]corticosterone, a steroid which is not effectively extruded by the MDR pump. Two additional MDR pump inhibitors, verapamil and quinidine, potentiate (30-fold) the dexamethasone-mediated transcriptional response as expected, but have no detectable effects on a corticosterone-mediated transcriptional response. Unlike immunosuppressive drugs, these ion channel blockers do not bind to receptor-associated immunophilins (FK506-binding proteins or cyclophilins). Collectively, these results suggest that immunosuppressants potentiate a dexamethasone-mediated transcriptional response in LMCAT cells by inhibiting efflux of this steroid. In contrast, these drugs appear to suppress a corticosterone-mediated transcriptional response by a different mechanism, perhaps one involving their binding to glucocorticoid receptor-associated immunophilins.

Keywords: FK506; Glucocorticoid receptors; Immunosuppressants; Immunophilins; P-glycoprotein; Transcription

1. Introduction

Glucocorticoid hormones mediate their responses by binding with high affinity and specificity to intracellular glucocorticoid receptors (GR). According to the currently accepted model, once the lipid soluble glucocorticoid hormone diffuses across the plasma membrane of

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a target cell and binds to its cytoplasmic GR, the hormone-receptor complex then undergoes a ligand-induced conformational change referred to as 'activation' or 'transformation' (reviewed in Schmidt and Litwack, 1982). This conformational change results in the dissociation of a dimer of the 90 kDa heat shock protein (hsp90) (Sanchez et al., 1987) and an increased affinity of these hormone–receptor complexes for DNA. Once activated, these cytoplasmic GR complexes translocate into the nucleus where they bind as homodimers (Drouin et al., 1992) to specific cis-acting DNA sequences, termed 'hormone response elements' (HREs), located within the promoters of target genes (Yamamoto, 1985). Binding to either positive or negative glucocorticoid response elements (GREs) results in the enhancement or repression, respectively, of the rate of transcription of target genes. This transcriptional regulation may occur in a very cell specific manner as evidenced by the fact that glucocorticoid-mediated induction of atrial natriuretic factor occurs only in cardiac myocytes (Argentin et al., 1991). In contrast, the mouse mammary tumor virus (MMTV) promoter contains four GREs and when linked to an appropriate reporter gene and transfected into essentially any GR-expressing cell line this promoter can be utilized to study GR-mediated transcriptional activation (Majors and Varmus, 1983; Beato, 1989).

In addition to hsp90, other non-hormone binding proteins have also been shown to be associated with the unliganded, heteromeric GR complex. Reconstitution experiments performed utilizing GR complexes immunoabsorbed from cytosols prepared from L929 mouse fibroblasts have demonstrated that hsp70 and a 60 kDa protein referred to as p60 are required for GR–hsp90 heterocomplex assembly in a rabbit reticulocyte lysate system (Dittmar et al., 1996; Dittmar and Pratt, 1997). Additional data have demonstrated that a widely distributed 23 kDa acidic protein referred to as p23 is also associated with the GR via its ability to bind directly to hsp90 in an ATP-dependent manner (Johnson and Toft, 1994). It has been proposed that although p23 may not be required for folding of the GR protein as once predicted (Hutchison et al., 1997), it may function to stabilize the GR–hsp90 heterocomplex and hence block rapid disassembly (Dittmar and Pratt, 1997). Owens-Grillo et al. (1995) have also demonstrated that there are at least two independent types of cytoplasmic GR–hsp90 heterocomplexes, one that binds hsp56, which is a 56–59 kDa heat shock protein also known as p59 (Renoir et al., 1990), and another that contains cyclophilin-40 (CyP-40), and that both of these proteins bind to a common site on hsp90. In addition to being a constitutively expressed heat shock protein, hsp56 also binds the immunosuppressive drugs FK506 and rapamycin and has therefore been identified as an immunophilin of the FKBP (FK506 binding protein) family (Sanchez, 1990). Since FKBP59, which is also referred to as FKBP52 (Peattie et al., 1992) binds to hsp 90 it is also referred to as a heat shock protein-binding immunophilin (Callebaut et al., 1992). Likewise CyP-40 is a 40 kDa protein that also qualifies as an immunophilin and binds cyclosporin A (CsA) with high affinity and specificity (Kieffer et al., 1992). Although GR-associated hsp90 appears to mediate a variety of functions including masking of the DNA binding domain (Howard et al., 1990) and stabilization of the hormone binding site in the unliganded GR (Nemoto et al., 1990), less information is available concerning the potential role(s) of hsp56 or CyP-40 in regulating GR function(s). Czar et al. (1995) have utilized an antibody directed against hsp56 to demonstrate that this heat shock protein-binding immunophilin is required for trafficking of GR from the cytoplasm to the nucleus in mouse L-929 fibroblasts. Several laboratories have also probed the potential roles of these immunophilins by investigating the effects of immunosuppressive drugs on key GR-functions including activation/transformation, nuclear translocation and agonist-mediated transcriptional responses (Hutchison et al., 1993; Ning and Sanchez, 1993; Czar et al., 1995; Ning and Sanchez, 1995; Renoir et al., 1995; Sanchez et al., 1995). Despite the fact that these studies have generated important new data, a clear understanding of the mechanism(s) underlying the effects of these drugs on GR functions is currently lacking.

In addition to their potential abilities to modulate GR functions by interacting directly with GR-associated immunophilins, these immunosuppressive drugs may also exert effects on glucocorticoid hormone action that are directly linked to the phenomenon of multidrug resistance (MDR). This MDR phenotype is frequently characterized by the presence of increased levels of a 170 kDa plasma membrane protein, referred to as P-glycoprotein, which is expressed not only in some tumor cells but also in normal human tissues including the luminal surface of transporting epithelia of the kidney proximal tubule, small intestine, colon, biliary hepatocytes and in capillary endothelial cells of the brain and testis as well as in the adrenal cortex (Thiebaut et al., 1987; Sugawara et al., 1988; Thiebaut et al., 1989). This location of the P-glycoprotein in non-tumor cells suggests that one of its physiological roles is to pump or secrete naturally occurring metabolites and toxic substances into bile and urine and directly into the lumen of the gastrointestinal tract (Ueda et al., 1992). This energy-dependent pump, which is a member of the ABC (ATP-binding cassette) transporter superfamily containing two transmembrane domains (each spans the membrane six times) alternating with two putative ATP binding sites, has been shown to export a wide range of biochemically unrelated anti-

cancer drugs out of tumor cells and by so doing lowering their intracellular concentrations to sublethal levels and inducing tumor resistance (Gottesman and Pastan, 1988; Bosch and Croop, 1996). Ueda et al. (1992) have demonstrated that P-glycoprotein also trans-epithelially transports cortisol (hydrocortisone), aldosterone and dexamethasone, thus lowering their effective intracellular concentrations. Hydrophobic steroids have been reported to bind tightly to this P-glycoprotein (Yang et al., 1989). However, some hydrophobic steroids, like progesterone, are not actively extruded from the cell despite their binding to this membrane transporter (Ueda et al., 1992). Thus different structural properties appear to determine whether a particular steroid molecule will merely bind to and inhibit the P-glycoprotein pump, or actually be extruded from the cell after it has bound to the transporter. Other studies, including one published by Hoof et al. (1993), have demonstrated that rapamycin, FK506 and CsA bind with high affinity to P-glycoprotein and by inhibiting its pumping activity these drugs elevate the intracellular concentrations of drugs such as adriamycin, colchicine and vinblastine, thus reversing resistance to these chemotherapeutic agents. Additional studies (Cornwell et al., 1987; Fojo et al., 1987; Ford and Hait, 1990) have shown that the MDR resulting from P-glycoprotein over-expression can be circumvented by the Ca^{2+} channel blocker, verapamil, and the Na^{+} channel blocker, quinidine. These channel blockers can thus function as chemosensitizing agents by competing for binding to the MDR-related membrane P-glycoprotein (Kessel and Wilderding, 1984; Cornwell et al., 1987; Akiyama et al., 1988; Ford and Hait, 1990). P-Glycoprotein expression has also been implicated in the acquisition of glucocorticoid resistance in murine thymoma cells and in this situation hormone-induced apoptosis can be restored by verapamil (Bourgeois et al., 1993). Finally, a recent report (Kralli and Yamamoto, 1996) has demonstrated that an FK506-sensitive transporter in yeast selectively decreases the intracellular levels and potency of glucocorticoid hormones. Thus membrane-associated transporters may be capable of modulating, perhaps in a cell-specific manner, the initial step in the glucocorticoid signaling pathway, which is the availability of the steroid for binding to intracellular GR (reviewed in Thompson, 1995).

