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What goes on behind closed doors: physiological vs. pharmacological steroid hormone actions

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Summary

Steroid hormone-activated receptor proteins are among the best understood class of factors for altering gene transcription in cells. Steroid receptors are of major importance in maintaining normal human physiology by responding to circulating concentrations of steroid in the nM range. Nonetheless, most studies of steroid receptor action have been conducted using the supra-physiological conditions of saturating concentrations (≥ 100 nM) of potent synthetic steroid agonists. Here we summarize the recent developments arising from experiments using two clinically relevant conditions: subsaturating concentrations of agonist (to mimic the circulating concentrations in mammals) and saturating concentrations of antagonists (which are employed in endocrine therapies to block the actions of endogenous steroids). These studies have revealed new facets of steroid hormone action that could not be uncovered by conventional experiments with saturating concentrations of agonist steroids, such as a plethora of factors/conditions for the differential control of gene expression by physiological levels of steroid, a rational approach for examining the gene-specific variations in partial agonist activity of antisteroids, and a dissociation of steroid potency and efficacy that implies the existence of separate, and possibly novel, mechanistic steps and cofactors.

Introduction

Steroid hormones are of major importance for the development, differentiation, and homeostasis of humans and vertebrates in general. Both agonist and antagonist steroids are involved, where antagonists or antisteroids are compounds that prevent the action of agonist steroids and are used in endocrine therapies to counter the actions of endogenous steroid. Five classes of steroid hormones are produced in humans (androgens, estrogens, glucocorticoids, mineralocorticoids, and progestins) and each preferentially binds to one of the 48 members of the steroid/nuclear receptor superfamily of intracellular proteins that are expressed in humans (1). Non-steroidal compounds, like 3,5,3'-triiodothyronine and vitamin D₃, are the common ligands for the nuclear receptors but most nuclear receptors are "orphan receptors" with no known ligands. The conventional mode of action of steroid hormones begins by its binding to the ligand binding domain (LBD) of the cognate steroid receptor (Fig. 1). The resulting receptor-steroid complex binds to specific, biologically active DNA sequences (called hormone response elements, or HREs) of the nuclear chromatin, where a variety of cofactors are recruited both to modify the chromosomal organization to varying extents and to interact with the transcription complex, thus altering the rates of transcription of relatively nearby DNA sequences (2–4). Antagonists act by preventing agonist binding to receptors and by forming a comparatively transcriptionally inactive receptor-steroid complex. The time scale of these transcriptional effects on mRNA synthesis is on the order of 15–30 min. More recently, it has become clear that steroid hormones can initiate even more rapid responses via membrane-

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bound receptors and “non-genomic” pathways, so called because the receptor does not interact with genomic DNA. The properties of these “non-genomic” pathways will not be considered here.

Most studies of steroid-regulated gene transcription have been conducted at saturating, or pharmacological, concentrations of agonist steroids. Such studies assure maximal response and require a minimum of experimental points (i.e., vehicle and a single high concentration of steroid). However, physiology occurs at subsaturating concentrations of ligand for the steroid/nuclear receptors. It has been tacitly assumed that what happens at saturating concentrations of steroid also occurs at subsaturating concentrations. However, recent studies in rats indicate a much more rapidly cycling nuclear localization of GR with physiological vs. higher concentrations of the natural glucocorticoid, corticosterone (5). Furthermore, it is obviously impossible for studies with a single high agonist steroid concentration to yield answers about physiologically important steps that depend upon steroid concentration. For example, steroid potency or the positioning of the dose-response curve, which gives the amount of response for steroid concentrations causing 0–100% occupancy of the cognate receptor, cannot be determined from experiments with a saturating concentration of steroid that bind all of the receptors. Likewise, the causes for the frequent observation that gene induction by GRs requires a 10-fold higher steroid concentration than gene repression, even though the same receptor-steroid complex is involved (6–9), will not be elucidated by studies at saturating steroid concentrations. Also, an antisteroid, by definition, blocks the action of an agonist steroid but usually retains some residual activity of its own. What dictates the amount of residual agonist activity of an antisteroid cannot be elucidated from studies with only an agonist steroid. The current model of steroid hormone action suggests that the dose-response curve for steroid binding to a receptor determines the dose-response curve for steroid-activated, receptor-mediated gene induction (or repression) (4,10,11). This predicts that all responses regulated by a given receptor-steroid complex in an organism will display both the same potency for an agonist and equal amounts of partial agonist activity for an antisteroid, when expressed as percent of maximal agonist activity under the same conditions (= percent partial agonist activity). Abundant evidence has established that this view is incorrect. The immediate question then is how can the same receptor-steroid complex produce different dose-response curves for agonists, or unequal amounts of percent partial agonist activities for antagonists?

An equally important question is how much difference in the positioning of the dose-response curve (or the amount of partial agonist activity) is required to elicit physiologically relevant changes? The answer appears to be “not much”. A central tenet of developmental biology is that relatively small differences in the concentrations of appropriate factors induce different developmental fates in cells [Wolpert, L, J. *Theor. Biology*, 25, 1–47 (1969) from *Nature*, July 26, 420–421 (2007) = news and views]. It has been calculated that a 10% difference in the concentration of Bicoid is sufficient for morphogenic effects in the *Drosophila* embryo (12), although arguments have been made for the necessity of slightly larger changes (13). Small differences in Myc levels are adequate for numerous cell-specific effects (14) with a 50% reduction in c-Myc causing teleomer-independent senescence in human fibroblasts (15). The developmental stages of *Drosophila* larvae are exquisitely sensitive to changes in the level of the hormone ecdysone (16). A three-fold variation in the concentrations of Decapentaplegic or activin is adequate for unequal developmental responses in *Drosophila* and *Xenopus* respectively (reviewed in 17) while a 2- to 5-fold alteration in fibroblast growth factor is sufficient to trigger the differentiation of presomitic mesoderm into somites in mouse embryos (18). Thus, there is ample precedent that small differences in protein expression, achieved with subsaturating concentrations of steroid hormone, can have significant physiological consequences.

The objectives of this article are to review some of the transcription properties that transpire behind the “closed doors” of high agonist steroid concentrations and are missed when looking only at these saturating steroid concentrations. We summarize the evidence that these parameters of gene induction (and repression) are not static but can be modulated. Advances in elucidating the mechanisms by which factors modulate these parameters are presented, with an emphasis on the last two years. Finally, the physiological relevance of this modulation is discussed. Most of the data involves the classical steroid hormone receptors but some with nuclear receptors are available.

