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EVIDENCE FOR A FUNCTIONAL INTERACTION BETWEEN CALMODULIN AND THE GLUCOCORTICOID RECEPTOR

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SUMMARY: In the signaling cascade of membrane-bound receptors, calmodulin (CaM) plays an important role. However, little is known about the role of CaM in the activation of intracellular steroid receptors, which are known to act as ligand-regulated transcription factors. We report here that CaM can interact in a calcium-dependent manner with the untransformed glucocorticoid receptor (GR) complex containing hsp90. In addition, we demonstrate that four unrelated CaM antagonists (trifluoperazine, compound 48/80, W7, and phenoxybenzamine) can inhibit GR-mediated gene expression in mouse L929 cells stably-transfected with the MMTV-CAT reporter gene. These results provide evidence that CaM may play an important role in the signal transduction pathways of steroid hormone receptors.

INTRODUCTION: The glucocorticoid receptor belongs to a class of proteins, the steroid receptors, that act as ligand-regulated transcription factors controlling specific gene expression (1). Although the mechanism of hormone-mediated activation of the GR is poorly understood, it is generally accepted that the first event in this process is the binding of hormone to the untransformed GR present as a cytoplasmic, heteromeric complex containing two heat shock proteins, hsp90 and hsp56 (2,3). Upon binding hormone, the GR dissociates from the hsps and the hormone-bound GR translocates to nucleus, where it acts as an enhancer of gene transcription.

Calmodulin is a highly conserved and ubiquitous mediator of calcium signaling in cells whose roles include regulation of target enzymes, modulation of cytoskeletal structures, and involvement in DNA replication and repair (4). Up until now, the role of CaM in signal transduction processes has been largely limited to regulatory cascades originating from plasma membrane receptors and ion-channels. However, a variety of studies have implicated Ca^{++} and/or CaM in the regulation of steroid receptor functions. For example, a GR-mediated increase in CaM gene expression has been reported (5), as well as evidence for CaM involvement in glucocorticoid-induced apoptosis in lymphocytes (5,6). Ca^{++}/CaM-dependent phosphorylation of the estrogen receptor has also been shown (7). Lastly, binding to actin filaments by purified hsp90 (8) or hsp90/GR complexes (9) can be inhibited by Ca^{++}/CaM, and direct binding to CaM by purified preparations of hsp90 (10,11), hsp70 (11,12) and hsp56 (13) has been reported. Two of these hsps, hsp90 and hsp56, are known to be components of untransformed GR complexes (2,3),while

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hsp70 is associated with untransformed and transformed progesterone receptor complexes (14). Taken together, these observations suggest that CaM may impinge upon the steroid receptor signal pathway through a direct interaction with receptor-associated heat shock proteins. In the present study, we provide in vitro and in vivo evidence for a functional interaction between CaM and the glucocorticoid receptor.

MATERIALS AND METHODS

CaM-Sepharose 4B was obtained from Pharmacia. [^{125}I]conjugated goat anti-mouse IgG (11.8 μCi per μg) was obtained from ICN Radiochemicals. [^{3}H]triamcinolone acetonide (42.8 Ci per mmole) was obtained from NEN. Protein A-Sepharose, horse radish peroxidase-conjugated rabbit anti-goat IgG, iron-supplemented newborn calf serum, and DMEM powdered medium were obtained from Sigma Chemical Company, as were the CaM antagonists trifluoperazine (TFP), compound 48/80 and the naphthalenesulfonamide, W7. Phenoxybenzamine (POBA) was obtained from Smith, Kline and French. The BuGR2 anti-GR monoclonal antibody (15) was a gift from Drs. William Hendry and Robert Harrison. The UPJ56 antiserum (16) against hsp56 was a gift from Drs. Martin Diebel, Jr. and Karen Leach. The D7α monoclonal antibody (17) against hsp90 was a gift from Dr. Joan Brugge. The rabbit antiserum to hsp70 was kindly provided by Dr. Etorre Apella. This antiserum was made against a C-terminal peptide of human hsp70 by Ehrhart *et al* (18) and is known to cross-react with hsp90.

