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The road less traveled: new views of steroid receptor action from the path of dose-response curves

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Abstract

Conventional studies of steroid hormone action proceed via quantitation of the maximal activity for gene induction at saturating concentrations of agonist steroid (i.e., $A_{\max}$). Less frequently analyzed parameters of receptor-mediated gene expression are EC_{50} and PAA. The EC_{50} is the concentration of steroid required for half-maximal agonist activity and is readily determined from the dose-response curve. The PAA is the partial agonist activity of an antagonist steroid, expressed as percent of $A_{\max}$ under the same conditions. Recent results demonstrate that new and otherwise inaccessible mechanistic information is obtained when the EC_{50} and/or PAA are examined in addition to the $A_{\max}$. Specifically, $A_{\max}$, EC_{50} , and PAA can be independently regulated, which suggests that novel pathways and factors may preferentially modify the EC_{50} and/or PAA with little effect on $A_{\max}$. Other approaches indicate that the activity of receptor-bound factors can be altered without changing the binding of factors to receptor. Finally, a new theoretical model of steroid hormone action not only permits a mechanistically based definition of factor activity but also allows the positioning of when a factor acts, as opposed to binds, relative to a kinetically defined step. These advances illustrate some of the benefits of expanding the mechanistic studies of steroid hormone action to routinely include EC_{50} and PAA.

Keywords

Steroid hormone action; Potency (EC_{50}); Efficacy ($A_{\max}$); Partial agonist activity (PAA); New insight for steroid receptor mechanism

1. Introduction

The mechanism of steroid hormone action has been studied for many years both for its immediate clinical relevance and as a paradigm for the differential control of gene transcription during development, differentiation, and homeostasis. These studies have been very productive and led to the general model in which steroids enter the cell by passive diffusion and bind to a specific intracellular receptor protein to form a receptor-steroid complex. After a still poorly understood step called activation, the activated complex

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associates with biologically active DNA sequences, called hormone response elements or HREs, and recruits a large variety of transcriptional cofactors. Some cofactors cause chromatin reorganization while others increase or decrease the rates of transcription of the target genes to eventually alter the levels of specific proteins (Metivier et al., 2006, Lonard and O'Malley, 2007, Wu and Zhang, 2009). All of this has been accomplished over the last 50 years with innumerable elegant studies of how various factors alter the maximal amount of gene expression with saturating concentrations of steroid, which we call $A_{\max}$ (Fig. 1A; see also Section 2.1)

More recently, it has become apparent that there are additional rewards from a broader view in which two other properties of steroid-regulated gene expression are examined. These are the dose-response curves of agonists, which gives the steroid concentration required for half-maximal gene expression (EC_{50}), and the amount of residual agonist activity displayed by almost all antisteroids, which we call the partial agonist activity or PAA (Figs. 1A and C; see also Section 2.1) (Simons; Jr., 2003, Simons; Jr., 2006, Simons; Jr., 2008, Simons; Jr., 2010). Two benefits of dose-response curves are well-known. First, these curves define the transcriptional responses over a range of steroid concentrations including physiological levels. This is the basis of steroid endocrinology and pharmacology and cannot be determined from studies with pharmacological concentrations of steroid that saturate the receptor. Second, it is now clear that the position of the dose-response curve, or the EC_{50} , is not the same for all genes regulated by a specific receptor-steroid complex in different tissues (Mercier et al., 1983, May and Westley, 1988). Initially, it was thought that the EC_{50} was determined by the affinity of steroid binding to its cognate receptor (Munck and Holbrook, 1984). In fact, such close correlations were initially interpreted as confirming that steroid-induced responses proceeded via binding to the receptor protein (Hackney et al., 1970, Rousseau and Baxter, 1979, Varmus et al., 1979). The underlying causes for tissue-specific differences in EC_{50} for the same receptor/steroid interactions are not fully understood but they are clearly relevant for the differential control of gene expression.

The PAA of an antisteroid, like the EC_{50} of an agonist for gene induction or repression, was initially thought to be an invariant property of each antisteroid. More recently, the PAA has also been found to vary with the gene and cell or tissue. Thus, the antiestrogens tamoxifen and raloxifene often display different activities for different genes in assorted cell lines (Zajchowski et al., 2000). Such observations have given rise to the concept of selective receptor modulator (SRM) steroids, which have different gene-selective properties in different cells and tissues. Again, the reasons for this behavior are poorly understood. Nevertheless, much research is now focused on finding new SRMs that only repress defined genes in selected tissues. This behavior would greatly reduce the undesirable side-effects of blocking all genes regulated by endogenous agonist steroids and should dramatically increase patient tolerance to endocrine therapies involving antisteroids.

Over the last decade, evidence has emerged that, in addition to tissue- or cell-specific properties that determine the EC_{50} of agonists and the PAA of antagonists, processes exist by which the EC_{50} and PAA of an individual gene can be altered within the same cell. This intracellular variation has been shown to be effected by changing the concentration of many of the numerous cellular factors (Simons; Jr., 2003, Simons; Jr., 2006, Simons; Jr., 2008, Simons; Jr., 2010) that are thought to participate in the multimeric complexes driving gene transcription (Metivier et al., 2006, Rosenfeld et al., 2006). Thus factor concentration acts as a molecular rheostat that adjusts the EC_{50} , and PAA, to any position in a continuum of activity values (Simons; Jr., 2003, Simons; Jr., 2006, Simons; Jr., 2008, Simons; Jr., 2010). Importantly, even greater mechanistic information is available from studies of the dose-response curves under these conditions. Not only can factor concentration modify the EC_{50} , PAA, and $A_{\max}$ in a gene-selective manner within a cell but the modulatory activity of a

factor is not constant and can vary with the parameter examined. Therefore, one or more of the three parameters can change and not necessarily in the same direction. These observations dramatically extend the possibilities for differential control of gene expression during development and for selective gene repression during endocrine therapy. At the same time, a new theoretical model of steroid receptor action is providing previously inaccessible mechanistic information when one quantifies the EC_{50} and/or PAA in combination with A_{max} . By examining how these quantities change with different concentrations of cofactor, one can now deduce the mechanism and location of action of a given cofactor or set of cofactors. Here we review several of these recent developments and consider the advances that they permit. The examples discussed are predominantly from our studies of glucocorticoid receptors because similar studies with other receptors are currently rare. However, the ability of the same factors to influence the induction parameters of other steroid receptors under less exacting conditions (Herdick et al., 2000, Wan et al., 2005, Yoon and Wong, 2006) suggest that the phenomena are general for all steroid receptors.