In the present series of experiments the murine LMCAT cell line, which has been stably transfected with the MMTV promoter-chloramphenicol acetyl transferase (CAT) reporter gene construct (Howard et al., 1990), has been utilized as a model system in which to evaluate the potential effects of known P-glycoprotein inhibitors on the transcriptional response elicited by a variety of GR agonists. The data presented in this report suggest that the abilities of these immunosuppressive drugs to potentiate the transcriptional responses induced by some agonists, such as dexamethasone, is related to the known ability of these drugs to inhibit the MDR pump or another related membrane transporter. In contrast, the ability of these drugs to suppress the transcriptional responses induced by other agonists, such as corticosterone, appears to occur via a different mechanism(s), perhaps one involving their binding to GR-associated immunophilins.

2. Materials and methods

2.1. Materials

FK506 was a gift from Fujisawa Pharmaceutical (Osaka, Japan and Melrose Park, IL). Rapamycin and (±)-verapamil were purchased from CalBiochem (La Jolla, CA), cyclosporin A from the pharmacy at The University of Iowa Hospitals and Clinics, and quinidine (sulfate salt dihydrate) from Sigma Chemical (St. Louis, MO). These reagents were dissolved in either ethanol (FK506, rapamycin, (±)-verapamil), glycerol/ethanol (CsA) or methanol (quinidine) and stored as concentrated stocks. Non-radioactive dexamethasone, corticosterone, deoxycorticosterone and acetyl CoA were obtained from Sigma (steroids stored as concentrated stocks in ethanol). [1,2,6,7-^{3}H]corticosterone ([^{3}H]corticosterone; 88 Ci/mmol), [1,2,4-^{3}H]dexamethasone ([^{3}H]dexamethasone;44 Ci/mmol), and D-*threo*-[dichloroacetyl-1-^{14}C] chloramphenicol (53 Ci/mmol), were purchased from Amersham (Arlington Heights, IL). All tissue culture components, including the Dulbecco's Modified Eagle's Medium (DMEM), were purchased from the University of Iowa Tissue Culture/Hybridoma Facility.

2.2. CAT reporter cell line

The LMCAT cell line was generously supplied by Dr Edwin Sanchez (Medical College of Ohio). This cell line, which expresses endogenous GR but not mineralocorticoid receptors, was derived from mouse L929 cells (fibroblast-like) that had been stably transfected with the pMMTV-CAT reporter gene construct (Howard et al., 1990) using the Lipofectin reagent (Felgner et al., 1987). These cells were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and penicillin (100 U/ml)-streptomycin (100 μg/ml), gentamycin (0.05 mg/ml), fungizone (2.5 μg/ml), 10 mM HEPES, and L-glutamine (2 mM). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and were subcultured weekly via trypsinization.

2.3. CAT assay

Quantitation of CAT enzyme activity was performed in duplicate essentially following the protocol described by Gorman et al. (1982) using [^{14}C]chloramphenicol as substrate. Following thin layer chromatography (TLC) the silica gel plates (J.T. Baker, Phillipsburg, NJ) were exposed for 3 days to Kodak X-Omat X-ray film (Sigma). After development of the resulting audioradiograms, the individual spots corresponding to the non-acetylated substrate and acetylated product were separately excised for counting in aqueous counting scintillant (Beckman Ready Gel). For each treatment the dpm corresponding to the acetylated spot were expressed as a percentage of the total dpm (acetylated plus non-acetylated). Within each experiment normalization of the data was performed by arbitrarily setting the percent acetylation detected in the ethanol-treated control group to 1.0. The normalized data from identical repeat experiments were pooled and plotted.

2.4. Binding Assays

Whole cell binding assays were performed in duplicate using 1×10^7 LMCAT cells that had been pretreated for 2 h with ethanol, or FK506 (10 μM) or verapamil (50 μM). After preincubation, the cells were resuspended in DMEM containing increasing concentrations of either [^{3}H]dexamethasone or [^{3}H]corticosterone in the absence (uncompeted) or presence (competed) of 500-fold molar excess of the appropriate unlabeled steroid. Cells were incubated for 1 h at 37°C in a humidified CO_2 incubator, and then washed three times with phosphate buffered saline (PBS) and subsequently resuspended in 200 μl PBS. The bound radioactivity in 100 μl aliquots was then quantitated by liquid scintillation spectroscopy. Specific whole-cell binding was calculated by subtracting nonspecific (competed) from total (uncompeted) binding. For cytoplasmic binding assays, LMCAT cells were preincubated for 2 h with either ethanol (control) or FK506 (10 μM) and subsequently homogenized via sonication (Polytron, Brinkmann Instruments) in aliquots of ice-cold buffer (50 mM potassium phosphate, 10 mM Na_2MoO_4, 10 mM monothioglycerol and 2 mM dithiothreitol, pH 7.0 at 0–4°C). The crude homogenate was then centrifuged at $105000 \times g$ for 1 h at 4°C. Aliquots of the cytosolic extracts were then incubated overnight at 4°C with increasing concentrations of [^{3}H]dexamethasone in the presence (competed) and absence (uncompeted) of a 500-fold molar excess of unlabeled dexamethasone. Quantitation of specific cytosolic binding was then performed in triplicate by the hydroxylapatite batch assay (Schmidt et al., 1985) followed by liquid scintillation spectroscopy.

2.5. Nuclear translocation assay

To measure the extent of agonist-induced nuclear translocation of GR, LMCAT cells were first pretreated for 2 h with either ethanol (control) or FK506 (10 μM), and then resuspended in serum-free DMEM containing either 30 nM [^{3}H]dexamethasone alone (uncompeted) or [^{3}H]dexamethasone plus a 500-fold molar excess of unlabeled dexamethasone (competed). The whole cell suspensions were then incubated at 37°C for 1 h. Cells were subsequently washed several times with PBS, and aliquots of the suspensions were then counted to determine total (nuclear plus cytoplasmic) cellular specific binding. Additional aliquots of the cells from both the ethanol and FK506 pretreatment groups were lysed by repeated freezing on dry ice and thawing at 1–4°C. The cytosolic fractions and nuclear pellets of the lysates were then separated by brief centrifugation in 1.5 ml Eppendorf tubes. Specific cytoplasmic (cytosolic) binding was then quantitated via liquid scintillation spectroscopy. The tips of the Eppendorf tubes containing the crude nuclear pellets were then cut off and assayed for specific binding. The percentage of dexamethasone-induced nuclear translocation in the various treatment groups was then calculated by expressing the specific binding detected in the nuclear pellet as a percentage of the total cellular specific binding.

2.6. Statistical analysis

Experimental results are expressed as means $\pm$ S.E. All CAT assays were performed in duplicate and the number of independent repetitions of a specific experiment is indicated in the appropriate figure legend. Statistical analyses were performed using the Microsoft Excel version 4.0 software package. The Student's t-test was used to determine statistical significance between treatment groups and the individual P values are indicated in the figure legends. P Values less than 0.05 were considered significant.