Cell Lines and Cytosol Preparation: Mouse L929 fibroblast cells and the derivative cell line LMCAT were grown in DMEM containing 10% charcoal-extracted newborn calf serum. The LMCAT cell line was established by stably-transfecting L929 cells with the MMTV-CAT reporter gene as previously described (19). Cytosols were prepared by Dounce homogenization in hypotonic buffer (10 mM Hepes, 5 mM EDTA, 10 mM sodium molybdate, pH 7.4) and centrifugation at 16,000 g.

CaM Affinity Chromatography: CaM-Sepharose 4B containing 1.0 mg/ml of calcium-free CaM was equilibrated in Buffer A (50 mM Tris, 1 mM DTT, 1 mM EDTA, 2.5 mM $MgCl_2$, 10 mM sodium molybdate, 50 mM NaCl, 0.25 mM PMSF, 1 μM pepstatin, 1 μM leupeptin, pH 7.4) containing 3 mM $CaCl_2$. In the experiments of Fig. 1, 0.3 ml aliquots of mouse L929 cell cytosol prepared in Buffer A were incubated in batch with 0.3 ml of the CaM resin in the presence of 3 mM $CaCl_2$ for 3 hours on ice. After the resins were washed five times with Buffer A containing 3 mM $CaCl_2$, the bound proteins were eluted with Buffer A containing 3 mM $CaCl_2$ ("Ca++") or 5 mM EGTA ("EGTA").

CAT Assay: Measurement of CAT enzyme activity was performed as previously described (19). Briefly, cell lysates were prepared by freezing and thawing in 0.25 M Tris, 5 mM EDTA (pH 7.5). Aliquots of lysate containing equal amounts of protein were added to the reaction mixture and the acetylated forms of [^{14}C]chloramphenicol were separated by thin layer chromatography. Quantitation was achieved by separately excising the spots corresponding to substrate and product and performing liquid scintillation spectroscopy.

Immunoadsorption and Western Blotting: CaM resin eluates were immunoadsorbed by addition of the indicated antibodies and incubation in batch at 4°C with protein A-Sepharose. After washing with TEG buffer (10 mM TES, 5 mM EDTA, 50 mM NaCl, 10% v/v glycerol, pH 7.6), the protein A-bound proteins were resolved by electrophoresis in 7% SDS-acrylamide gels and Western-blotting, in which the indicated probe antibody was followed by both ^{125}I- and peroxidase-conjugated counter antibodies as previously described (20).

RESULTS AND DISCUSSION

We have examined the binding of hsp90, hsp70 and hsp56 to a CaM affinity resin (Fig. 1). Cytosols from mouse L929 cells were applied to the CaM resin in the presence of Ca++ and, after extensive washing, the bound proteins were eluted with EGTA and analyzed by SDS-PAGE and

immunoblotting. The results obtained demonstrate Ca++-dependent binding by hsp90, hsp70 and hsp56 to CaM (Fig.1A). Moreover, immunoadsorption of the eluates with an antibody against hsp90 (D7α) resulted in the co-purification of hsp70 and hsp56 (Fig. 1B), suggesting that at least a portion of these hsps are retained by the CaM resin as the hsp complex previously implicated in the assembly of hormone competent GR complexes (21).

To determine if the untransformed GR complex was also retained by the CaM affinity resin, the eluates were immunoadsorbed with the BuGR2 monoclonal antibody against the GR (Fig. 2). Ca++-dependent CaM binding by the GR was found (Fig. 2A), and reprobing of this blot with an antibody against hsp90 and hsp70 showed the apparent continued association of hsp90 with this GR, along with trace amounts of hsp70 (Fig. 2B). Because of the large yields of hsp90, hsp70 and hsp56 seen in the EGTA eluates of the CaM resins (Fig. 1A), it is possible that the results of Fig. 2B did not accurately reflect the macromolecular state of the CaM-retained GR. For this reason, we performed the experiments of Fig. 3 in which the EGTA eluates were immunoadsorbed with BuGR2 ("B") or non-immune mouse IgG ("N") as a negative control, followed by Western-blotting using a mixture of rabbit polyclonal antibodies against hsp90, hsp70 and hsp56. The results of the blotting (Fig. 3A) and the quantitative analysis for hsp90 (Fig. 3B) showed the immune-specific presence of hsp90 ("EGTA B" lane), but no such presence for hsp70 and only trace amounts of hsp56. The lack of GR-associated hsp70 was consistent with our prior observation that the mouse L929 GR complex contains little or no hsp70 (22). Similarly, the