Theoretical background

The experimental protocols used over the last 40+ years to unravel the mysteries of steroid hormone action almost invariably employed a single saturating, or pharmacological, concentration of agonist steroid. This approach yields only the maximum amount of steroid-regulated gene expression, as shown in Fig. 2A for the induction of two genes A and B. For the purposes of this review, we call the maximum activity the A_{\max} . This value is also called the efficacy of the steroid but due to some confusion as to its precise meaning we will use A_{\max} here. More information is obtained if lower concentrations of agonist steroid are included to examine steroid potency (Fig. 2B). Under these circumstances, the concentration of steroid required for half-maximal induction, or EC_{50} , can be determined. As was found many years ago, the concentration of circulating cortisol in humans (80–600 nM) is about the same as the EC_{50} for some cortisol-induced effects (19). Importantly, changing the EC_{50} for steroid-regulated gene expression will obviously alter the response of that gene. Thus, Gene B, with a lower EC_{50} , will be maximally induced at a lower steroid concentration than gene A, which is important for the differential induction of gene B vs. A during development, differentiation, and homeostasis. This also means that the activity with 100 nM agonist should not be assumed to be maximal. A right shift in the dose-response curve can easily move the EC_{50} to values ≥ 100 nM (20,21). It should also be noted that there is no observed, or theoretical, correlation between A_{\max} (Fig. 2A) and EC_{50} (Fig. 2B) so that studies organized as in Fig. 2A are unable to give any information about the EC_{50} of the gene of interest.

Additional information is obtained if pharmacological concentrations of an antagonist, or antisteroid, are included (Fig. 2B). Antisteroids are used at these concentrations during endocrine therapies to block the actions of agonist steroids. However, the amount of residual agonist activity of each antisteroid is currently impossible to predict and must be experimentally determined. A useful parameter, when discussing the residual agonist activity of antisteroids, is the percent partial agonist activity.

While the precise values for the EC_{50} , or potency, and percent partial agonist activity can be calculated from plots of the raw data, as in Fig. 2B, it is much easier if the data are first replotted as percent of maximal induction for each gene, as in Fig. 2C. In this case, the EC_{50} and percent partial agonist activity can be readily read off of the graph. Such plots also clearly demonstrate that the EC_{50} is independent of the A_{\max} of gene induction. The importance of comparing the activities of antagonist and agonist under the same conditions cannot be overemphasized. The residual activity of the antisteroid is about the same for genes A and B (Fig. 2B) but that the percent partial agonist activity is much greater with gene B than with gene A (Fig. 2C). Replotting the data as in Fig. 2C will also immediately clarify whether or not reports of increased sensitivity (i.e, more activity at low concentrations of agonist), or more response with high levels of antisteroid, actually reflect a change in EC_{50} , or percent partial agonist activity respectively.

The current model of steroid hormone action says little about the determinants of the EC_{50} . Early observations of a close correlation between the K_d for steroid binding to receptors and

the EC₅₀ for steroid-regulated gene induction were viewed as evidence that the steroid was acting via binding to its cognate receptor. In fact, it has been known for many years that such a correlation is not general (22 and reviewed in 23,24). It should also be noted that high steroid binding affinity does not assure high efficacy of biological response (A_{max}). RU486 has a high affinity for GR (25) but a negligible A_{max} for gene induction under most conditions (26,27). Under conditions where the affinity label Dexamethasone 21-mesylate (Dex-Mes) covalently binds to >90% of the receptors, it displays ≤7% of maximal activity seen with Dex (28). Thus, detailed studies of the EC₅₀ with sub-saturating concentrations of agonists, and the percent partial agonist activity with saturating concentrations of antagonists, are required to gain an understanding of the determinants of these relevant parameters. Finally, as is described below, such experiments have revealed that the EC₅₀, and percent partial agonist activity, are not constant even for a single gene but can be modulated by a variety of factors.

Modulatory factors and their mechanism of action

Glucocorticoid modulatory element (GME)

The first example of a modulator of the EC₅₀, and percent partial agonist activity, of GR-regulated gene induction was a *cis*-acting 21 bp element, called the GME, at -3.6 kb of the rat TAT gene (29). Interestingly, the GME modulates the EC₅₀, and percent partial agonist activity, for GR induction of an exogenous reporter (GREtkLUC) in transiently transfected mouse mammary adenocarcinoma cells (1470.2 cells) but has no effect on these parameters for progesterone receptor (PR)-mediated induction of the same gene in the same cells (30). Thus there is receptor-selectivity of GME actions in 1470.2 cells.

GME-binding proteins 1 and 2 (GMEB-1 and GMEB-2)

An oligomeric complex of two proteins, GMEB-1 and -2, was found to participate in the actions of the GME (31). Increasing amounts of each GMEB, either alone or in combination in CV-1 and 1470.2 cells, cause a progressively larger right-shift to higher steroid concentrations for the GR dose-response curve of a GMEGREtkLUC reporter, and decrease in percent partial agonist activity, presumably due to squelching (30,32). In contrast, low concentrations of GMEB-2 with PRs produce a left-shift in the dose-response curve to lower steroid concentrations and an increase in the percent partial agonist activity of antiprogesterin. At higher GMEB-2 concentrations, PR and GR respond similarly (30). This suggests that PR is less sensitive to GMEB-2 than GR and needs higher GMEB-2 concentrations to see similar effects. This would also explain why the GME, with endogenous levels of GMEB-2, affects GRs but not PRs.

Changing concentrations of steroid receptor

The K_d for steroid (S) binding to receptor (R) is defined as $[S][R]/[RS \text{ complex}]$. Just as the concentration of steroid receptor does not affect the binding affinity of steroid, different receptor concentrations would not be expected to alter the EC₅₀, and percent partial agonist activity, for gene induction. This prediction, however, is wrong. Since the initial report that GR concentration influences not only the A_{max} but also the EC₅₀, and percent partial agonist activity, of GR-induced transcription (33), the same phenomenon has been observed for each of the classical steroid receptors (reviewed in 23,24). Gene repression by steroid receptors usually proceeds via a slightly different mechanism involving receptor-steroid complexes that are tethered to DNA-bound proteins as opposed to binding directly to DNA, as seen in induction (34,35). Nonetheless, it has been shown that increasing GR protein also causes the same decrease in EC₅₀ to lower steroid concentrations, and increase in percent partial agonist activity of antiglucocorticoids, in gene repression (36,37) as observed for gene induction.

