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Effects of acetylation, polymerase phosphorylation, and DNA unwinding in glucocorticoid receptor transactivation

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Abstract

Varying the concentration of selected factors alters the induction properties of steroid receptors by changing the position of the dose–response curve (or the value for half-maximal induction = EC_{50}) and the amount of partial agonist activity of antisteroids. We now describe a rudimentary mathematical model that predicts a simple Michaelis–Menten curve for the multi-step process of steroid-regulated gene induction. This model suggests that steps far downstream from receptor binding to steroid can influence the EC_{50} of agonist-complexes and partial agonist activity of antagonist-complexes. We therefore asked whether inhibitors of three possible downstream steps can reverse the effects of increased concentrations of two factors: glucocorticoid receptors (GRs) and Ubc9. The downstream steps (with inhibitors in parentheses) are protein deacetylation (TSA and VPA), DNA unwinding (CPT), and CTD phosphorylation of RNA polymerase II (DRB and H8). None of the inhibitors mimic or prevent the effects of added GRs. However, inhibitors of DNA unwinding and CTD phosphorylation do reverse the effects of Ubc9 with high GR concentrations. These results support our earlier conclusion that different rate-limiting steps operate at low and high GR concentrations versus high GR with Ubc9. The present data also suggest that downstream steps can modulate the EC_{50} of GR-mediated induction, thus both supporting the utility of our mathematical model and widening the field of biochemical processes that can modify the EC_{50}.
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1. Introduction

Steroid-regulated gene induction involves numerous steps that are unique to steroid receptors. The first step of steroid binding to intracellular receptor proteins is followed by a process (activation) that permits the binding of receptor-steroid complexes to specific DNA sequences in the genome, called hormone response elements or HREs [1,2]. The subsequent steps of steroid hormone action are thought to be shared by many transcription factors and include recruitment of transcription co-factors (including coactivators, corepressors, RNA pol II, and pol II associated components), RNA transcription initiation, elongation of the RNA transcripts with the help of elongation factors, termination of transcription, removal of non-coding sequences (or introns), and addition of a polyadenylation tail to the 3′ end of the newly synthesized mRNA [3].

Most studies of steroid-regulated gene induction examine the total level of product at maximal levels of induction. We have focused on two other important properties that are relevant for the differential control of gene expression and for endocrine therapies with antisteroids. They are the sensitivity of gene induction (i.e., the concentration of agonist steroid required for half maximal induction or the EC_{50}) and the partial agonist activity of antisteroids, respectively

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[4–18]. It was long thought that the EC_{50} for gene induction was determined by the affinity of steroid binding to receptor. However, evidence has been accumulating for many years that the determinants for the positioning of dose–response curves are much more complex [19–21]. Nevertheless, it was tacitly assumed that only the early steps in steroid hormone action affected the EC_{50}. An even more basic and unanswered question is why the dose–response curves nicely fit a simple Michaelis–Menten curve despite the fact that steroid induction is a vastly more complicated reaction than the simple one-step reaction of Michaelis–Menten kinetics. Thus, any studies about the positioning of dose–response curves would be greatly assisted by a further understanding of the parameters controlling the shape of these curves.

We have determined that at least two mechanistically distinguishable pathways exist in cells for modulating the EC_{50} of agonists and partial agonist activity of antisteroids in glucocorticoid receptor (GR)-induced processes. One pathway, which responds to increasing concentrations of GR [6], coactivators like TIF2 [7], or a cis-acting element called the GME [4,5], proceeds through a rate-limiting step or intermediate "X" [10] to reduce the EC_{50} of agonists and increase the partial agonist activity of antisteroids. Another pathway, which is regulated by Ubc9, appears to act at some point downstream of "X" [12,18]. Thus, Ubc9 modifies the above GR induction parameters only in the presence of high concentrations of GR, apparently by saturating the rate-limiting step "X" to uncover previously inaccessible downstream steps. A third potential pathway involves hSur2 [14], which is a component of the Mediator complex [22].

A variety of chemical agents are known to affect the total level of receptor-mediated gene transactivation. Some of the more commonly used agents inhibit histone deacetylase (HDAC) activity, RNA pol II C-terminal domain (CTD) phosphorylation, and topoisomerase I (Topo I). Histone acetylation and deacetylation are important determinants of nucleosome structure [23–25] and steroid receptor-regulated gene expression [26–28]. Two useful inhibitors of HDAC activity that have been used to preserve and/or increase histone/protein acetylation are tricostatin A (TSA) and valproic acid (VPA). TSA is thought to be a specific inhibitor of histone acetylation [29]. VPA appears to be a more general inhibitor as it can block the deacetylation of both histone and non-histone proteins [30]. The C-terminal domain (CTD) of RNA pol II is required for enhancer-driven transcription [31]. CTD phosphorylation is important in controlling the transition from initiation to elongation of mRNA transcripts [3]. Also, GR repression of various genes is associated with GR reducing the level of Ser-2 phosphorylation in the CTD of RNA-pol II [32]. Thus, CTD phosphorylation may play a major role in steroid hormone-regulated gene expression. Two inhibitors of CTD phosphorylation are 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) [33] and *N*-[2-(methylamino)ethyl]-5-isoquinoline-sulfonamide (H8) [34]. However, there are useful differences between the two reagents. For example, the concentrations of DRB that block P-TEFb activity are 3–10 fold lower than those for TFIIH [35–37]. Finally, topoisomerases are enzymes that cause reversible single strand (Topo I) or double strand (Topo II) cleavage of DNA via the formation of covalent protein–DNA complexes, thus allowing the relief of torsional stress that accumulates during transcription due to the unwinding of double stranded DNA [38,39]. Camptothecin (CPT) blocks Topo I by stabilizing the covalent intermediate between Topo I and DNA [40,41]. Topo I has been identified as the sole target of CPT [42,43]. However, it is not known whether any of the above reagents that affect steps downstream of steroid binding to receptor can also affect the EC_{50} of agonists and partial agonist activity of antisteroids.