2. Results and Discussion

2.1 Data analysis

An important component of analyzing the A_{max} , EC_{50} , and PAA of steroid-regulated gene expression is the presentation and interpretation of data. When only the A_{max} is examined, it is customary to show both the basal activity without added steroid and the fully induced (or repressed) level. An alternative mode of conveying the same information is to give the fold induction along with the basal activity. While the EC_{50} can be determined from a plot of the raw data (including basal activity) with multiple subsaturating concentrations of agonist steroid, it is difficult to readily compare the EC_{50} for the induction of one gene under two conditions (or multiple regulated genes) when the raw data are plotted. One must subtract the basal level from the A_{max} , divide this number by two, and add the resulting quotient to the basal level. The amount of steroid that corresponds to this calculated value is the EC_{50} for that dose-response curve. This has to be repeated with the other dose-response curve before one can compare EC_{50} s (Fig. 1A). When the parameter of interest is the EC_{50} , the task is much simpler (especially for readers of a paper) if the raw data are first transformed into percent of maximal activity, or A_{max} , above the basal level, as is common in pharmacology. When this is done, each data point is reduced by the amount of the basal activity, divided by ($A_{max} - \text{basal activity}$), and multiplied by 100. The resulting data for two conditions gives two parallel curves, for which the EC_{50} is simply determined by finding the steroid concentrations corresponding to 50% induction (Fig. 1B). The difference in EC_{50} s is even easier to determine. It is the distance between the two curves at the level of 50% of maximal activity. The A_{max} and basal levels are lost in this mode of presentation but can be presented in the figure legend.

The residual activity of antisteroids is often given as a raw value along with the basal level activity (Fig. 1C). In many instances, though, this antisteroid activity is displayed without reference to the maximal activity of the agonist under the same conditions (A_{max}). This approach can lead to erroneous conclusions because the activity of the antisteroid under condition B is often compared to that of the full agonist under condition A. To determine the PAA, the activity of the antisteroid must be compared to that of the full agonist under the same conditions. For example, the raw amount of agonist activity of the antisteroid in Fig. 1C under condition B is the same as that of the full agonist under condition A. This, however, does not make the antisteroid a full agonist. Under condition B, the activity of the full agonist increases more than that of the antagonist under the same conditions. Therefore, the antisteroid has less agonist activity, compared to the full agonist, under condition B than it has under condition A. The PAA has decreased under condition B. Again, this is most easily recognized if, as above for the dose-response curves, the data are expressed as percent

of maximal activity above basal level (Fig. 1D). Now the PAA, simply read off of the graph without the necessity of any mental gymnastics, is clearly lower under condition B than condition A. It should be remembered that the PAA, not the absolute activity of an antisteroid, determines how effective (or ineffective) an antisteroid is in blocking the activity of endogenous agonist steroid. For these reasons, all of the EC_{50} and PAA results presented here will be in the above formats of percent of the A_{max} above basal, which is the same as percent of maximal fold induction.

2.2 A_{max} , EC_{50} , and PAA can be separately modulated

Glucocorticoid receptors (GRs) affect almost every tissue in the body – from the induction of surfactant in the lungs of premature infants to the repression of the immune system. For this reason, GRs are an excellent model for examining the conditions under which the A_{max} , EC_{50} , and PAA of steroid-induced gene expression can be modified. For GRs, as for the other steroid receptors, it was long suspected that studies of A_{max} would simultaneously yield answers to what controlled the EC_{50} and PAA. This belief stemmed from the initially close correlation between EC_{50} and steroid affinity for receptor and the observations that the higher affinity steroids yielded greater A_{max} values (Raynaud, 1980). At the same time, it was long thought that the PAA of antisteroids simply reflected a reduced efficiency of whatever factor(s) or process controlled that A_{max} of full agonist steroids. Therefore, it was surprising to find that A_{max} , EC_{50} , and PAA could be separately regulated in primary human peripheral blood mononuclear cells (PBMCs) (Luo and Simons; Jr., 2009). The levels of endogenous coactivator TIF2 in PBMCs were significantly reduced 48 hr after transient transfection of siRNAs targeting TIF2 or the control protein, Lamin (Fig. 2A). Steroids were added during the last 8 hr of tissue culture, after which the levels of three glucocorticoid inducible genes (GILZ, CD163, and THBS1) were determined by qRT-PCR using SybrGreen. Because only relative levels of each gene are available with SybrGreen, the fold induction was calculated. The fold induction is closely related to the A_{max} when the basal levels are relatively constant. At the same time, the EC_{50} and PAA with and without TIF2 siRNA were determined by the graphing procedures outlined above. As seen in Fig. 2B, the effect of reduced endogenous TIF2 protein varies with the gene examined. None of the parameters of CD163 are significantly affected. Interestingly, only some of the parameters of GILZ and THBS1 are influenced by lowering the level of TIF2. Furthermore, the affected parameters are not the same for the two genes. With THBS1, only the EC_{50} is altered by a decrease in TIF2 protein. With GILZ, only the EC_{50} is not changed, although the decrease in fold induction is not quite statistically significant. It thus appears that changing levels of cofactors can alter the A_{max} , EC_{50} , and PAA of endogenous genes under conditions that are physiologically relevant for humans. Furthermore, the ability of a factor to selectively modulate one or more induction parameters indicates that one set of molecular interactions can control A_{max} and that, under at least some conditions, different interactions determine the EC_{50} or PAA. A corollary is that the modulation of EC_{50} and PAA will most likely require some protein surfaces and factors that are different from those involved in the modulation of A_{max} . These new surfaces and proteins cannot be revealed by studies of A_{max} and will require analyses of EC_{50} and/or PAA.