Posttranslationally modified receptors

The possibility that posttranslational modification of receptors and factors could influence the dose-response curve (or potency or EC_{50}) and/or percent partial agonist activity is a very attractive hypothesis that would dramatically expand the number and variety of control mechanisms. We are aware of only one possible example in which the hepatitis B virus nonstructural protein, X protein (HBx), causes an increase in both EC_{50} and A_{max} . HBx did not physically interact with nuclear androgen receptor (AR)-ligand complexes. Instead, the authors speculate that increased AR activity occurs by elevating AR phosphorylation through HBx-mediated activation of the c-Src kinase signaling pathway, although a direct response with increased AR phosphorylation remains to be established (38).

Mutant receptors

Receptor mutations that affect steroid binding affinity would be expected to alter the EC_{50} of gene expression in a parallel fashion. However, the K303R mutation at the junction of the hinge and LBD of the human estrogen receptor alpha ($ER\alpha$) produces a left-shift in dose-response curve for cell growth and increased affinity for the coactivators (TIF2 and AIB1) without any change in steroid binding affinity (39). Several mutations in the GR LBD have recently been described that increase the EC_{50} of induction of exogenous and endogenous genes by ≥ 100 -fold while increasing the K_d of steroid binding by < 6 -fold (40). This dissociation of EC_{50} and K_d is not unique to the classical steroid receptors. The L422R mutation in the LBD of thyroid receptor beta 1 ($TR\beta 1$) alters the EC_{50} for gene induction independent of steroid binding affinity but dependent on the DNA sequence of the thyroid response element (41). $TR\beta 2$ has a longer N-terminal domain than $TR\beta 1$ but is otherwise identical. Both isoforms bind thyroid hormone with the same affinity. Nevertheless, the dose-response curve for $TR\beta 2$ induction of a transfected reporter is ~ 3 fold left-shifted from that of $TR\beta 1$. This appears to be due, at least in part, to $TR\beta 2$ having an ~ 3 fold higher affinity than $TR\beta 1$ for the coactivator TIF2 (42). Thus not only variations in the concentration of receptors, but also changes in receptor sequence, can differentially alter the properties of transactivation.

Coactivators and corepressors with classical steroid receptors

The p160 coactivators (SRC1, TIF2/GRIP1, and AIB1) are defined as factors with intrinsic transactivation activity that increase the A_{max} of gene induction by receptor-agonist complexes (43). The corepressors NCoR and SMRT were discovered from their ability to repress the transactivation of enhancer-bound, ligand-free nuclear receptors and were found to have high affinity for antagonist-bound receptors. Subsequently it was determined that coactivators and corepressors do not selectively bind to receptor-agonist and receptor-antagonist complexes respectively. Instead, coactivators and corepressors can each associate with both agonist -and antagonist-bound receptors. Consequently, the ratio of coactivators to corepressors is thought to be a critical determinant of final A_{max} activity (44–46).

Coactivators and corepressors also modulate the EC_{50} of agonist-induced transcription and the percent partial agonist activity of antagonists. Exogenous GRIP1/TIF2 and SRC-1 each causes a left-shift in the dose-response curve of AR (47,48), GR (26,49), mineralocorticoid receptor (MR) (50), and PRs (51,52). The behavior with $ER\alpha$ is more complex. Overexpression of SRC-1 (but not TIF2 or AIB1) made tamoxifen (but not raloxifen) an agonist in MCF-7 cells while SRC-1 small-interfering RNA (siRNA) eliminated tamoxifen but not estradiol agonist activity in Ishikawa cells (53). More recently, SRC-1 increased the total activity of tamoxifen in Cos-7 cells but not as much as for estradiol so that the percent partial agonist activity actually decreased (54). An attractive mechanistic explanation for the seemingly general behavior of coactivators (23,24) may be the ability of coactivator to increase receptor-steroid complex binding to HREs. The coactivator PGC-1 β has been found to increase both the EC_{50} and A_{max} of reporter gene induction by $ER\alpha$ in Cos-7 cells (Anastasia Kralli, personal

communication). It has recently been shown that TIF2 stabilizes the cooperative binding of agonist-bound PR-A complexes to a tandem repeat of PREs to cause a 10-fold left-shift in the binding isotherm (55) while no TIF2 binding is observed to ligand-free GRs bound to GREs (21). The M770I rat GR mutation displays increased affinity for GRIP1 and produces a left-shift in the dose-response curve (56). The corepressors NCoR and SMRT interact with both agonist- and antagonist-bound ER α s, GRs, and PRs (44,45,57–60) to shift the position of the dose-response curve of agonists and amount of percent partial agonist activity of antisteroids (26,44,58,60–62). Interestingly, the direction of the changes produced by the corepressors can be receptor- and cell-dependent (26,62). Inhibition of the class III histone deacetylase, Sirt1, by either nicotinamide or Sirt1 siRNA was found to increase the A_{\max} and decrease the EC_{50} of AR gene induction of an exogenous reporter in LNCaP cells (63).

There are several differences between gene induction and repression, the most obvious of which being that repression decreases, while induction increases, gene expression. This suggests at least some divergent mechanistic pathways for the two processes. Nevertheless, five modulators (coactivators TIF2 [GRIP1, SRC-2] and SRC-1 \pm the comodulator STAMP, corepressor SMRT, and comodulator Ubc9) altered the A_{\max} , EC_{50} , and percent partial agonist activity of endogenous and exogenous repressed genes similarly to that for GR-regulated gene induction. These five factors interact with different domains of GR and thus are sensitive topological probes of GR action. This suggests that GR-mediated induction and repression share many of the same molecular interactions and that the causes for different levels of gene transcription arise from more distal downstream steps (37). The actions of SRC-1 by itself appear to be promoter and/or cell dependent as exogenous SRC-1 is reported to cause a decrease (64) or increase in EC_{50} (37) in two different systems.

