In Vivo Evidence for the Generation of a Glucocorticoid Receptor-Heat Shock Protein-90 Complex Incapable of Binding Hormone by the Calmodulin Antagonist Phenoxybenzamine

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The glucocorticoid receptor (GR) is a ligand-regulated transcription factor whose ability to bind hormone is thought to be dependent on association with the 90-kDa heat shock protein (hsp90). In the present study, we have generated a novel form of the GR, in which the receptor remains complexed to hsp90 but has lost its ability to bind hormone, by treatment of intact cells with the calmodulin (CaM) antagonist phenoxybenzamine (POBA). Treatment of these cells, mouse L929 cells stably transfected with the mouse mammary tumor virus-chloramphenicol acetyltransferase (MMTV-CAT) reporter construct, with increasing concentrations of POBA resulted in a concentration-dependent inhibition of dexamethasone (Dex)-induced CAT gene expression, with 100 μM POBA resulting in approximately 80% inhibition. This inhibitory effect of POBA was markedly reduced if POBA was added after a short incubation with Dex, suggesting that the primary effect of POBA was on hormone-induced transformation of the GR. Using a subcellular fractionation technique, POBA inhibition of CAT gene expression was found to correlate with an inhibition of Dex-induced GR nuclear translocation. However, inhibition of translocation was not the primary effect of POBA on the GR signal pathway, as POBA was found to reduce GR hormone-binding capacity after treatment of intact cells. The inhibitory effect of POBA on hormone-binding function correlated closely with the inhibitory effect of this drug on CAT gene expression and was not due to an oxidation of sulfhydryl groups, a condition known to reduce GR hormone-binding capacity. Incubation of cytosols from untreated cells with POBA did not decrease GR steroid-binding capacity, demonstrating that this inhibitory effect was not the result of a competitive antagonism at the ligand-binding site. Quantitation of GR protein in the cytosols of POBA-treated cells revealed that the decrease in steroid-binding function was not due to a loss of GR protein. Surprisingly, the amount of GR-bound hsp90 was also unaltered in response to POBA. Taken together, the above observations provide evidence for a novel state of the GR within intact cells in which hsp90 interaction is but one step in the generation or maintenance of hormone-competent receptors. In addition, these results point to the potential use of POBA, and possibly other CaM inhibitors, as antagonists of steroid receptor actions. (Molecular Endocrinology 10: 14–23, 1996)

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INTRODUCTION

The signal transduction pathway of steroid hormones can be divided into two major stages based on the functional state of the receptor protein. In the untransformed state, steroid receptors exist as large heteromeric complexes containing one molecule of receptor and two molecules of 90-kDa heat shock protein (hsp90); the accumulated evidence suggests that the receptor in this complex is fully competent for hormone-binding function (see Refs. 1 and 2 for review). In the transformed state, steroid receptors have dissociated from hsp90 in response to hormone and are able to bind DNA and enhance specific gene expression (1, 2). Thus, hsp90 appears to be a central component in the signal pathway of steroid hormones.

With regard to the glucocorticoid receptor (GR), a variety of reports have demonstrated a role for hsp90 in maintaining this receptor in the non-DNA-binding, hormone-competent state. The earliest evidence of this kind occurred when the laboratories of Pratt (3, 4) and Gustafsson (5) demonstrated a correlation between loss of GR-bound hsp90 and loss of hormone-binding function for GRs present in cytosolic preparations. Since then, *in vitro* translation systems have

been used by the same laboratories to show that hsp90 binding to the GR during the terminal stages of GR translation is required to generate the high affinity, steroid-binding state of the receptor (6, 7). The reticulocyte lysate systems employed in these studies were further exploited to demonstrate reconstitution of GR-hsp90 complexes (8). This reassociation of GR with hsp90 was shown to be a temperature-dependent process, resulting in the complete restitution of receptor hormone-binding function as well as inactivation of its DNA-binding function. Similar reconstitutions of progesterone receptor-hsp90 complexes have been demonstrated by the laboratories of Toft (9, 10) and Smith (11).

In contrast to the *in vitro* results described above, very little *in vivo* evidence exists for the involvement of hsp90 in the hormone-binding function of steroid receptors. A variety of papers describing the effects of glucocorticoid (12), progesterone (13), and estrogen (14) receptor mutations have shown a correlation between the loss of the hsp90-binding regions of these receptors and the acquisition of constitutive DNA-binding and transactivation functions. However, as these mutations also destroyed the hormone-binding function of the receptors, a direct effect of hsp90 on this function could not be proven. More convincing evidence was provided by Picard and co-workers when it was shown that expression of GR in yeast cells producing very low levels of hsp90 resulted in GR with a greatly reduced ability to bind hormone (15).