We are not aware of any other studies like Fig. 2 where endogenous genes and proteins are examined in primary human cells. However, there have been numerous reports in which various transcriptional cofactors that influence A_{max} can also alter the EC_{50} and/or PAA of androgen, estrogen, mineralocorticoid, progesterin, and vitamin D3 receptors along with estrogen receptor β and thyroid receptor $\beta 2$ (Simons; Jr., 2008, Simons; Jr., 2010). Thus, it is reasonable to propose that this behavior of both gene-selective modulation of induction parameters and the ability to separately modify A_{max} , EC_{50} , and/or PAA will be found to be general for all steroid/nuclear receptors.

2.3 Dissociation of receptor binding and biological function of cofactors

The discovery of coactivators, corepressors, and comodulators (NURSA web site, Lonard and O'Malley, 2007) demonstrated that the $A_{\max}$ of steroid receptors can be regulated by the association of these macromolecules. The above report that individual induction parameters can be differentially modulated, in some cases by mechanisms that may be different from those controlling $A_{\max}$, raises the question of whether the $A_{\max}$, EC_{50} , and PAA could be altered via mechanisms other than cofactor binding. More specifically, if small molecules/drugs can change the interactions of receptor-bound cofactor with other components of the transcriptional machinery without altering the binding of cofactor to receptor, this would provide an exciting new avenue to influence steroid hormone actions (Simons; Jr., 2010). Importantly, several reports have described small molecules that inhibit the changes in $A_{\max}$ caused by transcription factors (Estebanez-Perpina et al., 2007, Parent et al., 2008, Hwang et al., 2009, Moore et al., 2010).

The first suggestion that this approach would be possible with GRs came from studies on the effects of mutations in the ligand binding cavity that were selected, on the basis of x-ray studies (Bledsoe et al., 2002, Suino-Powell et al., 2008), to discriminate between the binding of two glucocorticoids with very different molecular structures: Dex and DAC (Fig 3A) (Tao et al., 2008). Some of these mutations have relatively mild effects on the receptor binding affinity of either steroid (<6-fold decrease), with the apparent affinity of DAC being reduced less than that of Dex. Surprisingly, these same mutations decreased the potency (i.e., increased the EC_{50}) for induction of two exogenous reporter genes (GREtkLUC and MMTVLuc) in a steroid-dependent manner that was 15- to 90-fold greater than expected from the changes in steroid binding affinity. In most cases, the discrepancy was the greatest for Dex-bound mutant receptors (Fig. 3B). These mutations are close to, and could perturb, the coactivator/corepressor binding pocket of GR to thereby alter cofactor binding. This might explain the changes in EC_{50} because coactivators and corepressors competitively bind to GRs to modify the EC_{50} of transactivation (Wang et al., 2004). However, this explanation was discarded when no differences in the binding of the coactivator TIF2, or the corepressor NCoR, to mutant vs. wild type GRs complexed with either Dex or DAC were detected in two-hybrid bioassays (Tao et al., 2008). A clue to the increase in EC_{50} came from studies with truncated GRs, in which the ligand binding domain (LBD) of GR is fused to the GAL4 DNA binding domain (DBD). The $A_{\max}$, EC_{50} , and PAA of this chimera are regulated by TIF2, and other cofactors, just like the full length receptor (Cho et al., 2005). This simplifies mechanistic studies because the AF1 transactivation domain of the N-terminal half of GR, which is much more active for determining the $A_{\max}$ than the AF2 transactivation domain of the LBD, is no longer present. When exogenous TIF2 was added to whole cell transactivation assays with the mutant GAL/GR LBD chimeras, there was usually a much greater decrease in the EC_{50} of DAC-bound than Dex-bound receptors (Fig. 3C) (Tao et al., 2008). These results, coupled with the above mentioned unchanged TIF2 binding, indicated that while the affinity of TIF2 for GR was not altered by the mutations in the LBD, another surface(s) of the GR-bound TIF2 was perturbed to modify the activity of TIF2. Furthermore, the data suggest that the alterations in tertiary structure of GR-bound TIF2 would be more severe with the Dex-bound than the DAC-bound mutants receptors. In turn, the proposed more disruptive conformational changes in TIF2 bound to the GR-Dex complexes would cause a greater decrease in those interactions of TIF2 with other transcriptional components that are critical for determining the EC_{50} for gene induction.

Similar results have recently been reported for Ubc9 (Lee and Simons; Jr., 2011), which is another modulator of GR induction properties (Kaul et al., 2002). Using the above wild type and A625I GAL/GR LBD chimeras, the DAC-bound mutant receptor was shown to be much more responsive to added Ubc9 than the Dex-bound complex (Fig. 3D) even though all complexes bound Ubc9 equally well in co-immunoprecipitation assays (Lee and Simons; Jr.,

2011). Thus, the A625I mutation not only causes a more dramatic decrease in $A_{\max}$ with Dex than with DAC but also results in a significantly smaller increase in $A_{\max}$ with added Ubc9. Similarly, the EC_{50} of the mutant receptor without added Ubc9 is greater than that of the wild type GR with both Dex and DAC. However, with transfected Ubc9, the EC_{50} of the A625I mutant decreases when bound with DAC while it increases with Dex. The greater effect of Ubc9 on DAC- vs. Dex-bound mutant GRs was also seen for several endogenous genes, although the differences are manifested here only for $A_{\max}$ (Fig. 3E). This gene- and parameter-selective effect for endogenous targets is reminiscent of the above behavior of TIF2 in PBMCs. Collectively, these results suggest that the transcriptional activities of GR-bound cofactors are sensitive to subtle changes in GR LBD tertiary structure in a manner that does not necessarily affect cofactor binding to GRs. Furthermore, it is reasonable to expect that this dissociation of factor binding and activity will occur with a wider diversity of GR-associated factors.