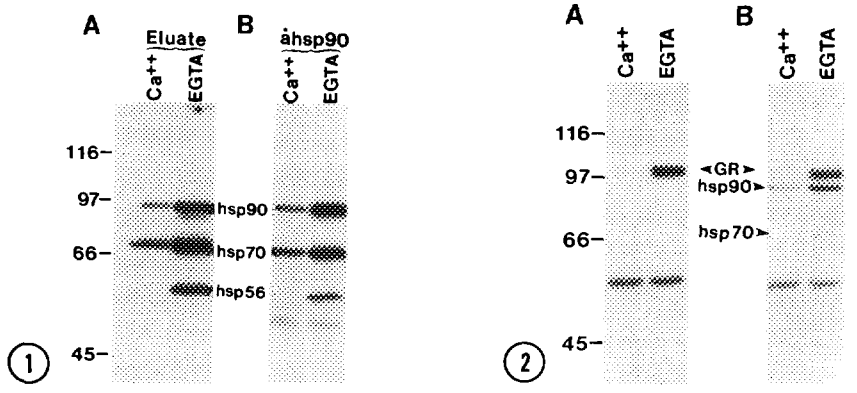

Figure 1. Ca++-dependent binding to CaM by hsp90, hsp70, and hsp56. Aliquots (0.3 ml) of mouse L929 cell cytosol were incubated in batch with CaM resin as described under Materials and Methods and the bound proteins were eluted with $CaCl_2$ ("Ca++") or EGTA ("EGTA"). The eluates were either lyophilized and redissolved in SDS sample buffer (Panel A) or were immunoadsorbed with the D7α monoclonal antibody (17) against hsp90 (Panel B). The "Eluate" and "åhsp90" samples were then resolved by SDS-PAGE and immunoblotting using a rabbit serum against hsp70 and hsp90 (18) and the UPJ56 rabbit serum against hsp56 (16) as probes.

Figure 2. Ca++-dependent binding to CaM by the untransformed GR complex. Aliquots (0.3 ml) of mouse L929 cell cytosol were incubated in batch with CaM resin as described under Materials and Methods and the bound proteins were eluted with $CaCl_2$ ("Ca++") or EGTA ("EGTA"). Panel A: The "Ca++" and "EGTA" eluates were immunoadsorbed with BuGR2 monoclonal antibody against the GR (15), and the bound proteins were resolved by SDS-PAGE and immunoblotting using the BuGR2 antibody. Panel B: The blot of Panel A reprobed with the antiserum against hsp90 and hsp70.