This relative lack of *in vivo* data on hsp90 and steroid-binding function has been due in large part to a lack of drugs or ligands, other than steroid hormones, that can directly or indirectly affect receptor-hsp90 interactions. However, a series of recent publications demonstrating structural and/or functional interactions between calmodulin (CaM) and hsp90 or between CaM and steroid receptors may now provide a novel basis by which to modify the intracellular interactions of receptors and hsp90. For example, direct Ca^{2+}-dependent binding to CaM by hsp90 has been demonstrated (16, 17) as well as the inhibition of hsp90 binding to actin filaments by CaM (18). These studies suggested that CaM may be involved in steroid receptor functions, perhaps through an interaction of CaM with receptor-associated hsp90. Several observations lend support to this hypothesis. Yahara and co-workers have shown that in addition to inhibition of hsp90 binding to actin filaments (18), CaM can also inhibit actin binding by GR-hsp90 complexes (19). A variety of investigators studying the estrogen receptor have shown Ca^{2+}-dependent binding of CaM to the estrogen receptor (20, 21) as well as CaM-dependent phosphorylation of this receptor (22). A report by McConkey *et al.* (23) has provided evidence for the involvement of CaM in glucocorticoid-induced apoptosis in lymphocytes, and this observation was expanded upon by Dowd *et al.* (24) when they demonstrated that apoptosis in lymphocytes occurs through a mechanism involving GR control of CaM gene expression. In the latter study, treatment of cells with a CaM antagonist was found to inhibit glucocorticoid-induced cell death, providing further evidence for CaM involvement in this process.

At a more fundamental level, the above results suggest the possibility that functional CaM is a necessary factor in the general activation of the glucocorticoid signal pathway. We have previously pursued this question by testing the effects of various CaM antagonists on GR-mediated gene expression (25). We found that treatment of mouse L929 cells with any of four unrelated CaM antagonists resulted in inhibition of dexamethasone (Dex)-induced expression of a stably transfected reporter gene [mouse mammary tumor virus-chloramphenicol acetyltransferase (MMTV-CAT)]. In the present study, we have explored the mechanism of action by which one of these CaM antagonists, phenoxybenzamine, inhibits activation of the GR *in vivo*. We demonstrate that the primary effect of this drug on the receptor is to inactivate the hormone-binding function of the GR. Surprisingly, this inactivation of hormone-binding function results in GR that is still complexed to hsp90, providing *in vivo* evidence that hsp90 association with the GR is not the only requirement in the generation or maintenance of mature GR complexes capable of binding hormone.

RESULTS

Phenoxybenzamine (POBA) Inhibition of GR Transactivation Occurs before Hormone-Mediated Transformation of the GR

As mentioned above, we have previously assessed the effects of various CaM antagonists on Dex-induced CAT gene expression in an L929 cell line (LMCAT) stably transfected with the MMTV-CAT reporter construct (25). We found dose-dependent inhibition of this GR function by POBA, trifluoperazine (TFP), compound 48/80, and the naphthalenesulfonamide, W7. In these experiments, we treated LMCAT cells for 2 h with CaM antagonist before the addition of 1 μM Dex and subsequent incubation for an additional 20 h. As an initial step in identifying the stage of the GR signal pathway affected by these antagonists, we tested the idea that POBA may be acting by inhibiting hormone-induced transformation of the GR. In the experiments shown in Fig. 1, LMCAT cells were treated with 1 μM Dex for 2 h before the addition of POBA and subsequent incubation for 20 h. As we have previously shown that a 2-h treatment of LMCAT cells with 1 μM Dex results in near-complete nuclear translocation of the GR (26), addition of POBA after this step should not result in inhibition of GR-induced transcription activity if the primary effect of the CaM antagonist occurs at an earlier stage in the GR activation process. From the results obtained, it is clear that POBA inhibition of GR-mediated CAT gene expression in the LMCAT cells is not as effective when this drug is given after

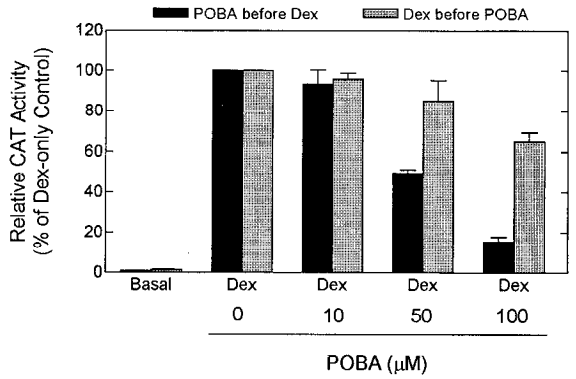

Fig. 1. POBA Inhibition of GR Transactivation Occurs before Acquisition of GR Transcription Enhancement Function

Replicate flasks of LMCAT cells were left untreated (basal) or were pretreated either with the indicated concentrations of POBA (■) for 2 h or with 1 μM Dex for 2 h (▨). After pretreatment, the cells were incubated with 1 μM Dex (■) or with the indicated concentrations of POBA (▨) for an additional 20 h. CAT activities were determined and expressed as percentages of the 1 μM Dex-only control. The data are the mean ± SEM of five independent experiments.