A variety of reports suggest that this action at a distance, with no obvious alteration of intervening structures, is more common than previously suspected (Clarkson et al., 2006, Hilser and Thompson, 2007). For example, DNA binding of GRs stabilizes the GR AF-1 domain (Kumar and Thompson, 2003) while the DNA sequence can act as an allosteric ligand for transactivation by different mutant GRs independent of the DNA binding affinity of each GR (Meijsing et al., 2009). The $A_{\max}$ of GR-regulated gene induction is sensitive to altered ligand structure without affecting the binding of TIF2 peptides (Biggadike et al., 2009). The Jun dimerization protein 2 (JDP2) binds to progesterone receptor LBD but affects activity of the progesterone receptor AF-1 domain (Hill et al., 2009). Thus it seems likely that the binding and modulation of GR transactivation properties can be separated for a variety of transcriptional cofactors. The generality of this behavior with other receptors remains to be established but including determinations of the EC_{50} (and PAA) is a critical element in detecting the effects.

2.4 Why does the dose-response curve follow a first-order plot?

When considering steroid dose-response curves, two major questions are rarely addressed. First, what determines the shape of the sigmoidal dose-response curve? Second, why does the position, but not the shape, of the curve change with different concentrations of modulatory cofactor? With regard to the shape of the dose-response curve, steroid-regulated gene induction usually gives curves on semi-log plots that look like the standard sigmoidal curve of Michaelis-Menten kinetics. More precisely, such sigmoidal curves are first-order Hill-plot dose-response curves (FHDCs). It is easy for a one step reaction, like steroid binding to receptors, to give a FHDC. It is extremely difficult for a multi-step process in which factors combine to form larger complexes, as expected in steroid induction of gene transcription, to give a FHDC. In an effort to address these issues in a semi-quantitative manner, we have constructed from first principals a mathematical model for steroid hormone action that involves a series of complex forming reactions (Kim et al., 2006, Ong et al., 2010). The only precondition of the model is that complexes are relatively weakly bound and/or exist only transiently, both of which are biologically reasonable (Nagaich et al., 2004, Stavreva et al., 2004). This model faithfully reproduces the FHDCs that are seen both for simple gene induction experiments and for more complex responses, such as seen with different concentrations of exogenous GR and the comodulator Ubc9 where the position but not the shape of the curves change (Ong et al., 2010, Chow et al., 2011).

2.5 Information regarding where and how a factor acts as determined from studies of EC_{50} and $A_{\max}$

A significant consequence of the above model is that it can accommodate the addition of other reaction steps, as long as each reaction step being inserted has an output of product

that defines a FHDC with respect to its input. With this restriction, any number of steps can be inserted anywhere in the overall sequence and the new sequence will still follow a FHDC. Furthermore, the model indicates that there will usually be one step after which the concentration of bound cofactor is negligibly small compared to the free concentration of that factor. This step is called the concentration-limiting step (CLS). The CLS is a mathematically defined step that can be loosely considered analogous to the more familiar rate-limiting step in enzyme kinetics but the CLS is not necessarily a rate-limiting step *per se*. Because the model analyzes a steady-state system with no input or output flux, such as steroid-mediated gene induction in cells, it is not capable of revealing information about kinetic rates.

Analysis of the underlying equations for this model revealed that graphical methods can be used to determine the site of action (relative to the CLS), and kinetic nature of action, of any factor that changes the EC_{50} and/or A_{max} of the net reaction output (Ong et al., 2010, Chow et al., 2011). The most frequently used plots are A_{max}/EC_{50} and EC_{50}/A_{max} . The different possibilities, depending on whether linear or non-linear plots are observed, are listed in a decision tree format in Fig. 4A. Additional decision points are (1) whether the y-axis intercept of the linear A_{max}/EC_{50} plot is equal to zero or is greater than zero and (2) whether the non-linear EC_{50}/A_{max} plot approaches a greater than zero value G or not. Given these graphical behaviors, the factor is identified as an activator (A) or one type of inhibitor (competitive [C], linear [L], partial uncompetitive [PU], partial non-competitive [PN], or partial mixed [PM]). In many cases, the site of action of the factor, relative to the CLS, is also determined. Three points about this graphical analysis should be noted. First, one does not need to have any prior knowledge of how a factor works in order to use the graphical methods to determine the nature of factor activity and the position of its action relative to the CLS. Second, this information about a factor cannot be obtained by determining just the A_{max} . EC_{50} data are an obligatory component of the analysis. Third, to the best of our knowledge, this information is not yet obtainable by any other method. Assays such as chromatin immunoprecipitation, by itself (ChIP) and in combination with genome-wide sequencing of the binding sites (ChIP-seq), are powerful methods for determining when and where a factor binds (Metivier et al., 2003, John et al., 2008). However, these and other methods are unable to determine when and where a factor acts. As is clearly shown by paused RNA polymerase II (Gilchrist et al., 2008, Price, 2008, Fuda et al., 2009), the binding of a factor is often temporally separated from when it exerts its biological activity.