The observations of equilibrium interactions of coactivators and corepressors with GRs have led to the hypothesis that the ratio, as opposed to the absolute amount, of coactivators and corepressors is a major determinant of the final EC_{50} and percent partial agonist activity values (26,58,59). These competitive interactions do not appear to be unique for GRs (23,24). The coactivator TIF2 inhibits PR association with the corepressor NCoR in two-hybrid assays (59). Endogenous SMRT co-immunoprecipitates with both agonist and antiandrogen-bound AR while TIF2 and SMRT competitively inhibit the actions of each other with agonist- but not antagonist-bound ARs (65). The coactivators SRC-1 and CBP functionally compete with NCoR in transactivation, 3-hybrid, and nuclear colocalization assays with ARs in a manner that depends upon the ratio of coactivators to corepressors (46). Similar competitive binding to agonist-bound ARs has been inferred from the increased binding of the coactivator SRC-1 to the promoter region of three endogenous AR-regulated genes in ChIP assays with siRNAs to NCoR and SMRT (66). Interestingly, corepressors are recruited equally to the enhancers of these genes (66), and of a transfected reporter gene (60), by both agonist- and antagonist-bound ARs and PRs respectively. Coactivators and corepressors were initially found to associate with a common region in the LBD of thyroid receptors (67–69). Recent studies have confirmed competitive binding of coactivators and corepressors to the GR LBD (70,71) and extended this competitive binding to include a second region in the N-terminal domain of both GRs and PRs (60).

IL-1 β causes the loss in ChIP assays of NCoR and all NCoR-associated factors with antiandrogen-bound ARs, apparently by MEKK1 recruitment to TAB2. IL-1 β also increases the percent partial agonist activity of ER α and PR antagonist complexes (72).

Coactivators and corepressors with nuclear receptors

Reports of modulatory activity with coactivators and corepressors was initially restricted to the classical steroid receptors but are becoming more common with the nuclear receptors. Equilibrium competitive binding of coactivators and corepressors to thyroid receptors (TRs)

and retinoic acid receptors (RARs) *in vivo* has been described along with SMRT binding to a RAR response element in ChIP assays \pm retinoic acid in a manner that is competed by added SRC-1 (73). These observations can explain the ability of SMRT to decrease the amount of thyroid hormone (T_3)-induced transactivation by TRs. Furthermore, column-purified steroid-free TR/retinoid X receptor (RXR) complexes contain corepressors that are not eliminated by the addition of the agonist T_3 (74).

Unexpectedly, SMRT but not NCoR appears to be required for full activity of agonists in a receptor- and cell-specific manner. With exogenous reporter genes, SMRT siRNA decreases ER α transactivation in MCF-7 and HeLa cells (but not HepG2 cells) but increases transactivation by vitamin D receptors, (VDRs), TRs, and ARs in HeLa cells. With MCF-7 cells, SMRT siRNA reduces expression of many but not all of the estrogen-regulated endogenous genes examined and reduces cell growth (75).

RIP140 usually functions as a corepressor. In an example of equilibrium binding, the ratio of RIP140 to the coactivator PCAF was found to determine amount of each factor binding to complexes of RAR/RXR/coactivator GRIP1 \pm retinoids (76). Collectively, the above reports illustrate that the concept of competitive cofactor binding, determined by the ratio of competing cofactors, is more general than initially proposed for GRs (26) and also occurs with a variety of the classical steroid receptors and the nuclear receptors. Whether it is a property of, and will affect the EC₅₀ and partial antagonist activity of, all steroid/nuclear receptors remains to be established.

When a coactivator is not a coactivator

Coactivators, as described above, were initially defined by their ability to increase gene transactivation by a receptor-agonist complex. When discussing receptor-mediated repression, where an agonist steroid causes a decrease in the total amount of gene expression, the expected behavior of a coactivator may not be immediately obvious. For the purposes of this review, we define a coactivator as a transcriptionally active cofactor that augments the activity of a receptor-agonist complex, whether it be to cause a further decrease (as in repression) or increase (as in induction) in gene expression. Thus, by this definition, the report of TIF2 accentuating the repression by GR-agonist complexes of an AP-1 induced gene in U2OS.rGR cells is an example of TIF2 acting as a coactivator in gene repression (77).

Coactivators can bind to receptors but not exert the usual responses of increased A_{max} , decreased EC₅₀, or increased percent partial agonist activity. The coactivator SRC-1 appears to associate with ARs bound by the antiandrogens cyproterone acetate and bicalutamide. However, SRC-1 increases the percent partial agonist activity of only cyproterone acetate-bound ARs (78). Point mutations in the GR LBD have been described to eliminate the activity of added TIF2 in the presence of the agonist dexamethasone (Dex) while maintaining the ability of TIF2/GRIP1 to interact with GR-Dex complexes (40). In both cases, the data support a mechanism where the steroid or point mutation causes minor changes in receptor-coactivator interactions that have little effect on binding but are magnified by the time they are propagated to the outer surface of the coactivator and thus have major consequences on its ability to modify transcriptional processes. This scenario is similar to the report that phosphorylation of the human ER α at Ser305, which is just before the LBD, alters the orientation (and presumably activity) of bound coactivator SRC-1 without affecting the affinity of SRC-1 binding (79).

Other factors that modulate EC₅₀ and percent partial agonist activity of receptors

Ubc9 was initially found to modulate the properties of GR (23,24) and was recently shown to affect PRs (30). β -catenin increases the percent partial agonist activity of antiandrogens with ARs (80). Squirrel monkey FKBP51 causes an \approx 10-fold right-shift in the dose-response curve

of both human and squirrel monkey GRs (81). Vav3 is localized to membranes and does not bind to ARs or alter AR protein levels. However, Vav3 causes a left-shift in AR dose-response curve in a manner that requires the AR AF1 domain along with the PH domain of Vav3 (82). STAMP is a new co-modulator that binds SRC-1 and TIF2 and enhances their ability to alter the EC₅₀ and percent partial agonist activity of AR, GR, and PR complexes in gene induction (30,77). The activity of STAMP with GRs requires TIF2 and STAMP binding to GR via their respective receptor interaction domains (RIDs). STAMP (in the presence of TIF2) and Ubc9 both retain the same modulatory activity in GR-mediated induction and repression (37). The dose-response curve for androgen induction of three transfected reporters is left-shifted to a lower EC₅₀ in C4-2B cells (an androgen independent subline of LNCaP cells) compared to LNCaP cells even though the AR levels are the same (83). The factors responsible for this subline-dependent behavior are not yet known.