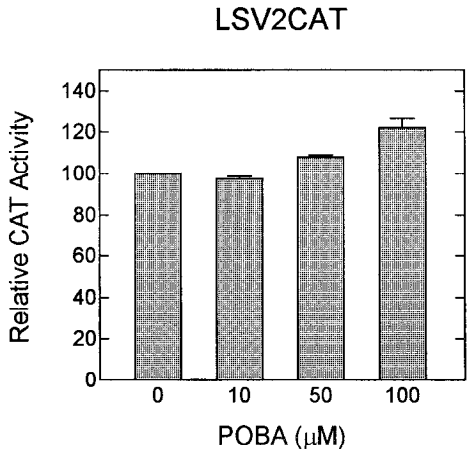

Fig. 2. POBA Has No Effect on CAT Enzyme Levels in LSVCAT Cells

Replicate flasks of LSVCAT cells were untreated or treated with the indicated concentrations of POBA for 22 h. CAT activity was assayed and expressed as a percentage of the untreated control value. The data are the mean ± SEM of four independent experiments.

hormone treatment. These results suggest that the inhibitory effect of POBA occurs primarily during the transformation process and not at the level of transcription, translation, or protein (CAT) stability.

As a further test of the above hypothesis, we measured the effects of POBA on L929 cells stably transfected with the pCAT-control reporter (LSVCAT cells), in which constitutive expression of CAT is controlled by the simian virus 40 (SV40) early promoter. The results of these experiments are shown in Fig. 2, and they demonstrate no reduction in CAT gene expression in response to POBA treatment. Thus, it appears unlikely that the actions of POBA on CAT gene expression in LMCAT cells are the result of a decrease in general gene transcription or effects on posttranscriptional events.

The results presented in Figs. 1 and 2 suggest that the inhibitory effect of POBA on the GR occurs at a stage before the activation of transcriptional enhancement activity. One way in which this could happen is by preventing the nuclear translocation and/or DNA-binding functions of the GR. We have tested this possibility by measuring the amounts of cytosolic and nuclear GR in cells treated with various combinations of Dex and POBA (Fig. 3). The results show a POBA concentration-dependent decrease in Dex-induced nuclear translocation of the GR.

POBA Inhibition of GR Transactivation Results from Loss of Hormone-Binding Function

Although the findings presented in Fig. 3 implicate the translocation process as a potential target stage for the effects of POBA, it remained possible that an effect of POBA on GR hormone-binding function could have the same result. For this reason, we performed the experiments shown in Fig. 4A, in which steroid-binding capacities were measured in cytosols derived from LMCAT cells treated with increasing concentrations of POBA. From the results obtained, it is clear that treatment with POBA can result in a large decrease in the hormone-binding function of these cells, and that the magnitude of this decrease approximates the POBA-induced decreases seen in GR translocation (Fig. 3) and CAT gene expression (Fig. 1). We have also observed decreases in hormone binding after treatment of these cells with TFP, compound 48/80, and calmidzolium (data not shown). Given that TFP and compound 48/80 both decreased Dex-induced CAT expression in LMCAT cells (25), it is likely that the primary effect of these CaM antagonists on the GR signal pathway is to inhibit the ability of GR to bind hormone.

As it is well known that reduced sulfhydryl groups are required for a variety of GR functions, including steroid-binding activity (27), and that an endogenous NADPH-dependent, thioredoxin-mediated, thiol-disulfide exchange system exists that is responsible for maintaining the rat liver GR in the steroid-binding state (28), we tested the possibility that the inhibitory effect of POBA on GR steroid-binding function was the result of sulfhydryl group oxidation. In the experiments shown in Fig. 4B, LMCAT cells were treated with POBA, and cytosols were prepared in the presence or absence of dithiothreitol (DTT), an agent capable of reducing disulfide bridges within the GR generated by a variety of oxidizing agents (27). Cytosols made in this fashion were measured for steroid-binding capacities, and the results demonstrate that DTT was not capable of reversing the inhibitory effects of POBA on GR steroid-binding function. Although these results indicate that sulfhydryl oxidation is not the mechanism by

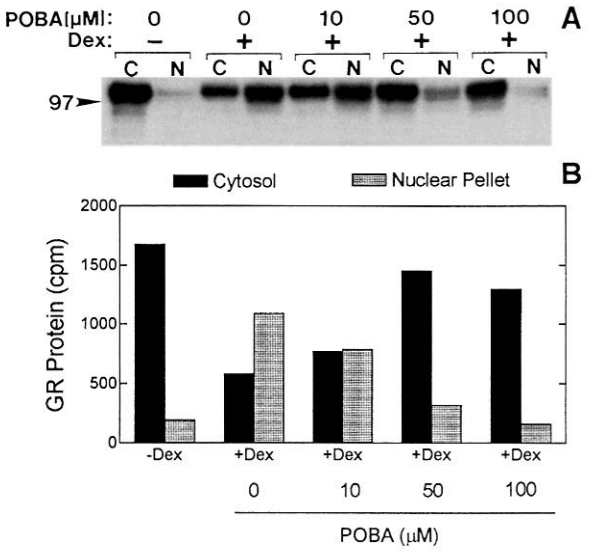

Fig. 3. POBA Inhibits Dex-Induced GR Translocation to the Nucleus

Replicate flasks of LMCAT cells were pretreated with POBA at the indicated concentrations for 2 h. After pretreatment, the cells were either left untreated or treated with 1 μM Dex for an additional 2 h. Cytosolic (C) and nuclear pellet (N) fractions were obtained by Dounce homogenization, and each fraction was extracted with 0.5 M NaCl and immunoadsorbed with BuGR2 antibody against GR. Relative amounts of GR protein were measured by quantitative Western blotting using the BuGR2 antibody as probe and both peroxidase- and ^{125}I-conjugated counter antibodies as described in *Materials and Methods*. A, Autoradiogram of a typical Western blot. B, Quantitation of GR protein in the cytosolic and nuclear fractions.

which POBA inhibits GR steroid-binding function, the possibility still remains that POBA-induced sulfhydryl oxidation is occurring and that it leads to GR misfolding that cannot be corrected by reducing agents acting under energy-deficient (4 C) conditions.