The purpose of this study was first to see if a mathematical model could be constructed that would predict a simple Michaelis–Menten curve for the multi-step process of steroid-regulated gene induction and, consequently, would guide our efforts to determine what steps might participate in altering the EC_{50} of GR-agonists and partial agonist activity of GR-antisteroids. The model suggested that steps significantly downstream from steroid binding to receptor can, under some conditions, modify the EC_{50} and partial agonist activity. We therefore asked if the actions of modulators of GR transactivation properties can be reversed by any of the above well-characterized agents that inhibit steps downstream of GR binding to steroid: protein deacetylation (with TSA and VPA), CTD phosphorylation (with DRB and H8), and DNA unwinding (with CPT). It should be noted that no one factor can always change the GR induction properties. Similarly, no one inhibitor would be expected to alter GR induction properties under all conditions. Results with inhibitors of DNA unwinding and CTD phosphorylation confirm this prediction and support our hypothesis that steps far downstream of steroid binding to receptors can contribute to the modulation of the EC_{50} and partial agonist activity of GR-complexes.

2. Materials and methods

Unless otherwise indicated, all operations were performed at 0 °C.

2.1. Chemicals

Dexamethasone (Dex) was purchased from Sigma (St. Louis, MO). Dexamethasone-21-Mesylate (Dex-Mes) was prepared as described previously [44].

2.2. Plasmids

GREtkLUC [45] and Ubc9/pSG5 and hSA/pSG5 [12] have been described previously. hSA/pcDNA was created by Jack Blackford (Steroid Hormones Section, NIDDK/NIH) by excising the 2.3-kb hSA cDNA (Stratagene Liver catalog #937224) cloned into the *Eco*RI and *Xho*I sites of pBlue-

script SK. hSA was then cloned into the *Eco*RI and *Xho*I sites of pcDNA3 (Invitrogen). pSVLGR (expressing rat GR) and XPD/pcDNA3 (encoding XPD) were received as generous gifts from Keith Yamamoto (UCSF, San Francisco) and David Levens (NCI, Bethesda, MD), respectively. The Renilla-TS reporter, an internal control for transient transfection efficiency, was a gift from Drs. Nasreldin M. Ibrahim and Otto Fröhlich (Department of Physiology) and Dr. S. Russ Price (Department of Medicine) at Emory University School of Medicine (Atlanta, GA) and contains the thromboxane synthetase promoter inserted into the *Hin*dIII site of the pRL-null plasmid (Promega).

2.3. Cell culture and transfections

CV-1 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Carlsbad, CA) with 10% fetal calf serum (Biofluids Inc.; Rockville, MD). SV40-immortalized XPD fibroblasts (R683W) (received from Jan Hoeijmakers, Center for Biomedical Genetics, Rotterdam, Netherlands) were grown in Dulbecco's modified Eagle's medium (Quality Biological Inc.; Gaithersburg, MD) with 10% FCS. Cells were kept at 37 °C in a humidified incubator with 5% CO_2. Cells were transiently transfected with GR plasmid, 100 ng of GREtkLUC reporter (unless otherwise noted), and 10 ng of Renilla TS as an internal control for 18–24 h with FuGENE 6 (Roche Diagnostics; Indianapolis, IN) in Opti-MEM I (Invitrogen) without serum to maintain a FuGENE:DNA ratio of 2:1. When varying the amount of Ubc9 and XPD, the total molar amount of vector plasmid was kept constant by the addition of either hSA/pSG5 or hSA/pcDNA3, respectively. The total transfected DNA was adjusted to either 300 ng or 500 ng/well of a 24-well plate with pBluescriptII SK+ (Stratagene; La Jolla, CA). Cells underwent induction for 24 h with steroid treatment and then lysed. Reporter gene activity was detected using the Dual-Luciferase assay system (Promega; Madison, WI) according to manufacturer's instructions. All luciferase values with different concentrations of steroid are normalized to co-transfected Renilla activity to correct for differences in transfection efficiency and expressed as a percentage of maximal response with 1 μM Dex. The Renilla values always show a steroid-dependent decrease with increasing steroid (maximum of ≈50% decrease) that is very similar under all conditions and disappears with increasing inhibitor concentration (data not shown). Therefore, this consistent behavior is unrelated to the ability (or lack of ability) of some, but not other, treatments to reverse the effects of individual treatments on the position of the dose–response curve or the amount of partial agonist activity. For the dose–response curves, the average value (±S.D. of triplicate samples) at each steroid concentration is plotted. The partial agonist activity of a steroid A (expressed as percent) is defined as follows: 100 × [the activity with 1 μM steroid A − the basal level seen in the absence of hormone]/[the activity with 1 μM Dex − the basal level seen in the absence of hormone].