Sur2 is another modulator of GR transcriptional properties but no new data has appeared since the original report (84). Kinases have long been suspected of altering receptor transcriptional properties and several substantial effects have been documented (85). CDK5 and p25 together repress GR transactivation but with no obvious change in the EC₅₀ of either exogenous (MMTV) or endogenous (SGK) genes (86).

Selectivity of Modulatory Factor Actions

Receptor-selective effects

The same DNA sequence is capable of mediating gene induction by most of the classical steroid receptors (i.e., AR, GR, MR, and PR). Thus, much of the specificity gained by steroids complexing with cognate receptors appears to be lost after binding DNA. The first indication that modulatory factors could restore some of the lost specificity was seen with the opposite responses of GRs and PRs to the corepressors NCoR and SMRT. Thus, in the same cell with the same exogenous reporter gene, NCoR induces a decrease in the EC₅₀, and an increase in percent partial agonist activity, with PRs but an increase in EC₅₀, and a decrease in percent partial agonist activity with GRs (62). Similar diversities in response of PRs vs. GRs are seen with Ubc9 and GMEB-2 and with the GME DNA element (30). The behavior of PR and GR with STAMP ± TIF2 are the same but separate regions of STAMP are involved, suggesting that activity differences may be revealed under other conditions (30). hZimp7 increases AR transactivation, with an apparent left-shift in dose-response curve but not with GRs or PRs (87). COBRA1 causes a right-shift in the dose-response curve of AR gene induction. COBRA1 binds ARs and GRs in a co-IP assay much more avidly than PRs (88). Thus, COBRA1 is predicted not to significantly alter the EC₅₀ for PR-regulated induction in the same cells. The factor ELL reduces the EC₅₀ for gene induction by MR by a factor of ≈10 and increases both the A_{max} for aldosterone and the percent partial agonist activity of the antagonist spironolactone while exhibiting no effect on ARs or PRs and decreasing the A_{max} for GRs (89).

Tissue selectivity of modulatory actions

The GME is active with GRs in Fu5-5 rat hepatoma (29), CV-1 (90) and 1470.2 cells but inactive with PRs in 1470.2 cells (30). GMEB-2 modifies the properties of GRs in CV-1 (32) and of GRs and PRs in 1470.2 cells. The initial activity of Ubc9 with GRs in CV-1 cells (21, 91) is reduced for GRs in 1470.2 cells but is very similar for PRs in both cell lines (30). STAMP modulatory activity with GRs has been reported in CV-1 and U2OS human osteosarcoma cells (77) and with PRs in CV-1 cells (30). Thus, the effects of these factors are not limited to a single steroid receptor or a single cell line.

Dissociation of A_{\max} , EC_{50} , and percent partial agonist activity

The current model of steroid hormone action gives no guidance as to whether changes in A_{\max} , EC_{50} , and/or percent partial agonist activity are linked. Recent reports suggest that it is possible to selectively modulate each parameter.

Separation of A_{\max} (efficacy) and EC_{50} (potency)

The S530A mutation of PR causes about 10-fold right-shift in dose-response curve with no change in maximal induction or progesterone binding affinity both in cell-free extracts and in whole cells at 37 °C (92). Amino acids 41–64 of human ER α influence the EC_{50} but not the A_{\max} with estradiol (93). The GR polymorphisms N363S and R23K cause increased or decreased transcription respectively of the endogenous GILZ gene (but not the repressed IL-2 gene) with no change in EC_{50} of the induced gene (94). Ubc9 increases the A_{\max} of GR-regulated induction of an exogenous reporter in the presence of both low and high concentrations of GR but apparently affects the EC_{50} of gene induction only with high GR levels (21,91). Removal of N- and C-terminal domains of TIF2 reduces the coactivator-mediated increase in A_{\max} more than the shift of the dose-response curve to lower steroid concentrations (95). The deacetylase inhibitor TSA increases the A_{\max} of transfected gene induction by GRs in the presence of the corepressor SMRT without altering the EC_{50} (58, see also references in 30). These results are consistent with the A_{\max} (efficacy) and EC_{50} (potency) of gene induction being controlled by different pathways, at least under some conditions.

Separation of A_{\max} and percent partial agonist activity

The S118A mutation in human ER α often blocks receptor phosphorylation by ERK1/2. With several endogenous genes for which ER α -mediated induction is decreased by the S118A mutation, the absolute amount of partial agonist activity is unchanged, which means that the percent partial agonist activity increases (96). Similar consequences were seen for the activity of the antagonist RU486 with the double mutant M770A/L771A in rat GR (27). Amino acids 41–64 of human ER α are necessary for percent partial agonist activity of antisteroids but not transactivation by estradiol (93). SMRT siRNA reduces the activity of ER α -agonist complexes but increases the activity of receptors bound by the antiestrogen 4-hydroxytamoxifen (75). Together these results suggest that the mechanisms governing the percent agonist activity of antisteroids can be different from those controlling the A_{\max} of agonists.

Separation of EC_{50} and percent partial agonist activity

The percent partial agonist activity goes up when the EC_{50} goes down, and vice versa, under a wide variety of conditions (Fig. 2C; reviewed in 23). As there is no theoretical reason for this correlation, it is not surprising that exceptions are being found. The M578A mutation in rat GR increases the EC_{50} for Dex (and reduces the A_{\max}) while converting the weak antigluocorticoid cyproterone acetate (97) to a full agonist with lower EC_{50} than Dex (98). The deacetylase inhibitors VPA and TSA in the presence of high, but not low, GR concentrations cause little change in EC_{50} but increase the percent partial agonist activity (99).

Separation of EC_{50} s in repression vs. induction

Receptor-regulated repression and induction are generally thought to proceed via two different mechanisms with repression employing receptors “tethered” to DNA by other DNA-bound proteins (34,35). However, gene repression is not a property of tethered receptors, as seen by the numerous examples of gene activation by chimeras of receptors fused to a different DNA-binding protein. For GRs, the EC_{50} for gene induction is usually 6–10 fold higher than that for repression by the same receptor-steroid complex in the same cells (6–9). Elucidating the

reasons for these differences will provide valuable insight into the mechanism of receptor-mediated gene expression in general and gene repression in particular.