Interestingly, hormone-binding capacity was unaffected when cytosol from untreated LMCAT cells was directly treated with POBA (Fig. 4C). The latter result has two important implications. First, the inability of POBA to inhibit steroid-binding under *in vitro* conditions eliminates the possibility that the actions of POBA are due to a competitive antagonism at the hormone-binding site of the GR. Second, the ineffectiveness of POBA *in vitro* suggests that its actions *in vivo* are to inhibit a dynamic equilibrium within the intact cell that is responsible for either the maintenance or the generation of the hormone-binding competent GR.

The results of saturation hormone binding experiments are presented in Fig. 5. In these experiments, cytosols from LMCAT cells treated with 100 μM POBA were assayed for steroid-binding function using increasing concentrations of [^{3}H]triamcinolone acetonide. The results show a large decrease in the number of steroid-binding sites (B_{max}) after POBA treatment (B_{max} = 21.6 fmol GR/mg protein) compared with that in control cells (B_{max} = 163.3). In contrast, the dissociation constant was slightly reduced by the POBA treatment (K_d = 1.5 nM) compared with the control value (K_d = 3.1 nM), indicating a slight increase in the hormone binding affinity for the GR of POBA-treated cells. Overall, the major effect of POBA on GR hormone-binding function is to reduce the number of binding sites, either by inactivating this GR function or by causing a loss of GR protein.

POBA Inhibition of Hormone-Binding Function Is Not the Result of Loss of GR Protein or GR-Associated hsp90

Although little is known about the intracellular processes that control the hormone-binding functions of the GR, there were two alternative possibilities that could be tested to explain the inhibitory effects of POBA on GR hormone-binding function. One possibility was that POBA treatment of the LMCAT cells was somehow promoting GR turnover, resulting in a decrease in GR protein levels. However, the results presented in Fig. 3 clearly show that the amount of GR protein in cytosols derived from POBA-treated cells is unchanged compared with that in control cells, proving that the loss of GR hormone-binding function in response to POBA is not due to the loss of GR protein. The second possibility was that GR levels remained unchanged, but that POBA treatment resulted in a structure/function change in the GR or GR complex components. Given the large body of *in vitro* evidence for the role of hsp90 in maintaining the GR in the hormone-binding competent state (3–8), we reasoned that this structural change in the GR could take the form of a destabilization of the GR-hsp90 complex. To test this possibility, we performed the experiments shown in Fig. 6, in which levels of GR-associated hsp90 were measured in cells treated with or without POBA. To our surprise, the amount of GR-associated hsp90 was also unchanged in the POBA-treated cells, suggesting that the CaM antagonist was not acting by causing a loss of hsp90 from the untransformed GR complex or by preventing association of hsp90 with GR during *in vivo* assembly.

The anti-hsp90 serum used for probing of the Western blots in the experiment presented in Fig. 6 recognizes hsp70 in addition to hsp90. In keeping with our prior experience with the GR complex of L929 cells (29), very little, if any, hsp70 was found specifically bound to the untransformed GR complex, and the amount of GR-bound hsp70 did not change in response to POBA treatment (quantitative data not shown). We have also analyzed these samples for the presence of GR-associated hsp56 using a polyclonal antibody, but no change in the GR-hsp56 ratio was observed after POBA treatment (Fig. 6). Taken as a whole, these results demonstrate that the GR complex from POBA-treated cells does not appear to have undergone significant quantitative changes for the