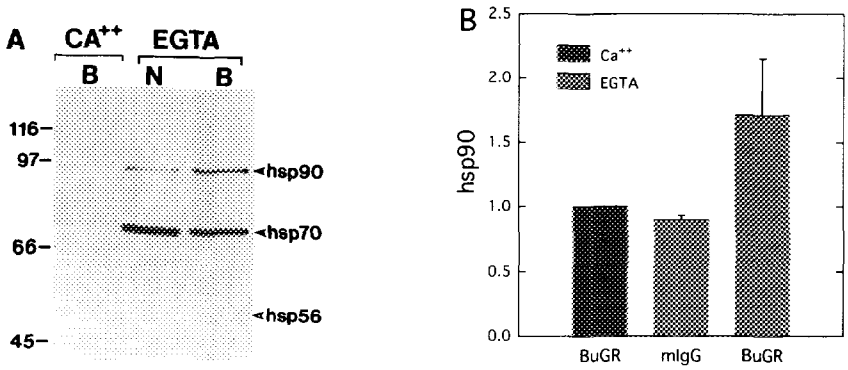

Figure 3. The CaM-bound GR complex contains hsp90, but not hsp70 or hsp56. Aliquots (0.3 ml) of mouse L929 cell cytosol were incubated in batch with CaM resin as described under Materials and Methods and the bound proteins were eluted with $CaCl_2$ ("Ca++") or EGTA ("EGTA"). Panel A: The "Ca++" eluate was immunoadsorbed with BuGR2 monoclonal antibody against the GR (15), while the "EGTA" eluates were immunoadsorbed with BuGR2 ("B") or non-immune mouse IgG ("N"). The bound proteins were resolved by SDS-PAGE and immunoblotting using a rabbit serum against hsp70 and hsp90 (18) and the UPJ56 rabbit serum against hsp56 (16) as probes. Panel B: The relative amounts of GR-associated hsp90 in the "Ca++" and "EGTA" eluates as determined by a quantitative procedure employing ^{125}I- and peroxidase-conjugated counter antibodies (20). The ^{125}I cpm values obtained were normalized to hsp90 in the "Ca++" eluates and represent the means ± S.E.M. of three independent experiments.

relative lack of GR-associated hsp56 was not entirely unexpected, as the GR complex of L929 cells contains, in our experience (unpublished observations), hsp56 that is less tightly bound as compared to GR complexes from human IM9 cells (23), or the GR-expressing CHO cell line, WCL2 (24). However, hsp90 is clearly still bound to the GR under these conditions. Taken together then, the results of Figs. 1-3 provide evidence that the untransformed GR complex of L929 cells can interact with CaM, and that the binding of the untransformed GR complex to CaM is most likely occurring through the hsp90 component.

To determine a role for CaM in GR function, we stably-transfected L929 cells with a chloramphenicol acetyltransferase (CAT) reporter gene (pMMTV-CAT) under the control of glucocorticoid response elements, and a cloned dexamethasone (Dex)-responsive cell line (LMCAT) was obtained (25). Treatment of LMCAT cells with increasing concentrations of the CaM antagonist phenoxybenzamine (POBA) prior to addition of hormone resulted in a concentration-dependent decrease in Dex-induced CAT gene expression, with a large reduction (≈87%) in CAT activity occurring at 100 μM drug (Fig. 4A). Although POBA is well characterized as a CaM antagonist (26), it is also known to antagonize adrenergic receptors (26). Indeed, many if not all CaM antagonists engage in side reactions with protein targets other than CaM (26). We have attempted to overcome this lack of absolute specificity by measuring the effects of a variety of structurally different CaM antagonists on Dex-induced CAT gene expression (Figs. 4B-D). Reductions in Dex-induced CAT activities were observed in LMCAT cells treated with the phenothiazine , trifluoperazine (Fig. 4B); with the hydrophobic reagent, compound 48/80 (Fig. 4C); and with the naphthalenesulfonamide, W7 (Fig. 4D). [For a review of these antagonists see ref. (26)]. In contrast, W5, a chlorine-deficient analogue of W7 that is 10 times less potent