Parallel changes in A_{\max} and EC_{50}

A recently proposed mathematical model of gene induction by steroid hormones involving a series of linked, cyclical steps (Fig. 3) can explain most changes in both A_{\max} and EC_{50} as resulting from alterations in a single step downstream of steroid binding affinity for receptors (99) (Ong et al., in preparation). Only when the A_{\max} and EC_{50} both change in the same direction (e.g., increase in A_{\max} and right-shift in dose-response curve to higher steroid concentrations) does it appear that effects on two separate parameters or steps must be invoked. Such parallel changes in A_{\max} and EC_{50} have been reported with GRs with CBP (33), with SMRT in a cell-specific manner (62), with GMEB-2 and PR and the GMEGREtkLUC reporter (30), and for the R629Y mutant (rat GR) in a steroid-specific manner (40). Parallel changes have also been reported for ARs with COBRA1 (88) and hepatitis B virus protein X protein (HBx) (38), and for PR with NCoR (62) and with GMEB-2 in a manner that depends upon PR concentration (30).

Mechanistic considerations of independence of parameters

The separate regulation of EC_{50} and/or percent partial agonist activity from A_{\max} might have been predicted because graphical displays of EC_{50} and percent partial agonist activity omit any mention of A_{\max} (Fig. 2C). This plus the mathematical model for modifying A_{\max} and EC_{50} , suggests that different pathways or steps may be recruited under appropriate conditions to modulate these two parameters (Fig. 4). Thus, mechanisms and cooperating factors may emerge during investigations of the determinants of EC_{50} that would go undetected in studies of A_{\max} . Some of the pathways that appear to be influenced by transcription factors, and may participate in the dissociation of A_{\max} and EC_{50} , are steps downstream of receptor binding to DNA, including transcription initiation (100), RNA pol II elongation (99,101), relief of supercoiling (99), and mRNA splicing (100,102–104). This hypothesis is consistent with the view that transcription is not a series of individual, isolated steps but rather reflects the net product of a series of interconnected reactions (105,106).

Physiological relevance of modulating the EC_{50} and percent partial agonist activity

Examples of physiological consequences of modulating the concentrations of relevant factors, and thus the EC_{50} and percent partial agonist activity, are becoming more numerous in proportion to the frequency of studying these parameters. A two-fold increase in GR gene dosage in mice, achieved by using a yeast artificial chromosome, causes a 60% increase in receptor protein and GR mRNA but a >10-fold decrease in EC_{50} for induction of thymocyte apoptosis (107). The naturally occurring I747M mutation in human GR causes a two-fold decrease in affinity and A_{\max} and a 20–30 fold increase in EC_{50} . Thus the steroid concentration sufficient for 50% induction by the wild type GR affords 5-fold less activity with the mutant GR (49). Androgen resistance correlates with increased AR levels in prostate tumor xenograph models (108), as expected from tissue culture studies where increases in AR decrease the EC_{50} for gene induction and increased the percent partial agonist activity of antiandrogens. It should be remembered, though, that only 1/3 of androgen-insensitive prostate cancers are associated with elevated AR (109), so that other mechanisms are clearly involved (110). The estrogen-insensitivity of many breast tumors with increased levels of AIB1 (111) is consistent with the predicted ability of elevated AIB1 to decrease the EC_{50} for endogenous estrogens and increase the percent partial agonist of antiestrogens (50). However, it now appears that these effects of AIB1 involve being a coactivator of the cell cycle regulator E2F1 and are independent of ER α s (112). At least part of the syndrome of pituitary (or central) resistance to thyroid

hormone (RTH) can be explained by TR β 2, found primarily in the pituitary/hypothalamus, displaying an enhanced response to T₃, relative to the TR β 1 isoform, due to increased affinity for the coactivator TIF2. This causes a lower EC₅₀, which makes TR β 2 respond more robustly to physiological levels of hormone (42). During development in *Drosophila* larvae, the temporal regulation of gene expression by the steroid hormone ecdysone is such that the first genes expressed have the lowest EC₅₀ (16). A more comprehensive discussion of physiologically relevant changes in factor concentration can be found in a recent review (24).

Conclusions

Dramatic advances have been made over the last decade in unraveling the mechanism of steroid hormone action. Nevertheless, our understanding of the differential control of gene expression by the single circulating concentration of steroid hormone during development, differentiation, and homeostasis remains fragmentary at best. These difficulties derive in part from the relative scarcity of experiments run with physiological (i.e., sub-saturating) concentrations of agonist steroid. Those studies that have been performed have yielded the attractive hypothesis that increasing concentration differences among cells of factor(s) that interact with receptor-steroid complexes act like a rheostat to progressively change the position of the dose-response curve for agonists, and the percent partial agonist activity of antagonists. Abundant support for this hypothesis is now available from tissue culture studies and physiological evidence is beginning to mount. These factor gradients could easily be established if the extra-cellular concentration of possible inducers (or repressors) of the genes for each factor, or regulators of factor mRNA stability, are found to change. This will require detailed studies of the type recently performed for the receptors themselves, where it was found that the level of steroid receptors varies both by at least a factor of 20 between tissues (113) and in a circadian rhythm (114). New techniques, such as ChIP, ChIP-reChIP, ChIP-on-chip, chromatin conformation capture, siRNA, and transgenic mice with knock-out and knock-in mutations provide powerful new tools to examine the mechanisms behind the changes in EC₅₀ and percent partial agonist activity. It is now possible to look at the specific factors that are recruited to specific regions of exogenous and endogenous regulated genes, to ask if two (or more) factors are present at a given region of the gene at the same time or sequentially, to determine whether the factors are covalently modified and/or move to new DNA locations under the different conditions, to investigate the possible communication between proteins bound at separated DNA sites, to define the precise nuances that are becoming commonplace between endogenous genes, and to examine the functional consequences of varying the amounts of specific factors (or their mutated forms) within the physiologically encountered levels. The net results from such studies should provide a quantum leap forward in our understanding of steroid hormone action at physiologically important levels of both steroid and protein factors.