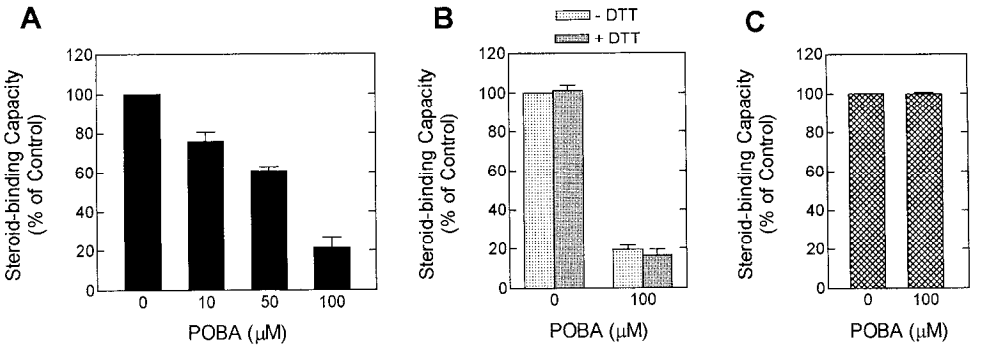

Fig. 4. POBA Treatment of Intact Cells Decreases GR Hormone-Binding Capacity

A, Effect of POBA on steroid-binding capacity in the intact cell. LMCAT cells were treated at 37 C with the indicated concentrations of POBA for 2 h, and cytosols were prepared. Steroid-binding capacities were measured using [^{3}H]triamcinolone acetonide and expressed as percentages of the POBA-untreated control value. Values shown are the mean ± SEM for four independent experiments. B, DTT does not reverse POBA-mediated inhibition of the GR hormone-binding capacity. Aliquots of the cytosol from LMCAT cells untreated or treated with 100 μM POBA were incubated on ice with or without 2 mM DTT for 2 h. Hormone-binding capacities were measured using [^{3}H]triamcinolone acetonide and expressed as percentages of the POBA-untreated control value in the absence of DTT. Values shown are the mean ± SEM for two independent experiments. C, POBA does not antagonize the binding of hormone ligand to GR. Cytosols from untreated LMCAT cells were incubated on ice with or without 100 μM POBA for 2 h. Hormone-binding capacities were measured using [^{3}H]triamcinolone acetonide and expressed as percentages of the POBA-untreated control value. Values shown are the mean ± SEM for two independent experiments.

three known steroid receptor-associated hsp: hsp90, hsp70, and hsp56.

DISCUSSION

We have provided evidence that the CaM antagonist POBA can efficiently inactivate the GR signal pathway in intact cells by a process that ultimately results in the loss of GR hormone-binding function. This decrease in hormone-binding function in response to POBA was not due to reductions in the amount of GR protein or receptor-associated hsp (hsp90, hsp70, and hsp56). Thus, by these criteria, the hormone-incompetent GR complex of POBA-treated cells appears be no different than the native untransformed complex of normal cells. What, then, can account for the loss of GR function within this complex? Clearly, we do not have the answer to this question at present, but a variety of intriguing possibilities exist.

First, it is possible that the GR complex of POBA-treated cells has changed with respect to components other than hsp90, hsp70, and hsp56. In addition to these hsp, a variety of other proteins have recently been shown to associate with steroid receptor complexes (for review, see Ref. 30). These include FKBP54, an immunophilin that, like FKBP59 (hsp56), can bind the immunosuppressive drugs FK506 and rapamycin (31); CyP-40, an immunophilin that binds cyclosporin A (32); p23, a unique acidic phosphoprotein (33); and p60, a protein that, along with hsp70, transiently associates with PR complexes during assembly (34). In theory, then, the effects of POBA on GR function could be due to changes in content for any of these proteins within the untransformed GR complex, and further studies on the macromolecular state of the POBA-generated, hormone-incompetent GR will be required to answer this question.

A second explanation for the effects of POBA on GR function derives from the idea that some of the receptor-associated proteins denoted above may be involved in the folding and assembly of functional steroid receptor complexes. All of these proteins, in addition to hsp70 and hsp56, have been shown to form complexes with hsp90 that are independent of

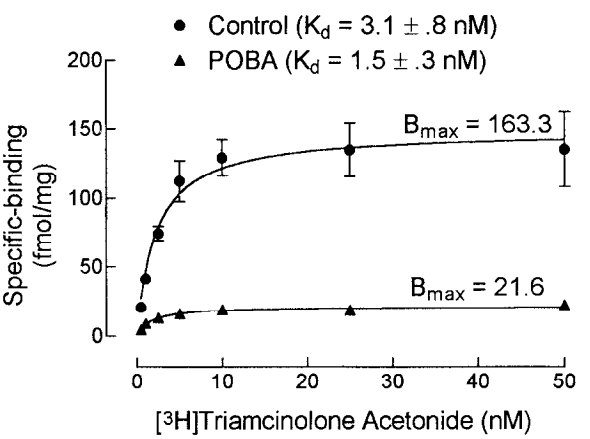

Fig. 5. Effect of POBA Treatment of Intact Cells on the Saturation Binding Curve for [^{3}H]Triamcinolone Acetonide

LMCAT cells were incubated with ethanol vehicle (●) or 100 μM POBA (▲) at 37 C for 2 h. Cytosols were prepared, and [^{3}H]triamcinolone acetonide binding was measured as described in *Materials and Methods*. The values shown are the mean ± SEM of three independent experiments. K_d and B_{max} values were derived from Scatchard transformation of the data.