3. Results

3.1. Effects of pretreatment with FK506, rapamycin or cyclosporin A on glucocorticoid agonist-mediated transcriptional response

In the first series of experiments the effects of pretreatment of LMCAT cells with different immunosuppressive drugs on subsequent glucocorticoid agonist-mediated enhancement of CAT reporter gene transcription were investigated. As shown in Fig. 1 (Panel A), pretreatment for 2 h with FK506 (10μM) resulted in significant potentiation ($\sim$12-fold) of CAT gene transcription mediated by subsequent incubation

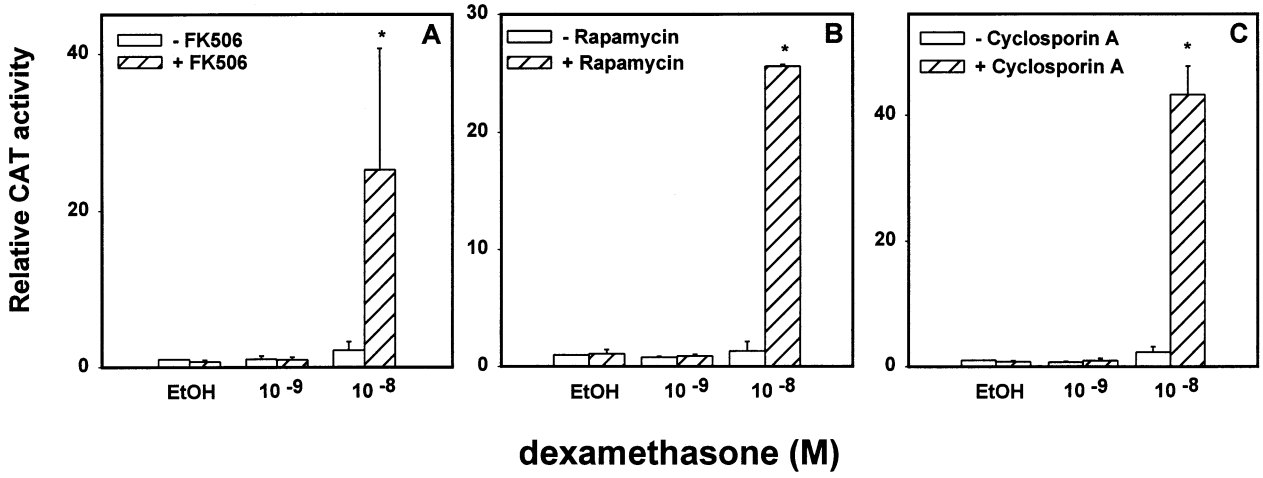

Fig. 1. Potentiation of dexamethasone-mediated transcriptional activation in LMCAT cells by immunosuppressive drugs. Cells were pretreated for 2 h at 37°C with 10 μM FK506 (panel A), rapamycin (panel B), or cyclosporin A (panel C). Following a 20 h incubation at 37°C with ethanol (control) or the indicated concentrations of dexamethasone, CAT reporter gene activity was quantitated as described in Section 2. Data presented are the means ± SEM for four (panel A) or two (panels B and C) independent experiments performed in duplicate. *, Significantly different from hormone-treated control ($P < 0.05$, panels A and C; $P < 0.01$, panel B).

(18 h) with a nonsaturating concentration (10^{-8} M) of dexamethasone, a potent synthetic GR agonist. Minimal potentiation was detected at a 10-fold higher concentration (10^{-7} M) of dexamethasone (data not shown). Pretreatment with other immunosuppressive drugs, including rapamycin (10 μM; Panel B) or cyclosporin A (10 μM; Panel C), also resulted in significant potentiation ($\sim$20-fold) of the transcriptional response mediated by a subsequent incubation with 10^{-8} M dexamethasone, but exerted little or no effect on the response detected after an incubation with 10^{-7} M dexamethasone (data not shown). Identical pretreatement with FK506 also resulted in significant potentiation ($\sim$6-fold) of the subsequent transcriptional response mediated by 5×10^{-8} M hydrocortisone as well as potentiation ($\sim$2.5-fold) of the transcriptional response mediated by 2.5×10^{-10} M triamcinolone acetonide (data not shown). In contrast, identical pretreatment with either FK506 (Fig. 2, Panel A), rapamycin or cyclosporin A (data not shown) resulted in significant suppression (2–8 fold) of the transcriptional response mediated by subsequent incubation with 10^{-7}M corticosterone. Identical pretreatment with FK506 also resulted in significant suppression of the transcriptional response mediated by a subsequent incubation with deoxycorticosterone (Fig. 2, Panel B), a weaker GR agonist that lacks a hydroxyl group at the 1 and 17 positions, or cortexolone (Fig. 2, Panel C), a GR antagonist that also functions as a weak agonist and has one hydroxyl group at position 17. In all of these experiments immunosuppressive drug-induced potentiation of a dexamethasone-mediated transcriptional response served as a positive control. The optimal concentrations of the various hormone at which these suppressive effects were detected reflects the relative affinities of these agonists for the GR. Examples of individual CAT assays that illustrate the opposite effects of FK506-pretreatment on subsequent dexamethasone- and corticosterone-mediated transcriptional responses are presented in Fig. 3.

3.2. Effects of FK506 pretreatment on whole-cell binding of agonists and subsequent nuclear translocation of GR-agonist complexes

In order to evaluate the potential effects of FK506 pretreatment on the subsequent binding of glucocorticoid agonists to intracellular GR, whole cell binding assays were performed utilizing [^{3}H]dexamethasone or [^{3}H]corticosterone. Intact LMCAT cells were again preincubated for 2 h with FK506 (10 μM) and subsequently incubated with increasing concentrations (10^{-10}–10^{-7} M) of either [^{3}H]dexamethasone or [^{3}H]corticosterone. Specific binding of tritiated hormone was then quantitated as previously described. As shown in the binding curves presented in Fig. 4 (Panel A), pretreatment with FK506 dramatically enhanced the subsequent specific binding detected at each of the designated concentrations of [^{3}H]dexamethasone. However, the fold enhancement of [^{3}H]dexamethasone binding was clearly higher at the lower concentrations of tritiated hormone (5.1-fold at 10 nM) than at the higher concentrations of tritiated hormone. These data are thus consistent with the fact that pretreatment with FK506 significantly potentiates a dexamethasone-mediated transcriptional response, but only at a nonsaturating concentration of the agonist. In contrast, pretreatment with FK506 had no discernible effect on the subsequent whole-cell specific binding of [^{3}H]cortico-

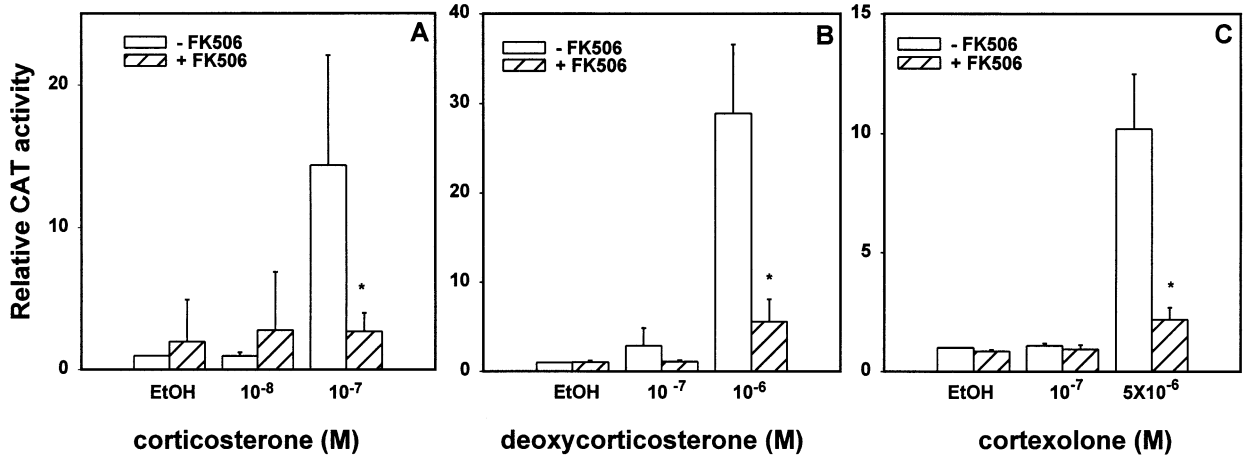

Fig. 2. Suppression of glucocorticoid agonist-mediated transcriptional activation in LMCAT cells by FK506. Following a 2 h preincubation with 10 μM FK506, the cells were incubated for 20 h at 37°C with ethanol (control) or the indicated concentrations of corticosterone (panel A), deoxycorticosterone (panel B), or cortexolone (panel C). CAT reporter gene activity was subsequently quantitated as described in Section 2. Data presented are the means $\pm$ SE for six (panel A) or three (panels B and C) independent experiments performed in duplicate. *, Significantly different from hormone-treated control ($P < 0.05$, panels A and C; $P < 0.01$, panel B).

sterone (Fig. 4, Panel B). In order to determine whether the enhanced whole cell binding of [^{3}H]dexamethasone was the result of an intracellular effect of FK506 on GR binding levels, perhaps occurring as a consequence of drug-mediated up-regulation of GR protein levels, or a more indirect effect of FK506 such as one mediated at the level of the plasma membrane, the potential effect of FK506 on the cytosolic binding of