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Abbreviations

A_{max}	maximal activity
AR	androgen receptor
Dex	

	dexamethasone
Dex-Mes	Dex-21-mesylate
DBD	DNA binding domain
EC₅₀	effective concentration for 50% of maximal response
ER	estrogen receptor
GME	glucocorticoid modulatory element
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HB_x	hepatitis B virus nonstructural protein, X protein
HRE	hormone response element
LBD	ligand binding domain
MR	mineralocorticoid receptor
MMTV	mouse mammary tumor virus
PR	progesterone receptor
RAR	retinoic acid receptor
RXR	retinoid X receptor
siRNA	small-interfering RNA
TR	thyroid receptor
TRβ1	thyroid receptor beta 1

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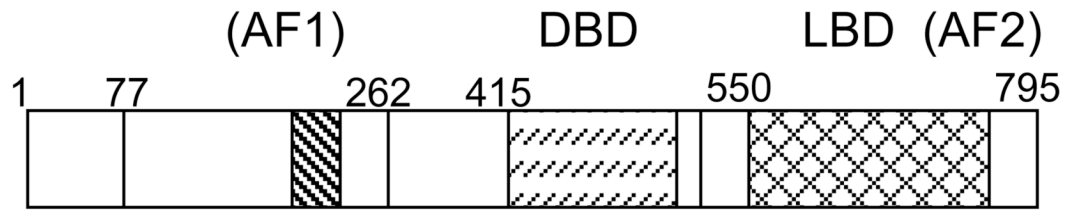
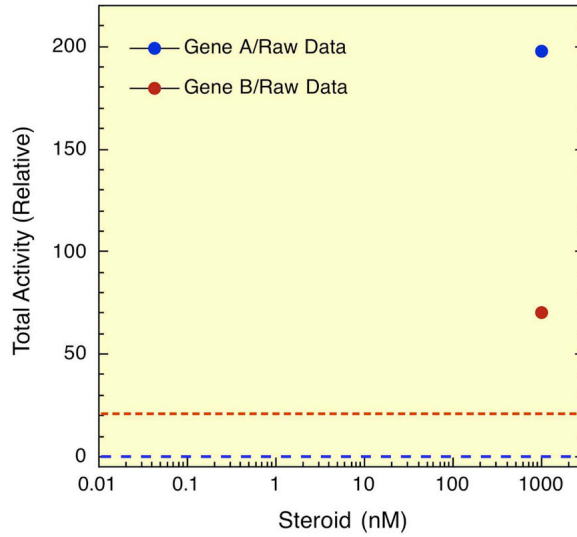


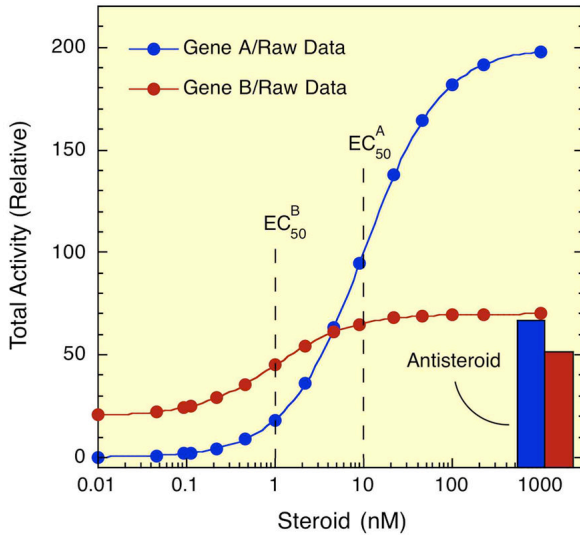
Fig. 1.

Schematic of domains of rat GR with numbers above the drawing indicating the amino acids of the LBD with AF2 (cross hatched), AF1 (core is striped), and DBD (dashes) domains. These domains are almost identical in GRs of other species and are similarly organized in the other steroid receptors (115).

A Pharmacological Steroid Concentration



B Various Steroid Concentrations (Raw Data)



C Various Steroid Concentrations (Normalized Data)

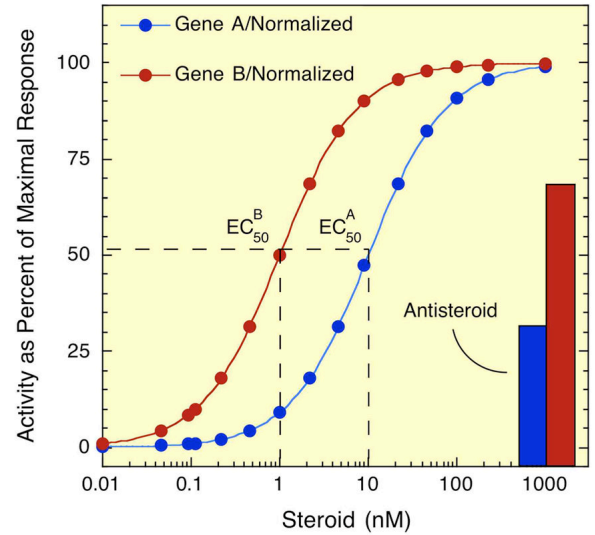


Fig. 2. Data plots for experiments on steroid hormone action. (A) Induction of gene expression of transiently transfected luciferase reporter genes A and B with a saturating concentration of agonist steroid. Raw luciferase data are plotted. Background levels with no added steroid are indicated by the dashed lines. (B) Dose-response curves for induction of transiently transfected luciferase reporter genes A and B by varying concentrations of agonist steroid, and bar graphs of residual agonist activity of an antisteroid with each gene. Raw luciferase data are plotted. (C) Normalized data for gene induction data of panel B. For each gene, the data are expressed as percent of maximal induction.