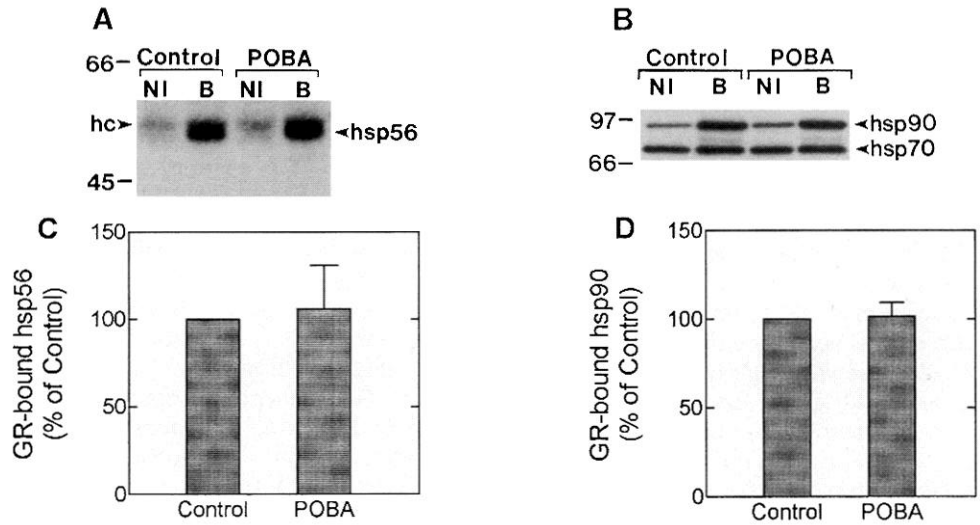

Fig. 6. The Hormone-Binding Incompetent GR of POBA-Treated Cells Remains Bound to hsp90 and hsp56

Replicate flasks of L929 cells were left untreated (control) or treated at 37 C with 100 μM POBA (POBA) for 2 h. Cytosols were prepared, and the GR-hsp90 complexes were immunoadsorbed with BuGR2 antibody against the GR (B) or with nonimmune mouse IgG (NI). The samples were analyzed for hsp56 (A) or GR-associated hsp90 and hsp70 (B) by Western blotting in which the UPJ56 antibody or a rabbit polyclonal against hsp90 and hsp70 were employed. The protein bands in A, migrating just above hsp56 and found in both the immune and nonimmune lanes, represent the heavy chains (hc) of the immunoprecipitating antibody. Quantitations of GR-bound hsp56 (C) or GR-bound hsp90 (D) were performed as described in *Materials and Methods*. GR-bound hsp values were obtained by subtracting the hsp56 or hsp90 values of the respective nonimmune conditions. The values shown are the mean ± SEM of three independent experiments.

steroid receptors, and the roles of these protein complexes in steroid receptor assembly have been extensively studied by the laboratories of Toft, Smith, and Pratt. A major common advancement from the work of these laboratories is the conclusion that hsp90 binding to receptors is required for the generation of functional (hormone-competent) steroid receptors, but that this process requires the participation of a variety of factors, some of which are the proteins denoted above. Indeed, there is evidence to suggest the existence of heterogenous hsp90-p23-immunophilin complexes, each of which may play a distinct role in the assembly of mature untransformed steroid receptors. For example, based on PR reconstitution studies and elution profiles from FK506 affinity resins, Smith *et al.* (31) have suggested that FKBP54 and FKBP59 (hsp56) can exist in distinct complexes. Similarly, Renoir *et al.* (35), using FK506 and cyclosporin A affinity resins, have provided evidence for the presence of separate FKBP59-hsp90 and CyP-40-hsp90 complexes. Thus, the inhibitory effect of POBA on GR hormone-binding function could result from an alteration of any of a diverse group of protein complexes involved in the assembly of untransformed steroid receptor oligomers.

An alternative possibility to account for the inhibitory effect of POBA on GR transactivation is an alteration in the function or state of the associated hsp90. Although an enzymatic function for hsp90 has not been established, the presence of an ATP-binding site within this protein (36) and its ability to hydrolyze ATP and undergo autophosphorylation (37) suggest that its role in maintaining the hormone-competent GR may be more complicated than simple association with the receptor. Indeed, Smith *et al.* (10) have shown that the processes of *in vitro* assembly and activation of the PR are facilitated by ATP, whereas Shumacher *et al.* (38) have shown that the ability of hsp70-hsp90 complexes to renature proteins (chaperoning activity) is an ATP-dependent event. *In vivo* evidence in support of this hypothesis comes from two sources. Cadepond *et al.* (39), using an expression system in baculovirus-infected insect cells, tested a series of hsp90 deletion mutants, some of which formed complexes with GR that were unable to bind hormone. Bohen and Yamamoto (40) showed that point mutations of hsp90 could be generated which, when expressed in yeast, resulted in loss of GR signaling. Moreover, all of the hsp90 mutants retained their ability to interact with the untransformed GR, but failed to generate GR capable of efficient hormone-binding function. These two studies thus provided the first evidence that hsp90 involvement in the generation of hormone-competent receptors is more complicated than simple target recognition. Both of these works relied on exogenous expression of GR and hsp90 in cells of lower eukaryotic organisms, the results of which could be questioned due to the potential for artifact in exotic expression systems. In our study, we have found further evidence for this dual role of hsp90 in a mammalian cell line and for endogenous gene products. Most importantly, these results reinforce the hypothesis that hsp90, either alone or in concert with other GR-associated proteins, plays an active role in the mainte-

nance of receptors capable of high affinity ligand binding.