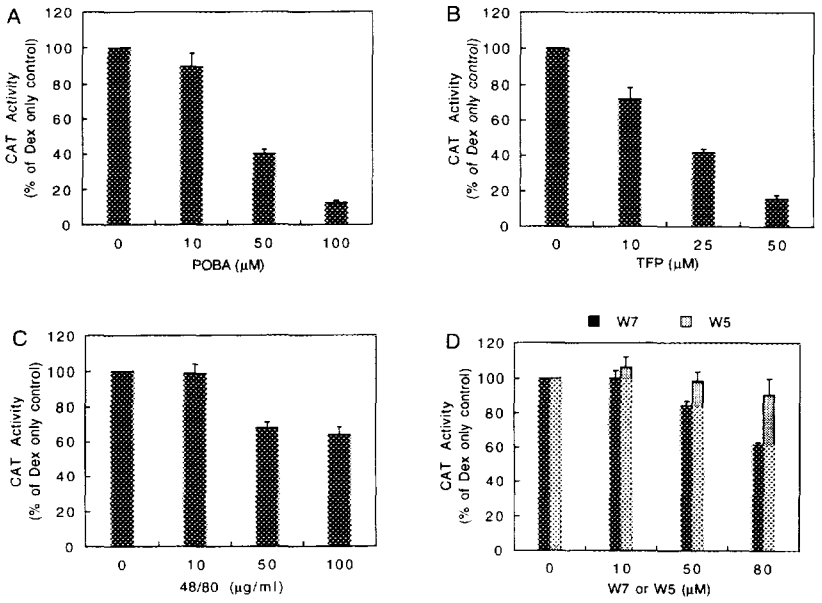

Figure 4. *Inhibition of hormone-induced, GR-mediated MMTV-CAT expression by CaM* antagonists. L929 cells stably-transfected with the MMTV-CAT reporter gene (LMCAT cells) were pretreated for 2 hours with the indicated concentrations of phenoxybenzamine (POBA) [Panel A], trifluoperazine (TFP) [Panel B], compound 48/80 [Panel C] and the naphthalenesulfonamides, W7 and W5 [Panel D]. Following pretreatment, 1 μM Dex (final concentration) was added to all flasks and the cells were cultured for an additional 20 h. After harvest, lysates were prepared and assayed for CAT activities, expressed as percentages of the 1 μM Dex controls. The results represent the means +/- S.E.M. of at least three independent experiments. Basal (no treatment) CAT levels averaged 1.5 % of the 1 μM Dex controls.

and that has been used as a control for unspecific effects of hydrophobic compounds (27), resulted in little or no reduction in CAT expression as compared to W7 (Fig. 4D), consistent with reports dealing with CaM function and the W5/W7 family of antagonists (28). POBA, TFP, 48/80 and W7 have in common either an ability to directly bind CaM, or an ability to inhibit CaM-dependent activation of target enzymes (e.g., phosphodiesterase). As none of these antagonists have non-CaM targets that are known to overlap, it is likely that the observed effects of these drugs on Dex-induced MMTV-CAT expression reflect their common actions on CaM.

The inhibition of Dex-induced CAT expression by POBA and the other CaM antagonists could be the result of a generalized effect of these drugs on gene transcription or protein synthesis. However, POBA had no effect on CAT expression in L929 cells stably-transfected with the constitutively active pSV2-CAT reporter (data not shown). In addition, the inhibitory effect of POBA could not be due to an augmentation of ligand-induced GR down-regulation, as no decrease in GR levels were observed in LMCAT cells subjected to combined POBA and Dex treatment (data not shown). Taken together, these observations suggest that the inhibitory effect of CaM antagonists on CAT expression is mediated through the GR signal transduction pathway.

Although we do not know at present the precise mechanism by which CaM exerts its intracellular effects on GR actions in vivo, it is possible that this effect of CaM is enzyme-mediated. In support of this speculation is a report demonstrating a correlation between activation

of estrogen receptor hormone-binding function and Ca++/CaM-dependent phosphorylation of the receptor (7). The latter report points to the existence of a CaM-dependent kinase capable of direct phosphorylation of steroid receptors. It is also possible that CaM may directly bind and control the functions of the receptor-associated heat shock proteins, hsp90, hsp70 or hsp56. As demonstrated in this work and elsewhere (10,12), these hsps are capable of binding CaM. In addition, all three of these hsps have been shown to have a variety of activities. For example, both hsp90 (29) and hsp70 (30) have ATPase activities, and, in the case of hsp70, ATP hydrolysis is required for release of unfolded substrate proteins (30); while peptidyl-prolyl cis-trans isomerase (rotamase) activity has been measured in purified preparations of hsp56 (31). Although it is interesting to speculate that CaM acts to control GR functions through a direct modulation of one or more of these hsp activities, no evidence as yet exists to suggest that these activities can be regulated by CaM. On the other hand, it is clear that CaM-dependent processes can effect these proteins. As already mentioned, binding of purified hsp90 (8) or GR/hsp90 complexes (9) to actin filaments has been reported and both of these interactions can be specifically inhibited by Ca++/CaM. Similarly, Ca++/CaM-dependent phosphorylation of hsp56 has been observed in vitro (32).

In this study, we have provided evidence for the interaction of CaM with the untransformed GR complex through its hsp90 component, and that CaM may play a functional role in vivo by maintaining the untransformed GR complex in a state responsive to hormone activation. Thus, the regulatory functions of CaM are not limited to signal pathways of membrane-bound receptors and ion channels, but may also include the intracellular steroid receptors.

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