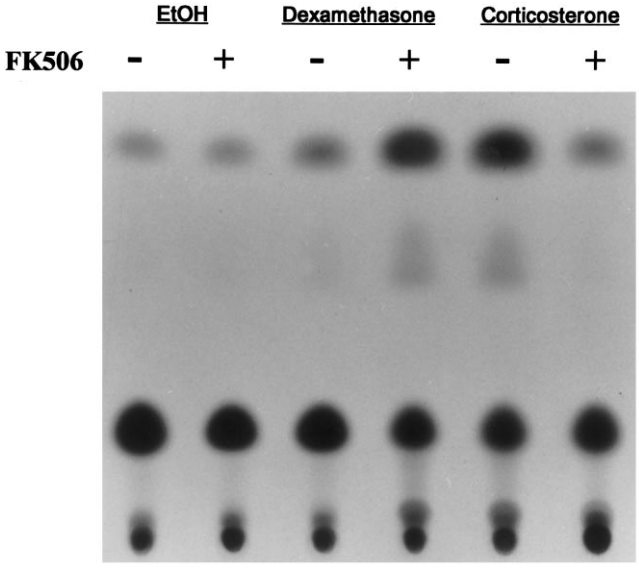

Fig. 3. Representative autoradiogram of CAT assays showing potentiation of a dexamethasone-mediated transcriptional response and suppression of a corticosterone-mediated transcriptional response following pretreatment with FK506. Cells were pretreated for 2 h with ethanol (−) or 10 μM FK506 (+). Following a 20 h incubation at 37°C with ethanol, dexamethasone (10^{-8} M), or corticosterone (10^{-7} M), CAT reporter gene assays were performed as described in Section 2.

[^{3}H]dexamethasone was evaluated. Intact cells were preincubated without or with FK506 (10 μM) for 2 h and cytosolic extracts from the two treatment groups were subsequently isolated and incubated with increasing concentrations (10^{-7}–10^{-10} M) of [^{3}H]dexamethasone. The data generated by these cytosolic binding assays (Fig. 5) indicated that FK506 pretreatment of intact cells does not elevate the subsequent specific binding levels of [^{3}H]dexamethasone if the tritiated agonist is added directly to cytosolic aliquots.

The possibility that FK506 may potentiate a dexamethasone-mediated transcriptional response by somehow driving a significantly higher proportion of cytoplasmic GR-dexamethasone complexes into the nuclei of LMCAT cells was next analyzed. Intact cells were preincubated for 2 h in the absence or presence of 10 μM FK506 and nuclear translocation assays were then performed using either 10^{-9} or 10^{-8} M [^{3}H]dexamethasone as described earlier. The data presented in Table 1 again demonstrate that FK506 pretreatment enhances [^{3}H]dexamethasone specific binding in intact cells, which is reflected in both the cytoplasmic and nuclear compartments. More specifically, using a final concentration of either 10^{-9} or 10^{-8} M [^{3}H]dexamethasone, significant increases ($\sim$8- and 6-fold, respectively) in specific nuclear binding were detected. However, although the absolute nuclear binding level increased, the percent nuclear translocation (nuclear binding expressed as a percentage of total cellular binding) was not increased by FK506 pretreatment. Nuclear translocation experiments were also performed using [^{3}H]corticosterone as the ligand. However, the relatively low binding affinity of the tritiated agonist

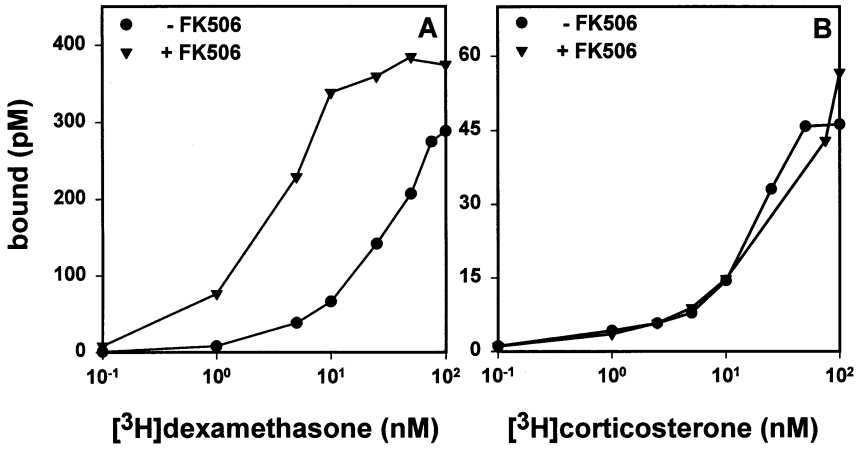

Fig. 4. Whole cell binding curves depicting the effects of preincubation with FK506. LMCAT cells were pretreated for 2 h with or without 10 μM FK506 and the subsequent whole-cell specific binding of [^{3}H]dexamethasone (panel A) and [^{3}H]corticosterone (panel B) were quantitated as described in Section 2. Each point represents the mean of duplicate binding assays and the experiment was performed twice with similar results.

combined with its high nonspecific binding precluded detection of specific nuclear binding.

3.3. Effects of pretreatment with verapamil or quinidine on glucocorticoid agonist-mediated transcriptional response

As seen in Fig. 6, pretreatment (2 h) with either 50 μM verapamil (Panel A) or 12.5 μM quinidine (Panel B), which are known inhibitors of the MDR pump, resulted in significant potentiation ($\sim$ 30- and 22-fold, respectively) of reporter gene transcription mediated by a subsequent 18 h incubation with 10^{-8} M dexamethasone, while no significant potentiation was detected at a final concentration of 10^{-7} M dexamethasone (data not shown). Pretreatment (2 h) with 50 μM verapamil also enhanced subsequent whole cell specific binding of [^{3}H]dexamethasone (Fig. 7), and as detected after a preincubation with FK506 (see Fig. 4(A)), a higher fold enhancement of specific binding was detected at the lower concentrations of tritiated agonist. Thus the effects of immunosuppressive drugs on potentiation of a dexamethasone (or hydrocortisone)-mediated transcriptional response appear to be mediated via inhibition of the MDR pump. In contrast to the effects of these channel blockers on a dexamethasone-mediated transcriptional response, identical pretreatment with either verapamil (Fig. 8, Panel A) or quinidine (Fig. 8, Panel B) had no significant effect on the transcriptional response mediated by a subsequent 18 h incubation with either 10^{-7} or 10^{-6} M corticosterone.

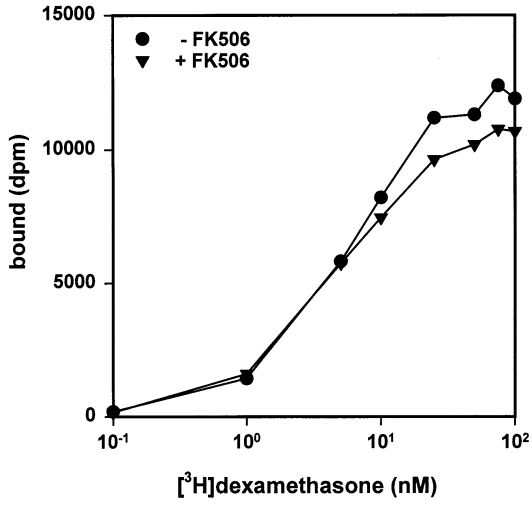

Fig. 5. Cytosolic binding curves depicting the effect of preincubation of intact LMCAT cells with FK506. LMCAT cells were pretreated for 2 h with or without 10 μM FK506 and cytosolic extracts were subsequently isolated and incubated overnight with increasing concentrations of [^{3}H]dexamethasone. Specific binding of [^{3}H]dexamethasone (dpm bound/100µl) was then quantitated using the hydroxylapatite batch assay as described in Section 2. Each point represents the mean of triplicate binding assays and the experiment was performed twice with similar results.