Given the recent evidence that both free (16, 17) and GR-associated hsp90 (25) can bind CaM under *in vitro* conditions, it is interesting to speculate that the alteration in GR function reported here in response to POBA treatment could be due to a lack of CaM interaction with hsp90 either before or after it becomes bound to the GR. In support of this idea is our observation that Ca^{2+}-dependent binding to CaM by both hsp90 and untransformed GR complexes can be inhibited by POBA as well as several other CaM antagonists (unpublished observations). However, other potential targets for CaM action on the GR signal pathway exist. For example, members of both the hsp56 and hsp70 protein families have been shown to contain highly conserved CaM-binding domains and to bind CaM in a Ca^{2+}-dependent manner (17, 41–43). As hsp70 plays an important role in the folding and assembly of mature steroid receptor complexes (44), an alteration of hsp70 function due to lack of CaM interaction could be responsible for the results reported here. In the case of hsp56, both the rabbit and human forms of this protein have been shown to contain consensus CaM kinase phosphorylation sites (45, 46), and its phosphorylation *in vitro* can be inhibited by the CaM antagonist calmidzolium (47). These observations present the intriguing possibility that the role, if any, of hsp56 in GR steroid-binding function may be regulated by CaM-dependent phosphorylation mechanisms.

As detailed in the introduction, a variety of reports exist providing evidence for the involvement of CaM in steroid receptor-mediated signaling. In two of these reports, a connection between glucocorticoid-mediated apoptosis and CaM was demonstrated (23, 24), and in each case, steroid-induced apoptosis was inhibited by CaM antagonists. A logical conclusion of these findings is that the CaM antagonists act to block the apoptotic actions of glucocorticoids by inhibiting CaM, whose synthesis is at least partially controlled by the GR (24). However, in light of our results, it must now be considered that CaM antagonism may also serve to inhibit glucocorticoid-mediated apoptosis by blocking activation of the GR signal pathway. Thus, it is possible that CaM antagonists act on two fronts to block steroid-induced cell death: 1) by direct inhibition of CaM as a mediator of apoptosis, and 2) by lowering CaM proteins levels via an inhibition of GR-mediated expression of the CaM gene. Of course, our observations also present the possibility that inhibition of GR activation by CaM antagonists could lead to decreases in expression of GR-regulated gene products, other than CaM, some of which may also be involved in apoptosis.

Although our results, using the CaM antagonist POBA, do not by themselves provide proof for the involvement of CaM in the maintenance or generation of the untransformed, hormone-competent state of the GR, they do provide strong evidence to this effect. Antagonists such as POBA have been widely used in the molecular analyses of CaM actions (see Refs. 48 and 49 for review). One possible drawback of the use of CaM antagonists is their potential for interaction with targets other than CaM. POBA, for example, was originally developed as an adrenergic antagonist, but it is unlikely that the effects of POBA described herein are via this mechanism, as we have not been able to prevent the POBA inhibition of GR activation with the adrenergic agonist norepinephrine (data not shown). In a prior study, we tested the effects of several, structurally unrelated antagonists (POBA, trifluoperazine, compound 48/80, and the naphthalenesulfonamide, W7) on Dex-induced GR transactivation, and each compound inhibited this process to varying degrees (25). As each of these compounds have different non-CaM side targets, their common actions on the GR signal pathway are a strong indication that these actions are mediated by a common target protein, namely CaM. Although proof of CaM involvement in the GR signal pathway awaits the development of the appropriate molecular approaches, our results nonetheless demonstrate the utility of phenoxybenzamine in identifying a novel stage of the GR signal pathway within intact cells, a stage in which the receptor is still complexed to hsp90 but can no longer bind hormone. Whether this stage of the GR represents a step in the assembly of hormone-competent GR after *de novo* synthesis, in the reassembly of GR after nuclear shuttling, or in a novel inactivation pathway remains to be determined.

MATERIALS AND METHODS

Materials

[^{3}H]Acetate (10.3 mCi/mmol) and ^{125}I-conjugated goat anti-mouse IgG (11.8 μCi/μg) were obtained from ICN Radiochemicals (Costa Mesa, CA). [^{3}H]Triamcinolone acetonide (49.5 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). Immobilon P membranes were obtained from Millipore Corp. (Bedford, MA). Phenoxybenzamine was obtained from Smith, Kline, and French. Dexamethasone, G418 (Geneticin) antibiotic, coenzyme A, acetyl coenzyme A synthetase, chloramphenicol, ATP, Tris, EDTA, protein A-Sepharose, horseradish peroxidase-conjugated rabbit anti-goat IgG, DMEM powdered medium, and iron-supplemented newborn calf serum were obtained from Sigma Chemical Co. (St. Louis, MO). The BuGR2 anti-GR monoclonal antibody (50) was purchased from Affinity Bioreagents (Golden, CO). A rabbit polyclonal serum against hsp70 that is also known to recognize hsp90 (51) was obtained from Dr. Ettore Apella. The UPJ56 antiserum (52) against hsp56 was a gift from Drs. Martin Diebel, Jr., and Karen Leach.