2.6 Characterization of factor activity via graphical analysis

An example of how this graphical analysis works is seen when considering the ability of the coactivator TIF2 to increase the A_{max} and decrease the EC_{50} of GR-mediated induction of luciferase protein from the exogenous reporter, GREtkLUC, in transiently transfected U2OS cells (Szapary et al., 1999, Simons; Jr., 2008, Chow et al., 2011). The luciferase activity seen with five concentrations of TIF2 plasmid, cotransfected with a constant amount of transfected GR plasmid and GREtkLUC reporter, is plotted against the different concentrations of the synthetic glucocorticoid steroid, dexamethasone (Dex). Exact curve fits of the data to a first-order Hill plot were performed by Kaleidagraph (Synergy Software) to obtain the EC_{50} and A_{max} for each set of reaction conditions. The ratio of A_{max}/EC_{50} gives a straight line when plotted against the amount of transfected TIF2 plasmid Fig. 4B. The table in Fig. 4A indicates that further interpretation of Fig. 4B involves the decision point of whether the linear plot of A_{max}/EC_{50} has a y-axis intercept of zero or greater than zero. This, in turn, requires knowledge of the position of the y-axis, i.e., when factor = 0. The underlying mathematics specifies that all references to “zero amounts of factor” mean when the total amount of factor (exogenous AND endogenous) is zero. To determine the amount of endogenous TIF2, in units of transfected TIF2 plasmid, we compared by Western

blotting the amount of TIF2 protein in U2OS cells without and with 20 ng of TIF2 plasmid (Fig. 4B insert). Densitometric analysis indicated that the total TIF2 with 20 ng of TIF2 plasmid was 1.6-fold greater than that with no added plasmid. From this it can be calculated that the point of no TIF2 in the cells corresponds to -12.7 ng of TIF2 plasmid and that the true y-axis is located as indicated by the dashed line in Fig. 4B. This means that the y-axis intercept is > 0 . From Fig. 4A, we can now conclude that TIF2 acts as an activator after the CLS.

It is well known that TIF2 acts as a coactivator but the precise kinetic mechanism of action has not yet been defined. Every factor that increases $A_{\max}$ is not necessarily an activator. For example, an inhibitor can be activating if it diverts the reaction scheme output from the ordinarily preferred, low efficiency/low yield step to a higher yielding step. The studies of Fig. 4 indicate that the coactivator activity of TIF2 in this system is indeed due to TIF2 acting as a kinetically defined activator. This is entirely consistent with the known properties of TIF2 (Bledsoe et al., 2002). The present studies further locate the position of TIF2 biological activity as being after the CLS. We note that the CLS is only defined within a zero flux steady state context and hence does not make any definitive statement about rates of product formation for which the concept of a rate-limiting step would be applicable. Hence, the fact that TIF2 acts after the CLS suggests, but does not necessarily imply, that TIF2 acts after the rate-limiting step of the gene induction of Fig. 4. All of the existing information regarding TIF2 action is limited to where in the reaction scheme TIF2 binds (e.g., Metivier et al., 2003). Because the site of factor binding is not equivalent to the site of factor action (viz. paused RNA polymerase II), it is not yet possible to compare this new information to what previously has been described for TIF2. More precise localization of the site of TIF2 action is currently prevented by a lack of knowledge regarding the nature of the CLS. It has also not yet been demonstrated that the CLS is the same under all reactions conditions. Under most circumstances, we suspect that the CLS remains constant. In this case, similar analyses of other cofactors for GR-regulated gene induction in the same cell will be able to construct an ordering of the actions of the different factors relative to the CLS.

3. Conclusions

This review discusses new mechanistic information about glucocorticoid receptor action that has recently been obtained by considering steroid potency (or EC_{50}) of agonists and the partial agonist activity of antagonists (PAA) in addition to the classical endpoint of $A_{\max}$ for agonists. First, these three parameters can be independently influenced by factor concentration for endogenous genes in primary human cells. This suggests that different interactions, and probably different factors, can modulate $A_{\max}$, EC_{50} , and PAA under conditions that are relevant to human physiology. Thus, substantial realms of GR action will be missed if only changes in $A_{\max}$ are followed. Conversely, these results suggest that one parameter, such as the PAA of an antisteroid being used in endocrine therapy, can be selectively modified. This would be a major advance in efforts to use antagonists to block agonist steroid induction of one gene, or in one cell type, while preserving most of the desired functions of the endogenous hormone. An example would be in limiting antiestrogen action to only preventing breast cancer tumor growth. Second, factor binding is not the only method by which the final activity of GRs can be modulated. Studies with mutant GRs suggest that changes in cofactor conformation, independent of cofactor binding to GRs, can modify the final induction parameters. These results offer the possibility of cofactors, and other downstream participants of GR-regulated gene expression, being targets for therapeutic intervention (Simons; Jr., 2010). This greatly expands the number of available targets for pharmaceuticals, which in turn significantly increases the probability of finding new clinically useful compounds. Third, a new theoretically based model of steroid hormone

action that correctly predicts several properties of GR action (Ong et al., 2010) is shown to be able to deduce both the kinetic mechanism of action of a factor and where it acts, relative to the CLS. This was demonstrated for the known coactivator TIF2 and should work for any factor that modifies the $A_{\max}$ and/or EC_{50} of GR-regulated gene expression. To the best of our knowledge, this information is not obtainable by any other existing methodology. This approach will also be able to put on a firm mechanistic footing those questions that are not easily resolved by studies of $A_{\max}$ alone, such as the precise activity of a factor that increases gene repression. An additional benefit would be in suggesting drug targets that would have fewer side-effects. Clearly, inhibiting a factor acting after the CLS would evoke fewer unwanted reactions than blocking a factor functioning at an earlier step, before the CLS.

It has long been known that the EC_{50} for gene induction is not constant in different cells for a given receptor-steroid complex (Simons; Jr., 2003, Simons; Jr., 2006, Simons; Jr., 2008, Simons; Jr., 2010). More recently, it has been established that changing levels of cofactors also alter the EC_{50} of the same gene in the same cell by the same receptor-steroid complex. These unpredicted differences in gene regulation are clearly important for understanding differential gene expression during development, differentiation, and homeostasis. Thus, it is very important to know “HOW” this modulation occurs. From the examples discussed in this review, it is evident that limited information is available from studies of steroid receptor action with saturating, pharmacological levels of steroid to determine the effects on $A_{\max}$. Not only are saturating concentrations of steroid rarely encountered in physiology but also additional studies of EC_{50} and PAA reveal mechanistic insight that cannot be abstracted from studies of $A_{\max}$ alone.