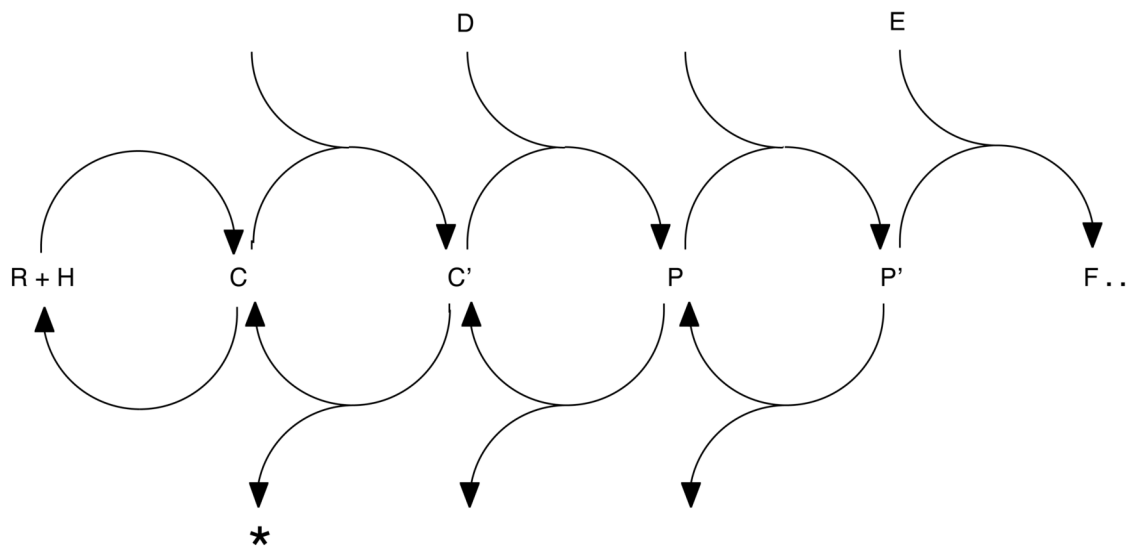


Fig. 3. Cyclical model of steroid hormone regulated gene transcription. Each circle of two reactions (single semi-circular arrow) represents a proposed, but usually unidentified, reaction in the currently uncharacterized series of steps from steroid (H) binding to receptor (R) to give the initial receptor-steroid complex (C) to the production of mRNA (beyond F). Other species correspond to both known (e.g., C' = activated complex; * = biologically inert “activated complex”) and unknown (e.g., P', E, etc.) factors and intermediates. This cyclical model predicts sigmoidal dose-response curves under a variety of conditions (from ref. 99).

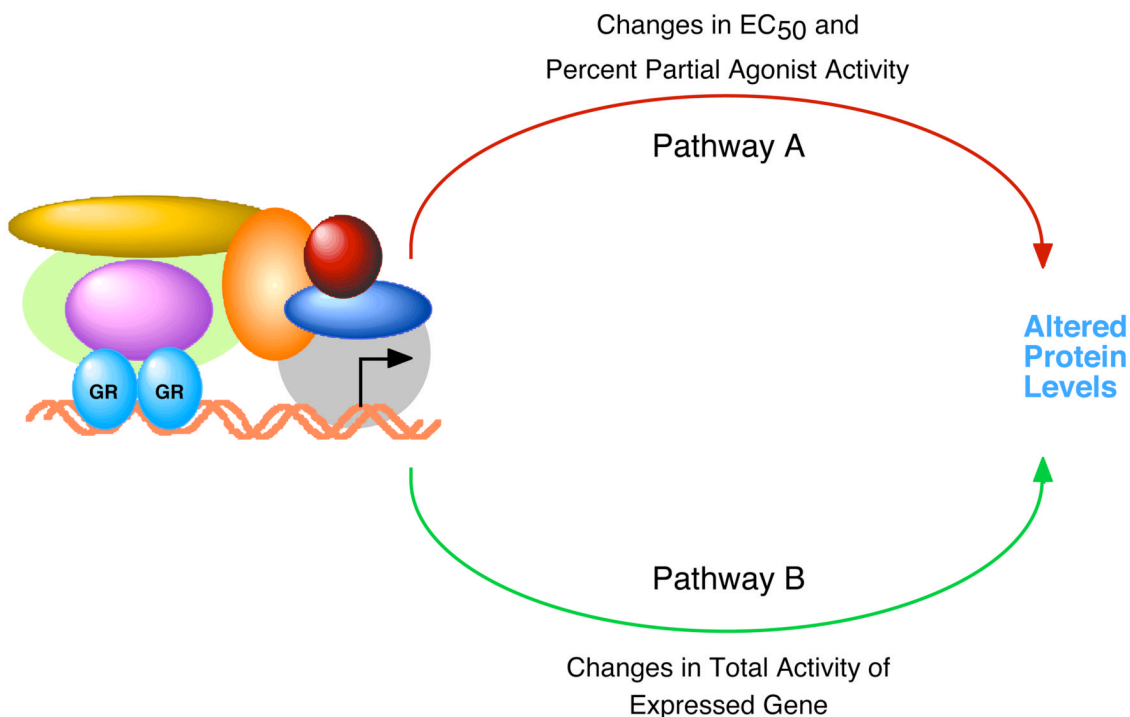


Fig. 4. Proposed model by which changes in total activity (A_{max}) and in EC_{50} and percent partial agonist activity can sometimes be expressed via different steps or pathways. The promoter-bound complex with GR is depicted as being a single multimeric species only for ease of drawing. The start of transcription is designated by the bent arrow. The mathematical model suggests that the various molecules, including GR, enter and leave the promoter regions sequentially so that only a few components are present at any one moment. The depicted arrows for pathways A and B may, depending upon the conditions, represent entirely different sequences in the reactions of Fig. 3 from receptor binding of steroid to production of protein product. Alternatively, A and B may represent other single cyclical steps in the overall pathway of Fig. 3, or anything in between. Similar different steps/processes may also dictate independent changes in percent partial agonist activity, although no mathematical model has yet been constructed for this.

Table 1Entities that modulate the EC₅₀ and/or percent partial agonist activity of receptors

Factor	Receptor	Reference
β-catenin	AR	(80)
Cell sub-line	AR	(83)
Coactivators	AR, ERα, GR, MR, PR, TR	(23,24,27,40,42,47,48,53,54,56,62,77,78,93)
COBRA1	AR	(88)
Corepressors	AR, ERα, GR, MR, PR	(23,24,44,45,57,60,61,75)
Deacetylase inhibitors	GR	(99)
ELL	MR	(89)
FKBP51	GR	(81)
GME	GR, PR	(29,30)
GMEB-2	GR, PR	(30,32)
Hbx	AR	(38)
IL-1β	ERα, PR	(72)
PGC-1β	ERα	(A. Kralli, personal communication)
Receptor mutations	ERα, GR, TR	(39–42,49,56,61,96,98)
STAMP	AR, GR, PR	(30,77)
Steroid receptor	AR, ERα, GR, MR, PR	(23,24,36)
Sur2	GR	(84)
Transcription mode *	GR	(6–9)
Ubc9	GR, PR	(23,24,30)
Vav3	AR	(82)
hZimp7	AR	(87)

* Induction vs. Repression