4. Discussion

The observation that specific immunophilins, including hsp56 and CyP-40, are associated with unliganded cytoplasmic GR-hsp90 heterocomplexes (Owens-Grillo et al., 1995) has led several laboratories to test the hypothesis that immunosuppressive drugs are capable of modulating GR functions, including transcriptional activation. Published studies have demonstrated that FK506 and rapamycin (Ning and Sanchez, 1993) as well as CsA (Renoir et al., 1995) significantly potentiate dexamethasone-induced MMTV-CAT activity in stably transfected LMCAT cells, but only when a non-saturating concentration (10^{-8} M) of agonist is used. With regard to the possible mechanism(s) by which these drugs mediate this potentiation, Ning and Sanchez

Table 1
Effects of FK506 pretreatment on nuclear binding of [^{3}H]dexamethasone-GR complexes

Concentration of [^{3}H]dex (M)	Pretreatment with FK506	Specific binding of [^{3}H]dexamethasone (dpm/10^7 cells)[a]			
		Total	Cytoplasmic	Nuclear	Percent of nuclear translocation
10^{-9}	–	3191 ± 345	1159 ± 46	1769 ± 105	55
10^{-9}	+	21 031 ± 9076 (6.6)[b]	8866 ± 563 (7.6)	12 648 ± 212 (7.1)	60
10^{-8}	–	23 054 ± 1691	8873 ± 414	15 049 ± 236	65
10^{-8}	+	110 026 ± 4023 (4.8)	47 832 ± 4965 (5.4)	61 699 ± 2040 (4.1)	56

[a] Data represent the means ± SE of triplicate determinations.
[b] Fold increase in specific binding induced by preincubation with FK506.

(1993) concluded from Western blotting experiments that FK506 enhances nuclear translocation of the GR protein in these cells. These same investigators (Ning and Sanchez, 1995) subsequently reported that in S49 lymphoma cells FK506 actually inhibits dexamethasone-induced activation of the GR to a DNA-binding form and stabilizes the unactivated GR-hsp90 heterocomplex. Using a series of CsA and FK506 analogues, including some of which are devoid of calcineurin phosphatase inhibitory activity, Renoir et al. (1995) concluded that the potentiating effects of these drugs on dexamethasone-induced gene expression in LMCAT cells do not appear to occur through a calcineurin (Ca^{2+}/calmodulin-regulated phosphatase)-mediated pathway. These data are important in light of the known abilities of these immunosuppressive drugs to inhibit T-cell proliferation via their abilities to bind another FKBP (FKBP12) and subsequently inhibit calcineurin activity (Liu et al., 1991).

In the present study the laboratory has investigated the mechanism(s) by which immunosuppressive drugs (FK506, rapamycin, and CsA) as well as channel blockers (verapamil and quinidine) modulate (potentiate or suppress) agonist-mediated transcriptional activation of CAT reporter gene activity in transfected LMCAT cells. The data presented demonstrate that pretreatment of these cells with any of these immunosuppressive drugs (Fig. 1) or channel blockers (Fig. 6) results in significant potentiation of the transcriptional response mediated by a non-saturating concentration of dexamethasone (10^{-8} M), hydrocortisone (10^{-7} M) (data not shown), and to a lesser extent, triamcinolone acetonide (2.5×10^{-10} M) (data not shown). One possible interpretation of these data is that by binding to and inhibiting an energy-dependent membrane transporter, each of these drugs is able to effectively block the efflux of these agonists and thus increase their intracellular concentrations and availability for GR binding. Both dexamethasone and hydrocortisone are steroids which contain –OH groups at both the 11 and 17 positions (see Fig. 9) and have been reported to be effectively exported out of cells by the MDR P-glycoprotein (Ueda et al., 1992; Bourgeois et al., 1993). Although triamcinolone acetonide (–OH group only at position 11) is a potent synthetic GR agonist, it is actively exported by the P-glycoprotein to a limited extent (Bourgeois et al., 1993).

In agreement with data published for L-929 cells (Kralli and Yamamoto, 1996) and consistent with this MDR pump inhibition mechanism are the facts that pretreatment of intact LMCAT cells with FK506 (Fig. 4, Panel A; Table 1) or verapamil (Fig. 7) enhanced the subsequent whole cell specific binding of [^{3}H]dexamethasone. The fact that FK506 pretreatment enhanced the [^{3}H]dexamethasone binding levels detected in both the cytoplasmic and nuclear compartments, but did not influence the percentage of specific [^{3}H]dexamethasone complexes translocated into the nucleus (Table 1), suggests that the drug-induced increase in nuclear binding was a direct consequence of increased GR activation resulting from the increased availability of the tritiated ligand within the cell. The observation that pretreatment of intact cells with FK506 failed to enhance subsequent cytosolic specific binding of [^{3}H]dexamethasone (Fig. 5) also argues that the potentiating effects of these immunosuppressants do not result from their abilities to increase cytoplasmic binding levels by potentially up-regulating GR gene expression. The facts that: verapamil blocks Ca^{2+} channels (Hondeghem and Mason, 1987); FK506 and rapamycin reverse the Ca^{2+} release channel-stabilizing effects of FKBP12 (Brillantes et al., 1994); and FK506 reduces cytosolic Ca^{2+} ion levels in liver cells (Kraus-Friedman and Feng, 1994), also raise the possibility that drug-induced alterations in intracellular Ca^{2+} ion levels might explain how these different drugs potentiate a dexamethasone-mediated transcriptional response. Several published reports have in fact implied that a decrease in intracellular Ca^{2+} concentration results in increased stability of GR (Kalimi et al., 1983; Rousseau

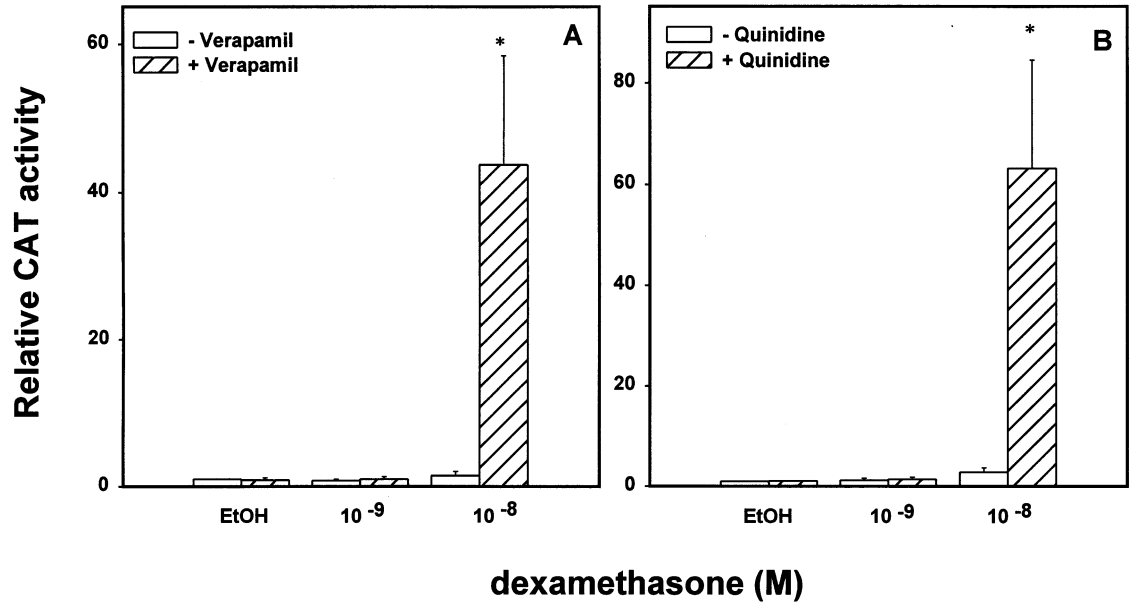

Fig. 6. Potentiation of dexamethasone-mediated transcriptional activation in LMCAT cells by verapamil or quinidine. Cells were pretreated for 2 h with either 50 μM verapamil (panel A) or 12.5 μM quinidine (panel B). Following a 20 h incubation at 37°C with ethanol (control) or the indicated concentrations of dexamethasone, CAT reporter gene activity was quantitated as described in Section 2. Data represent the means $\pm$ SE for five (panel A) or six (panel B) independent experiments performed in duplicate. *, Significantly different from hormone-treated control ($P < 0.005$, panel A; $P < 0.0005$, panel B).

and Bohemen, 1984; Bodwell et al., 1985), which theoretically could result in potentiation of an agonist-mediated transcriptional response. This potential mechanism, however, is clearly ruled out by the observation that quinidine, a known Na^+ channel blocker that is devoid of Ca^{2+} channel blocking activity (Hondeghem and Mason, 1987), is equally effective in potentiating this dexamethasone-mediated effect on CAT gene expression. The fact that both verapamil and quinidine inhibit the MDR pump (Bosch and Croop, 1996), but do not bind to immunophilins, also supports the conclusion that these drugs, like the immunosuppressive drugs, potentiate a dexamethasone-mediated transcriptional response by blocking efflux of the steroid, rather than by interacting with immunophilins such as those contained within the GR heteromeric complex.