Most studies of steroid hormone action have concentrated on $A_{\max}$ for two apparent reasons. First, they are simpler to conduct than those examining PAA and especially EC_{50} . Second, and more scientific, has been the assumption that anything reducing or increasing the $A_{\max}$ at saturating steroid concentrations would have similar effects at subsaturating concentrations. Numerous studies now challenge this assumption and indicate that very different effects via different mechanisms are often seen not only at subsaturating, physiological concentrations of steroid but also for the different parameters, $A_{\max}$, EC_{50} , and PAA. The methods of Fig. 4 have recently been dramatically expanded in a manner that appears capable of screening previously ignored factors for involvement in far downstream steps of GR-mediated transactivation (Chow et al, 2011). The closer these steps are to the final biological response for the gene of interest, the greater the prospect of selective control of hormonal therapies in the clinical setting. Thus, the major advantages of expanding one's experimental horizon to include EC_{50} and PAA may yet to be realized.

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References

- Biggadike K, Bledsoe RK, Coe DM, Cooper TW, House D, Iannone MA, Macdonald SJ, Madauss KP, McLay IM, Shipley TJ, Taylor SJ, Tran TB, Uings IJ, Weller V, Williams SP. Design and x-ray crystal structures of high-potency nonsteroidal glucocorticoid agonists exploiting a novel binding site on the receptor. *Proc Natl Acad Sci U S A*. 2009; 106:18114–18119. [PubMed: 19822747]
- Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, Lambert MH, Moore JT, Pearce KH, Xu HE. Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell*. 2002; 110:93–105. [PubMed: 12151000]

- Cho S, Kagan BL, Blackford JA Jr, Szapary D, Simons SS Jr. Glucocorticoid receptor ligand binding domain is sufficient for the modulation of glucocorticoid induction properties by homologous receptors, coactivator transcription intermediary factor 2, and Ubc9. *Mol. Endo.* 2005; 19:290–311.
- Chow CC, Ong KM, Dougherty EJ, Simons SS Jr. Inferring mechanisms from dose-response curves. *Methods in Enzymology*. 2011 in press.
- Clarkson MW, Gilmore SA, Edgell MH, Lee AL. Dynamic coupling and allosteric behavior in a nonallosteric protein. *Biochemistry*. 2006; 45:7693–7699. [PubMed: 16784220]
- Estebanez-Perpina E, Arnold AA, Nguyen P, Rodrigues ED, Mar E, Bateman R, Pallai P, Shokat KM, Baxter JD, Guy RK, Webb P, Fletterick RJ. A surface on the androgen receptor that allosterically regulates coactivator binding. *Proc Natl Acad Sci U S A*. 2007; 104:16074–16079. [PubMed: 17911242]
- Fuda NJ, Ardehali MB, Lis JT. Defining mechanisms that regulate RNA polymerase II transcription in vivo. *Nature*. 2009; 461:186–192. [PubMed: 19741698]
- Gilchrist DA, Nechaev S, Lee C, Ghosh SK, Collins JB, Li L, Gilmour DS, Adelman K. NELF-mediated stalling of Pol II can enhance gene expression by blocking promoter-proximal nucleosome assembly. *Genes Dev*. 2008; 22:1921–1933. [PubMed: 18628398]
- Hackney JF, Gross SR, Aronow L, Pratt WB. Specific glucocorticoid-binding macromolecules from mouse fibroblasts growing in vitro. A possible steroid receptor for growth inhibition. *Mol Pharmacol*. 1970; 6:500–512. [PubMed: 4318965]
- Herdick M, Bury Y, Quack M, Uskokovic MR, Polly P, Carlberg C. Response element and coactivator-mediated conformational change of the vitamin D(3) receptor permits sensitive interaction with agonists. *Mol. Pharmacol*. 2000; 57:1206–1217. [PubMed: 10825392]
- Hill KK, Roemer SC, Jones DN, Churchill ME, Edwards DP. A progesterone receptor co-activator (JDP2) mediates activity through interaction with residues in the carboxyl-terminal extension of the DNA binding domain. *J Biol Chem*. 2009; 284:24415–24424. [PubMed: 19553667]
- Hilser VJ, Thompson EB. Intrinsic disorder as a mechanism to optimize allosteric coupling in proteins. *Proc Natl Acad Sci U S A*. 2007; 104:8311–8315. [PubMed: 17494761]
- Hwang JY, Arnold LA, Zhu F, Kosinski A, Mangano TJ, Setola V, Roth BL, Guy RK. Improvement of pharmacological properties of irreversible thyroid receptor coactivator binding inhibitors. *J Med Chem*. 2009; 52:3892–3901. [PubMed: 19469546]
- John S, Sabo PJ, Johnson TA, Sung MH, Biddie SC, Lightman SL, Voss TC, Davis SR, Meltzer PS, Stamatoyannopoulos JA, Hager GL. Interaction of the glucocorticoid receptor with the chromatin landscape. *Mol Cell*. 2008; 29:611–624. [PubMed: 18342607]
- Kaul S, Blackford JA Jr, Cho S, Simons SS Jr. Ubc9 is a novel modulator of the induction properties of glucocorticoid receptors. *J. Biol. Chem*. 2002; 277:12541–12549. [PubMed: 11812797]
- Kim Y, Sun Y, Chow C, Pommier YG, Simons SS Jr. Effects of acetylation, polymerase phosphorylation, and DNA unwinding in glucocorticoid receptor transactivation. *J. Steroid Biochem. Molec. Biol*. 2006; 100:3–17. [PubMed: 16723222]
- Kumar R, Thompson EB. Transactivation functions of the N-terminal domains of nuclear hormone receptors: protein folding and coactivator interactions. *Mol. Endocrinol*. 2003; 17:1–10. [PubMed: 12511601]
- Lee G-S, Simons SS Jr. Ligand binding domain mutations of glucocorticoid receptor selectively modify effects with, but not binding of, cofactors. *Biochemistry*. 2011 in press.
- Lonard DM, O'Malley BW. Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation. *Mol Cell*. 2007; 27:691–700. [PubMed: 17803935]
- Luo M, Simons SS Jr. Modulation of glucocorticoid receptor induction properties by cofactors in peripheral blood mononuclear cells. *Human Immunology*. 2009; 70:785–789. [PubMed: 19665040]
- May FEB, Westley BR. Identification and characterization of estrogen-regulated RNAs in human breast cancer cells. *J. Biol. Chem*. 1988; 263:12901–12908. [PubMed: 2458337]
- Meijsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR. DNA binding site sequence directs glucocorticoid receptor structure and activity. *Science*. 2009; 324:407–410. [PubMed: 19372434]