The pMMTV-CAT plasmid DNA was obtained from Mark Danielsen and Gordon Ringold. The pMMTV-CAT plasmid contains the complete MMTV-long terminal repeat promoter upstream of the CAT reporter gene (53). Hormonally driven expression of CAT by this reporter is principally controlled by glucocorticoid response elements known to reside within the long terminal repeat region (54). The pCAT-control plasmid was obtained from Promega Corp. (Madison, WI). In this plasmid, high level, constitutive expression of the CAT reporter gene is controlled by the SV40 early promoter.

Cell Culture

The LMCAT cells were established as previously described (26). Briefly, mouse L929 cells were cotransfected with the pSV2neo and pMMTV-CAT plasmids, followed by selection for a stably transfected, cloned cell line using G418 (Geneticin) antibiotic. The LSVCAT cell line was similarly derived by cotransfecting L929 cells with the pSV2neo and pCAT-control plasmids. The LMCAT cells and LSVCAT cells were grown in an atmosphere of 5% CO_2 at 37 C in DMEM containing 0.2 mg/ml G418 and 10% iron-supplemented newborn calf serum. For all experiments, the newborn calf serum was stripped of endogenous steroids by extraction with dextran-coated charcoal. Typically, treatment of the cells with CaM antagonist was performed on cells that were at or near confluence.

CAT Assay

Measurement of CAT enzyme activity in the experiments shown in Figs. 1 and 2 was performed according to the method of Nordeen *et al.* (55) with minor modifications. Briefly, cell lysates were prepared by sequential freezing and thawing in 0.25 M Tris-5 mM EDTA (pH 7.5) and centrifugation at 13,600 × *g*. Aliquots of lysate containing equal protein content were added to the enzymatic reaction mixture and incubated at 37 C for 2 h. The reaction was stopped by the addition of ice-cold benzene, which also served the function of separating the acetylated forms of [^{3}H]chloramphenicol from the labeled sodium acetate substrate and intermediates. The benzene extracts were dried, and quantitation was achieved by liquid scintillation spectroscopy.

Cytosol Preparation, Cellular Fractionation, and Immunoadsorption of GR

In the experiments presented in Fig. 6, cytosols were prepared by Dounce A (Kontes Co., Vineland, NJ) homogenization of LMCAT cells in hypotonic buffer (10 mM HEPES, 3 mM EDTA, and 10 mM sodium molybdate, pH 7.4), followed by centrifugation at 13,600 × *g*. In the experiments shown in Fig. 3, LMCAT cells were fractionated into cytosolic and nuclear portions by Dounce A homogenization in hypotonic buffer, followed by centrifugation at 1,000 × *g*. The cytosolic fractions were saved, and the nuclear pellets were washed twice by resuspension and pelleting in hypotonic buffer. Hypotonic buffer was then added to both the pellet and cytosolic fractions to a final volume of 0.5 ml. Each fraction was made 0.5 M for NaCl by the addition of 0.5 ml of a 1-M stock solution in TEG (10 mM TES, 1 mM EDTA, 10% glycerol, 50 mM NaCl, and 10 mM sodium molybdate, pH 7.6) and incubated on ice with occasional vortexing for 1 h. After salt extraction, the nuclear pellets were centrifuged at 13,600 × *g*, and the supernatants were saved. For the experiments shown in Figs. 3 and 6, BuGR2 anti-GR monoclonal antibody (1.5 μg) was added to the regular cytosols or to the salt-extracted cytosols and nuclear fractions, and each sample was then adsorbed in batch to protein A-Sepharose, washed with TEG buffer, and eluted with 2 × SDS sample buffer, before gel electrophoresis.

Gel Electrophoresis and Quantitative Western Blotting

Samples were resolved by electrophoresis in 7% polyacrylamide-SDS gels, as described by Laemmli (56). The relative amounts of GR protein (Fig. 3) or GR-associated hsp (Fig. 6) were determined via a quantitative Western blotting technique previously described (57), employing primary antibody and both peroxidase- and ^{125}I-conjugated counterantibodies. After Western blotting and autoradiography, the peroxidase-stained bands were excised, and ^{125}I counts per min were determined via liquid scintillation spectroscopy. Receptor- or hsp-specific counts per min were derived by subtracting the counts per min measured in a background slice of comparable area.

Steroid Binding Assay

In the experiments depicted in Fig. 4, specific steroid-binding capacities were measured in replicate aliquots (45 μl) of 13,600 × *g* cytosols made by Dounce A homogenization in hypotonic buffer and incubated with 50 nM [^{3}H]triamcinolone acetonide (42.8 Ci/mmol) in the presence or absence of a 1000-fold excess of unlabeled Dex, as previously described (58). All hormone-binding capacities were calculated as specific counts per min/mg cytosol protein and expressed as percentages of the untreated control value.

In the experiments shown in Fig. 5, equilibrium saturation was performed at 4 C by incubating 45-μl replicate aliquots of cytosol with seven concentrations of [^{3}H]triamcinolone acetonide ranging from 0.5–50 nM for 12 h. Nonspecific binding was measured in the presence of 50 μM unlabeled dexamethasone. Specific binding values were then converted to moles of GR per mg cytosol protein. K_d and B_{max} values were obtained via computerized Scatchard analysis.

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