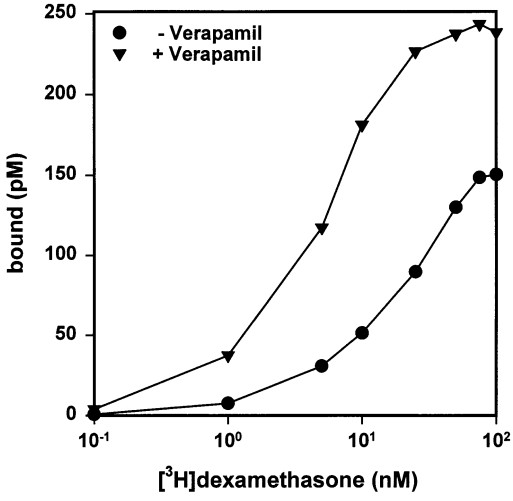

Fig. 7. Whole cell binding curves depicting the effect of preincubation with verapamil. LMCAT cells were pretreated for 2 h with or without 50 μM verapamil and the subsequent whole-cell specific binding of [^{3}H]dexamethasone was quantitated as described in Section 2. Each point represents the mean of duplicate binding assays and the experiment was performed twice with similar results.

Taken collectively these data indicate that LMCAT cells contain an FK506 (as well as rapamycin, CsA, verapamil and quinidine)-sensitive activity that reduces the intracellular accumulation of dexamethasone, hydrocortisone, and to a lesser extent, triamcinolone acetonide. The possibility that this activity may be energy-dependent is supported by the fact that L-929 cells have been reported to selectively extrude some steroids, including cortisol (hydrocortisone), via a process that is temperature- and energy-dependent (Gross et al., 1968, 1970). Thus it is logical to conclude that the potentiation observed in the present study is mediated by the MDR P-glycoprotein, which as previously mentioned is blocked by all of these immunosuppressive drugs and channel blockers. However, Kralli and Yamamoto (1996) recently used an antibody that recognizes Mdr1 and Mdr3, both of which have been shown to transport specific steroids including dexamethasone (Ueda et al., 1992; Bourgeois et al., 1993; Schinkel et al., 1995), to probe L-929 cell membranes and were unable to detect a cross-reacting protein. Since the LMCAT cell line was derived from L-929 cells it is

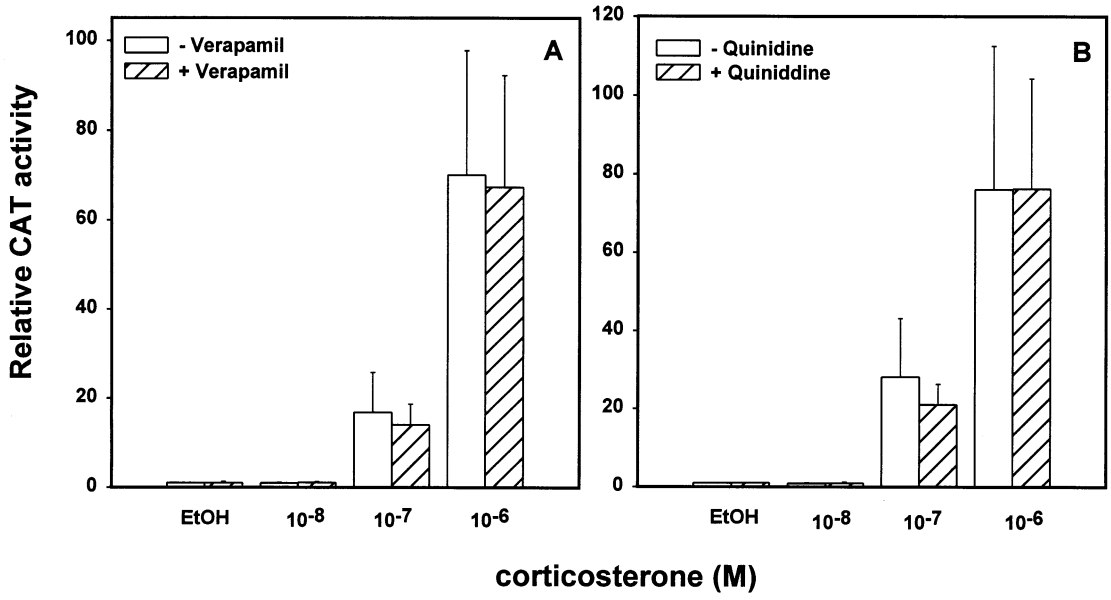

Fig. 8. Lack of an effect of preincubation with verapamil or quinidine on a subsequent corticosterone-mediated transcriptional response. Following a 2 h preincubation with either 50 μM verapamil (panel A) or 12.5 μM quinidine (panel B), LMCAT cells were incubated for 20 h at 37°C with ethanol (control) or the indicated concentrations of corticosterone. CAT reporter gene activity was subsequently quantitated as described in Section 2. Data are presented as the means $\pm$ SE of three (panel A) or six (panel B) independent experiments performed in duplicate.

possible that either: the P-glycoprotein is an efficient pump that is expressed at very low levels in both L-929 cells as well as the LMCAT cell line; expression of P-glycoprotein was induced during the stable transfection of L-929 cells to generate the LMCAT cell line; or that the potentiation observed in LMCAT cells is mediated by another distinct member of the ABC transporter superfamily that is not recognized by this anti-P-glycoprotein antibody. With regard to the last possibility, Abe et al. (1995) have reported that the MDR phenotype can occur in human tumors that overexpress the non-P-glycoprotein-mediated MDR gene, *MRP*. This gene encodes a 190 kDa membrane-associated ATP-binding protein that has minor sequence homology with P-glycoprotein but is also inhibited by verapamil (Abe et al., 1995).

The data presented here also demonstrate for the first time that pretreatment of LMCAT cells with specific immunosuppressive drugs results in significant suppression of the subsequent transcriptional activation mediated by GR agonists that include corticosterone (Fig. 2, Panel A), deoxycorticosterone (Panel B) or cortexolone (Panel C). It is not obvious why a previously published study detected no effect (potentiating or suppressive) of FK506 on a deoxycorticosterone-mediated transcriptional response in transiently transfected L-929 cells (Kralli and Yamamoto, 1996). One possibility is that a low concentration of this hormone was tested to evaluate the possibility of an FK506 potentiating effect and that a suppressive effect would not be detected at that hormone concentration. Although in the present study the precise mechanism(s) underlying this suppressive effect has not been fully identified, certain basic conclusions can be drawn from the data. First, it appears unlikely that inhibition of the MDR P-glycoprotein or another related membrane transporter with similar drug sensitivity is responsible for the observed suppression. Neither verapamil nor quinidine pretreatment resulted in subsequent suppression of a corticosterone-mediated response (Fig. 8, panel A and B), despite the fact that both of these ion channel blockers are effective inhibitors of the MDR pump. Secondly, although pretreatment with FK506 resulted in suppression of a transcriptional response mediated by deoxycorticosterone, this steroid lacks –OH groups at both the 11 and 17 positions (Fig. 9) and does not appear to be extruded from cells via the pump (Ford and Hait, 1990). Corticosterone (–OH group only at position 11) and cortexolone (–OH group only at position 17) have also been reported to be extruded from cells, but only to a very limited extent. Finally, pretreatment with FK506 has no effect on the subsequent whole-cell specific binding level of [^{3}H]corticosterone (Fig. 4, Panel B). Thus FK506 pretreatment does not appear to affect the intracellular availability of corticosterone for binding to the GR. Finally, it is important to point out that although an immunosuppressive drug has been previously reported to suppress a progesterone receptor-mediated transcriptional response in L cells transfected with an MMTV-luciferase reporter gene construct (Milad et al., 1995), this response was clearly drug-specific, and hence quite distinct from the agonist-specific responses reported here. Although Milad et al. (1995) found that cyclosporin A suppressed the transcriptional

response mediated by R5020, FK506 had no detectable effect on this response and rapamycin significantly potentiated the agonist-mediated transcriptional response. Because of these variable results obtained with different immunosuppressive drugs the authors of that study were unable to draw any firm conclusions concerning underlying mechanisms.