- Mercier L, Thompson EB, Simons SS Jr. Dissociation of steroid binding to receptors and steroid induction of biological activity in a glucocorticoid responsive cell. *Endocrinology*. 1983; 112:601–609. [PubMed: 6129134]
- Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F. Estrogen receptor- α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell*. 2003; 115:751–763. [PubMed: 14675539]
- Metivier R, Reid G, Gannon F. Transcription in four dimensions: nuclear receptor-directed initiation of gene expression. *EMBO Rep*. 2006; 7:161–167. [PubMed: 16452926]
- Moore TW, Mayne CG, Katzenellenbogen JA. Minireview: Not picking pockets: nuclear receptor alternate-site modulators (NRAMs). *Mol Endocrinol*. 2010; 24:683–695. [PubMed: 19933380]
- Munck A, Holbrook NJ. Glucocorticoid-receptor complexes in rat thymus cells. Rapid kinetic behavior and a cyclic model. *J. Biol. Chem*. 1984; 259:820–831. [PubMed: 6693398]
- Nagaich AK, Walker DA, Wolford R, Hager GL. Rapid periodic binding and displacement of the glucocorticoid receptor during chromatin remodeling. *Mol Cell*. 2004; 14:163–174. [PubMed: 15099516]
- NURSA web site.
<http://www.nursa.org/template.cfm?threadId=10222&dataType=Q-PCR&dataset=Tissuespecific%20expression%20patterns%20of%20nuclear%20receptors>
- Ong KM, Blackford JA Jr, Kagan BL, Simons SS Jr, Chow CC. A new theoretical framework for gene induction and experimental comparisons. *Proc Natl Acad Sci U S A*. 2010; 107:7107–7112. [PubMed: 20351279]
- Parent AA, Gunther JR, Katzenellenbogen JA. Blocking estrogen signaling after the hormone: pyrimidine-core inhibitors of estrogen receptor-coactivator binding. *J Med Chem*. 2008; 51:6512–6530. [PubMed: 18785725]
- Price DH. Poised polymerases: on your mark.get set.go! *Mol Cell*. 2008; 30:7–10. [PubMed: 18406322]
- Raynaud JP, Bouton MM, Ojasoo T. The use of interaction kinetics to distinguish potential antagonists from agonists. *TIPS*. 1980:324–327.
- Rosenfeld MG, Lunyak VV, Glass CK. Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev*. 2006; 20:1405–1428. [PubMed: 16751179]
- Rousseau GG, Baxter JD. Glucocorticoid receptors. *Glucocorticoid Hormone Action*. 1979:49–77.
- Simons SS Jr. The importance of being varied in steroid receptor transactivation. *TIPS*. 2003; 24:253–259. [PubMed: 12767725]
- Simons SS Jr. How much is enough? Modulation of dose-response curve for steroid receptor-regulated gene expression by changing concentrations of transcription factor. *Current Topics in Medicinal Chemistry*. 2006; 6:271–285. [PubMed: 16515481]
- Simons SS Jr. What goes on behind closed doors: physiological versus pharmacological steroid hormone actions. *Bioessays*. 2008; 30:744–756. [PubMed: 18623071]
- Simons SS Jr. Glucocorticoid receptor co-factors as therapeutic targets. *Current Opin. Pharmacology*. 2010; 10:613–619.
- Stavreva DA, Muller WG, Hager GL, Smith CL, McNally JG. Rapid glucocorticoid receptor exchange at a promoter is coupled to transcription and regulated by chaperones and proteasomes. *Mol. Cell. Biol*. 2004; 24:2682–2697. [PubMed: 15024059]
- Suino-Powell K, Xu Y, Zhang C, Tao YG, Tolbert WD, Simons SSJ, Xu HE. Doubling the size of the glucocorticoid receptor ligand binding pocket by deacylcortivazol. *Mol Cell Biol*. 2008; 28:1915–1923. [PubMed: 18160712]
- Szapary D, Huang Y, Simons SS Jr. Opposing effects of corepressor and coactivators in determining the dose-response curve of agonists, and residual agonist activity of antagonists, for glucocorticoid receptor regulated gene expression. *Mol. Endocrinol*. 1999; 13:2108–2121. [PubMed: 10598585]
- Tao Y-G, Xu Y, Xu HE, Simons SS Jr. Mutations of glucocorticoid receptor differentially affect AF2 domain activity in a steroid-selective manner to alter the potency and efficacy of gene induction and repression. *Biochemistry*. 2008; 47:7648–7662. [PubMed: 18578507]