Taken collectively, the data generated by the present study suggest that a mechanism(s) distinct from MDR pump inhibition may be responsible for the observed suppression. One possibility is that binding of an immunosuppressive agent like FK506 to a GR-associated immunophilin may be responsible for the subsequent suppression of agonist-mediated transcription. Although binding of FK506 to the receptor-associated FKBP59 may not alter (increase or decrease) binding of an agonist such as corticosterone, it may induce a subtle conformational change and interfere with a subsequent step in the GR pathway such as: agonist-induced dissociation of hsp90 and activation of the GR to a DNA-binding form; translocation of activated GR complexes into the nucleus; or binding of activated GR complexes to GREs upstream of target genes. Whether the response mediated by a specific GR agonist is potentiated or suppressed by preincubation with an immunosuppressive drug may reflect which mechanism predominates. For instance, dexamethasone is actively extruded by the MDR pump or related transporter and inhibition of this pump with any one of these drugs increases the intracellular concentration of this hormone and results in potentiation of its transcriptional response. In this situation the potentiating mechanism may mask or override any type of suppressive mechanism that may be the consequence of simultaneous binding of the drug to a GR-associated immunophilin. In contrast, deoxycorticosterone is not extruded from the cell by the pump and therefore inhibition of the pump fails to result in the potentiation of a deoxycorticosterone-mediated response. In this situation the lack of potentiation may facilitate detection of the suppressive effect mediated by preincubation with an immunosuppressive drug. Analysis of the effects of pretreatment with these drugs on the subsequent transcriptional responses mediated by additional GR agonists will contribute to the understanding of how structural features of the GR agonist dictate which of these mechanisms will predominate. Preliminary results (data not shown) indicate that progesterone (lacks –OH groups at positions 11 and 17) functions as a weak GR agonist in LMCAT cells and that transcriptional activation mediated by this steroid can also be significantly suppressed by a preincubation with FK506. These data are intriguing in light of the previously cited report that showed that progesterone binds tightly to the human P-glycoprotein but is not actively transported from cells (Ueda et al., 1992). Once again, in the absence of potentiation the suppressive mechanism appears to be manifested in LMCAT cells. In conclusion, the data presented here demonstrate that the potentiating and suppressive effects of immunosuppressive drugs on a glucocorticoid-mediated transcriptional response in stably transfected L-929 cells are agonist-specific and most likely mediated by different mechanisms.

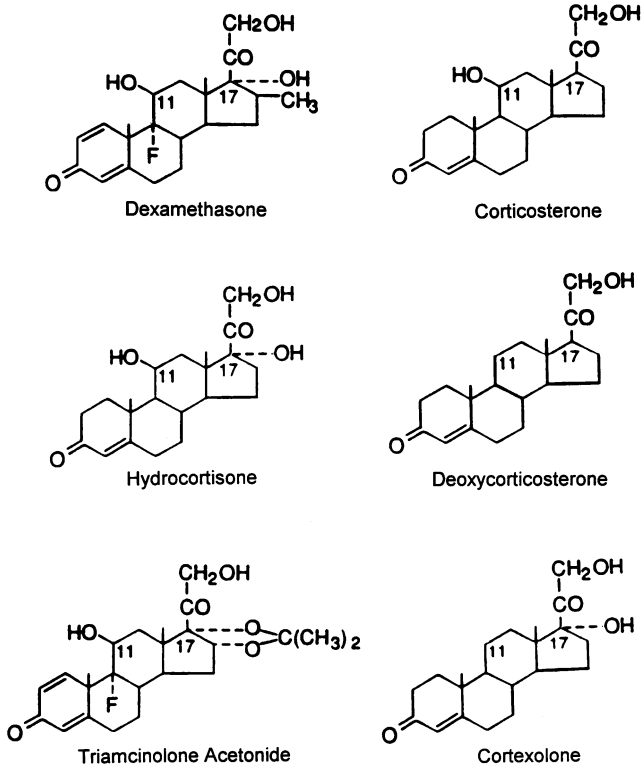

Fig. 9. Structures of glucocorticoid agonists. The transcriptional responses mediated in LMCAT cells by the agonists shown in the column on the left are all potentiated (decreasing order from top to bottom) by preincubation with an immunosuppressive drug while the transcriptional responses mediated by the agonists shown in the column on the right are all suppressed to similar extents by preincubation with an immunosuppressive drug.

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References

Abe, T., Koike, K., Ohga, T., et al., 1995. Chemosensitisation of spontaneous multidrug resistance by a 1,4-dihydropyridine analogue and verapamil in human glioma cell lines overexpressing MRP or MDR1. Br. J. Cancer 72, 418–423.

Akiyama, S.-I., Cornwell, M.M., Kuwano, M., Pastan, I., Gottesman, M.M., 1988. Most drugs that reverse multidrug resistance also inhibit photoaffinity labeling of P-glycoprotein by a vinblastine analog. Mol. Pharmacol. 33, 144–147.

Argentin, S., Sun, Y.L., Lihrmann, I., Schmidt, T.J., Drouin, J., Nemer, M., 1991. Distal cis-acting promoter sequences mediate glucocorticoid stimulation of cardiac atrial natriuretic factor gene transcription. J. Biol. Chem. 266, 23315–23322.

Beato, M., 1989. Gene regulation by steroid hormones. Cell 56, 335–344.

Bodwell, J.E., Holbrook, N.J., Munck, A., 1985. Stabilization of thymic glucocorticoid-receptor complexes by the calcium-activated protease inhibitor, calpastatin. J. Biol. Chem. 260, 2601–2604.

Bosch, I., Croop, J., 1996. P-glycoprotein multidrug resistance and cancer. Biochim. Biophys. Acta 1288, F37–F54.

Bourgeois, S., Gruol, D.J., Newby, R.F., Rajah, F.M., 1993. Expression of an mdr gene is associated with a new form of resistance to dexamethasone-induced apoptosis. Mol. Endocrinol. 7, 840–851.

Brillantes, A.-M.B., Ondrias, K., Scott, A., et al., 1994. Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. Cell 77, 513–523.

Callebaut, I., Renoir, J.-M., Lebeau, M.C., et al., 1992. An immunophilin that binds M(r) 90000 heat shock protein: main structural features of a mammalian p59 protein. Proc. Natl. Acad. Sci. USA 89, 6270–6274.

Cornwell, M.M., Pastan, I., Gottesman, M.M., 1987. Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. J. Biol. Chem. 262, 2166–2170.

Czar, M.J., Lyons, R.H., Welsh, M.J., Renior, J.-M., Pratt, W.B., 1995. Evidence that the FK506-binding immunophilin heat shock protein 56 is required for trafficking of the glucocorticoid receptor from the cytoplasm to the nucleus. Mol. Endocrinol. 9, 1549–1560.

Dittmar, K.D., Pratt, W.B., 1997. Folding of the glucocorticoid receptor by the reconstituted hsp90-based chaperone machinery. J. Biol. Chem. 272, 13047–13054.

Dittmar, K.D., Hutchison, K.A., Owens-Grillo, J.K., Pratt, W.B., 1996. Reconstitution of the steroid- receptor hsp90 heterocomplex assembly system of the rabbit reticulocyte lysate. J. Biol. Chem. 271, 12833–12839.

Drouin, J., Sun, Y.L., Tremblay, S., et al., 1992. Homodimer formation is rate-limiting for high affinity DNA binding by glucocorticoid receptor. Mol. Endocrinol. 6, 1299–1309.

Felgner, P.L., Gadek, T.R., Holm, M., et al., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA 84, 7413–7417.

Fojo, A.T., Shen, D.W., Mickley, L.A., Pastan, I., Gottesman, M.M., 1987. Intrinsic drug resistance in human kidney cancer is associated with expression of a human multidrug-resistance gene. J. Clin. Oncol. 5, 1922–1927.

Ford, J.M., Hait, W.N., 1990. Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol. Rev. 42, 155–199.

Gorman, C.M., Moffat, L.F., Howard, B.H., 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Mol. Cell. Biol. 2, 1044–1051.

Gottesman, M.M., Pastan, I., 1988. The multidrug transporter, a double-edged sword. J. Biol. Chem. 263, 12163–12166.

Gross, S.R., Aronow, L., Pratt, W.B., 1968. The active transport of cortisol by mouse fibroblasts growing in vitro. Biochem. Biophys. Res. Commun. 32, 66–72.

Gross, S.R., Aronow, L., Pratt, W.B., 1970. The outward transport of cortisol by mammalian cells in vitro. J. Cell Biol. 44, 103–114.

Hondeghem, L.M., Mason, J.W., 1987. Agents used in cardiac arrhythmias. In: Katzung, B.G. (Ed.), Basic and Clinical Pharmacology, 3rd Edition. Appleton and Lange, East Norwalk, CT, pp. 151–168.

Hoof, T., Demmer, A., Christians, U., Tummler, B., 1993. Reversal of multidrug resistance in Chinese hamster ovary cells by the immunosuppressive agent rapamycin. Eur. J. Pharmacol. 246, 53–58.

Howard, K.J., Holley, S.J., Yamamoto, K.R., Distelhorst, C.W., 1990. Mapping the HSP90 binding region of the glucocorticoid receptor. J. Biol. Chem. 265, 11928–11935.

Hutchison, K.A., Scherrer, L.C., Czar, M.J., et al., 1993. FK506 binding to the 56-kilodalton immunophilin (Hsp56) in the glucocorticoid receptor heterocomplex has no effect on receptor folding or function. Biochemistry 32, 3953–3957.

Hutchison, K.A., Stancato, L.F., Owens-Grillo, J.K., et al., 1997. The 23 kDa acidic protein in reticulocyte lysate is the weakly bound component of the hsp90 foldosome that is required for assembly of the glucocorticoid receptor into a functional hetero-complex with hsp90. J. Biol. Chem. 270, 18841–18847.

Johnson, J.L., Toft, D.O., 1994. A novel chaperone complex for steroid receptors involving heat shock proteins, immunophilins, and p23. J. Biol. Chem. 269, 24989–24993.

Kalimi, M., Hubbard, J., Ray, A., 1983. The effect of calcium on the hepatic glucocorticoid receptor. J. Steroid Biochem. Mol. Biol. 18, 665–671.

Kessel, D., Wilderding, C., 1984. Mode of action of calcium antagonists which alter anthracycline resistance. Biochem. Pharmacol. 33, 1157–1160.

Kieffer, L.J., Thalhammer, T., Handschumacher, R.E., 1992. Isolation and characterization of a 40 kDa cyclophilin-related protein. J. Biol. Chem. 267, 5503–5507.

Kralli, A., Yamamoto, K.R., 1996. An FK506-sensitive transporter selectively decreases intracellular levels and potency of steroid hormones. J. Biol. Chem. 271, 17152–17156.

Kraus-Friedman, N., Feng, L., 1994. Reduction of ryanodine binding and cytosolic Ca^{2+} levels in liver by the immunosuppressant FK506. Biochem. Pharmacol. 48, 2157–2162.

Liu, J., Farmer, J., Lane, W., Friedman, J., Weissman, I., Schreiber, S., 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 66, 807–815.

Majors, J., Varmus, H.E., 1983. A small region of the mouse mammary tumor virus long terminal repeat confers glucocorticoid hormone regulation on a linked heterologous gene. Proc. Natl. Acad. Sci. USA 80, 5866–5870.

Milad, M., Sullivan, W., Diehl, E., et al., 1995. Interaction of the progesterone receptor with the binding proteins for FK506 and cyclosporin A. Mol. Endocrinol. 9, 838–847.

Nemoto, T., Ohara-Nemoto, Y., Denis, M., Gustafsson, J.-A., 1990. The transformed glucocorticoid receptor has a lower steroid-binding affinity than the nontransformed receptor. Biochemistry 29, 1880–1886.

Ning, Y.-M., Sanchez, E.R., 1993. Potentiation of glucocorticoid receptor-mediated gene expression by the immunophilin ligands FK506 and rapamycin. J. Biol. Chem. 268, 6073–6076.

Ning, Y.-M., Sanchez, E.R., 1995. Stabilization in vitro of the untransformed glucocorticoid receptor complex of S49 lymphocytes by the immunophilin ligand FK506. J. Steroid Biochem. Mol. Biol. 52, 187–194.

Owens-Grillo, J.K., Hoffmann, K., Hutchison, K.A., et al., 1995. The cyclosporin A-binding immunophilin CyP-40 and the FK506-binding immunophilin hsp56 bind to a common site on hsp90 and

exist in independent cytosolic heterocomplexes with the untransformed glucocorticoid receptor. J. Biol. Chem. 270, 20479–20484.

Peattie, D.A., Harding, M.W., Fleming, M.A., et al., 1992. Expression and characterization of human FKBP52, an immunophilin that associates with the 90 kDa heat shock protein and is a component of steroid receptor complexes. Proc. Natl. Acad. Sci. USA 89, 10974–10978.

Renoir, J.-M., Radanyi, C., Jung-Testas, I., Farber, L.E., Baulieu, E.-E., 1990. The nonactivated progesterone receptor is a nuclear heterooligomer. J. Biol. Chem. 265, 14402–14406.

Renoir, J.-M., Mercier-Bodard, C., Hoffman, K., et al., 1995. Cyclosporin A potentiates the dexamethasone-induced mouse mammary tumor virus-chloramphenicol acetyltransferase activity in LMCAT cells: a possible role for different heat shock protein-binding immunophilins in glucocorticosteroid receptor-mediated gene expression. Proc. Natl. Acad. Sci. USA 92, 4977–4981.

Rousseau, G.G., Bohemen, C.G., 1984. Effects of membrane-active compounds on dexamethasone binding to the glucocorticoid receptor in intact cells. J. Steroid Biochem. Mol. Biol. 20, 37–41.

Sanchez, E.R., Meshinchi, S., Tienrungoj, W., Schlesinger, M.J., Toft, D.O., Pratt, W.B., 1987. Relationship of the 90 kDa murine heat shock protein to the untransformed and transformed states of the L cell glucocorticoid receptor. J. Biol. Chem. 262, 6988–6991.

Sanchez, E.R., Hu, J.-L., Ning, Y.-M., 1995. Potentiation of glucocorticoid receptor transactivation by immunophilin ligands and heat shock. In: Gametchu, B. (Ed.), Glucocorticoid Receptor Structure and Leukemic Cell Responses. R.G. Landes, Austin, TX, pp. 105–123.

Sanchez, E.R., 1990. Hsp56: a novel heat shock protein associated with untransformed steroid receptor complexes. J. Biol. Chem. 265, 22067–22070.

Schinkel, A.H., Wagenaar, E., van Deemter, L., Borst, P., 1995. Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. J. Clin. Invest. 96, 1698–1705.

Schmidt, T.J., Litwack, G., 1982. Activation of the glucocorticoid-receptor complex. Physiol. Rev. 62, 1131–1192.

Schmidt, T.J., Miller-Diener, H., Webb, M.L., Litwack, G.L., 1985. Thermal activation of the purified rat hepatic glucocorticoid receptor. Evidence for a two-step mechanism. J. Biol. Chem. 260, 16255–16262.

Sugawara, I., Kataoka, I., Morishita, Y., et al., 1988. Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16. Cancer Res. 48, 1926–1929.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I., Willingham, M.C., 1987. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc. Natl. Acad. Sci. USA 84, 7735–7738.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I., Willingham, M.C., 1989. Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: evidence for localization in brain capillaries and crossreactivity of one antibody with a muscle protein. J. Histochem Cytochem. 37, 159–164.

Thompson, E. B., 1995. Steroid hormones. Membrane transporters of steroid hormones. Curr. Biol. 5, 730–732.

Ueda, K., Okamura, N., Hirai, M., et al., 1992. Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. J. Biol. Chem. 267, 24248–24252.

Yamamoto, K.R., 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. 19, 209–252.

Yang, C.-P.H., DePinho, S.G., Greenberger, L.M., Arceci, R.J., Howitz, S.B., 1989. Progesterone interacts with P-glycoprotein in multidrug-resistant cells and in the endometrium of gravid uterus. J. Biol. Chem. 264, 782–788.