- Varmus HE, Ringold G, Yamamoto KR. Regulation of mouse mammary tumor virus gene expression by glucocorticoid hormones. *Glucocorticoid Hormone Action*. 1979;253–278.
- Wan W, Farboud B, Privalsky ML. Pituitary resistance to thyroid hormone syndrome is associated with T3 receptor mutants that selectively impair beta2 isoform function. *Mol Endocrinol*. 2005; 19:1529–1542. [PubMed: 15802373]
- Wang Q, Blackford JA Jr, Song L-N, Huang Y, Simons SS Jr. Equilibrium interactions of corepressors and coactivators modulate the properties of agonist and antagonist complexes of glucocorticoid receptors. *Mol. Endocrinol*. 2004; 18:1376–1395. [PubMed: 15016838]
- Wu SC, Zhang Y. Minireview: role of protein methylation and demethylation in nuclear hormone signaling. *Mol Endocrinol*. 2009; 23:1323–1334. [PubMed: 19407220]
- Yoon HG, Wong J. The corepressors silencing mediator of retinoid and thyroid hormone receptor and nuclear receptor corepressor are involved in agonist-and antagonist-regulated transcription by androgen receptor. *Mol Endocrinol*. 2006; 20:1048–1060. [PubMed: 16373395]
- Zajchowski DA, Kauser K, Zhu D, Webster L, Aberle S, White FAr, Liu HL, Humm R, MacRobbie J, Ponte P, Hegele-Hartung C, Knauth R, Fritzemeier KH, Vergona R, Rubanyi GM. Identification of selective estrogen receptor modulators by their gene expression fingerprints. *J. Biol. Chem*. 2000; 275:15885–15894. [PubMed: 10748166]

Fig. 1.

Graphical evaluation of A_{max} , EC_{50} , and PAA. (A) Raw data for agonist steroid induction of a luciferase reporter gene under two conditions (A and B). The position of the EC_{50} under each condition is indicated by the dashed vertical line. The maximum plateau value of luciferase activity for each condition is labeled A_{max} . (B) Normalized data for agonist steroid induction of a luciferase reporter gene under two conditions (A and B). The data of panel A are expressed as percent of maximal activity (A_{max}) under the same condition. (C) Raw data for induction of a luciferase reporter gene without or with saturating concentrations of agonist or antagonist steroid under two conditions (A and B). (D) Normalized data for agonist and antagonist steroid induction of a luciferase reporter gene under two conditions (A and B). The data of panel C are expressed as percent of maximal activity (A_{max}) under the same condition.

Fig. 2.

Level of coactivator TIF2 differentially affects the induction parameters of endogenous genes in PBMCs. (A) Transient transfection of TIF2 siRNA for 48 hr reduces endogenous TIF2 protein relative to control (Lamin siRNA). Western blotting with anti-TIF2 antibody reveals the level of endogenous TIF2 after the indicated treatments. (B) Reduced cellular levels of TIF2 alter the fold induction, EC₅₀, and PAA of the antiglycorticoid Dex-Mes (DM) for endogenous genes. The average values ($\pm$ S.E.M.) of fold induction (approximately equal to $A_{\max}$ because uninduced levels are very similar), EC₅₀, and PAA (% DM) activity of gene induction in PBMCs treated with TIF2 (+) or Lamin (-) siRNA for three endogenous genes from 6–7 independent experiments are plotted. Two-tailed p-values are * $P=0.038$, ** $P=0.0008$ (from Luo and Simons; Jr., 2009).

A

B

C

D

E

Fig. 3.

GR LBD mutations modify factor binding to GR much less than the transcriptional activity of GR-bound factor. (A) Structures of Dex and DAC. (B–D) Changes in induction parameters with receptor mutation for induction of exogenous reporter genes by different forms of GR plus added cofactors in transiently transfected CV-1 cells. EC₅₀ of GREtkLUC (S.E.M., n = 5–8) and MMTVLUC (S.E.M., n = 2) reporters induced by full length GR in (B) is altered by mutations in LBD in a manner that is sensitive to agonist structure (Dex vs. DAC) with no added cofactor. In (C), the EC₅₀ of a GAL-regulated reporter (FRLuc) induced by wild type and mutant GAL/GR525C receptors plus Dex or DAC is selectively modified by the presence of cotransfected TIF2 (S.E.M., n = 5). Similarly, in (D), the A_{max}, PAA of the antiglucocorticoid DM vs. Dex, and EC₅₀ of the FRLuc reporter induced by wild type and mutant GAL/GR525C receptors plus Dex or DAC is differentially sensitive to the presence of cotransfected Ubc9 (S.E.M., n = 7). Two-tailed p-values vs. wt GR are * P<0.05, ** P<0.005, *** P<0.0005. (E) Variation in induction parameters of endogenous genes by wild type and mutant full length GRs in U2OS cells with and without exogenous Ubc9 depends upon steroid structure (Dex, DM, or DAC; S.E.M., n = 5–7 with Dex and DM and n = 4 with DAC). (from Tao et al., 2008, Lee and Simons; Jr., 2011)

Fig. 4.

Graphical analysis of how and where cofactors modulate GR transactivation. (A) Decision tree for determining factor mechanism from plots of $A_{\max}$ and EC_{50} . The decision point “Approaches G” means that if the non-linear plot of $EC_{50}/A_{\max}$ approaches a plateau value G at infinite factor concentration (or $A_{\max}/EC_{50}$ approaches $1/G$), then one needs to determine the nature of the plot of the reciprocal of $[(A_{\max}/EC_{50}) - (1/G)]$. (B) Determination of TIF2 coactivator properties. The $A_{\max}$ and EC_{50} values for wild type GR transactivation of GREtkLUC with different concentrations of co-transfected TIF2 in U2OS cells were generated by a curve-fitting program (Kalediagraph, Synergy Software). These values were then used to plot $A_{\max}/(100 \times EC_{50})$. The straight line represents the computer-

generated best fit ($R^2 = 0.94$). The insert in B shows the Western blots with anti-TIF2 antibody of lysates from cells that had been transfected under the parallel conditions with 0 or 20 ng of TIF2 plasmid. From these data, it can be determined that the true y-axis is as indicated by the dashed vertical line (see text for details). (from Chow et al., 2011)