2.4. Statistics

All figures are representative of experiments carried out in triplicate at least two times. Best-fit curves ($R^2 \geq 0.95$) following Michaelis–Menten kinetics were obtained with KaleidaGraph (Synergy Software, Reading, PA). The values of n independent experiments were then analyzed for statistical significance by the two-tailed Student's t test using the program "InStat 2.03" for Macintosh (GraphPad Software, San Diego, CA). When the difference between the S.D.s of two populations was significantly different, then the Mann–Whitney test or the Alternate Welch t test was used.

3. Results

3.1. A mathematical model for GR-mediated induction

Several reports over the last few years have documented that the position of the dose–response curve for steroid hormone induction of gene transcription is not a fixed property of a given receptor-agonist complex but varies with the changing concentration of a variety of factors including the homologous receptor, coactivators, corepressors, and selected cofactors like Ubc9 [6–18]. In each case, the dose–response curves are sigmoidal and show an excellent fit with the curve generated by Michaelis–Menten kinetics. Thus, the kinetic properties of the simple bimolecular reaction of $A + B \leftrightarrows C$ appear, surprisingly, to be sufficient to describe the dose–response curve of the multi-step process of steroid-regulated gene induction that involves many molecules. Obviously, any advance in explaining why the dose–response curve for steroid-regulated gene expression is sigmoidal would assist in understanding how the position of the dose–response curve can change. As a first step, we have constructed and analyzed a multi-step model of GR-induced gene transcription. As is described in greater detail in Appendix A, this model is based upon an earlier model of Loeb and Strickland [46] involving coupled steps. This earlier model is the only attempt, of which we are aware, to construct a reaction sequence for hormone receptor action that affords Michaelis–Menten kinetics. However, our analysis of this model suggests that the conditions required for the mathematics to be valid corresponds to a situation that is rarely found for steroid receptors (i.e., receptor excess, where the total concentration of receptor $[R_T]$ is much greater than that of an intermediary interacting factor $[D_T]$). We show that this problem can be resolved if it is assumed that the product C (=receptor-steroid complex) is recycled (Fig. 1). In fact, a series of cascading, recycling steps can be constructed (Fig. 1) and shown to always be capable of giving sigmoidal dose–response curves (see Appendix A). Thus, Michaelis–Menten kinetics are obeyed as long as: (1) the products of each step are a low percentage of the reactants, (2) each step is part of a cyclical reaction, or (3) some combination of (1) and (2). Our present model also suggests that the simplest means for shifting the position of

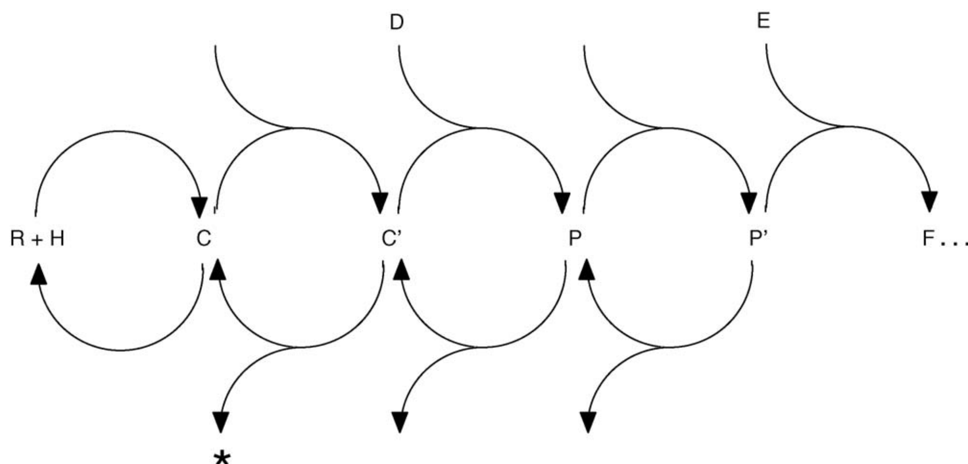

Fig. 1. Cyclical model of steroid hormone-regulated gene transcription. As described in the text (see Appendix A for details), this cyclical model predicts sigmoidal dose–response curves under a variety of conditions. R = receptor, H = steroid, C = RH complex, other species correspond to unspecified factors and intermediates.

the dose–response curve (or the value of the EC_{50}) is to vary either the equilibrium constant of any step of the overall reaction or the concentration of any component of the reaction step. This last conclusion is an extension of Loeb and Strickland's examination of systems dependent upon the generation of a secondary mediator [46]. Importantly, our analysis suggests that even changes near the end of the multi-step reaction pathway can affect the position of the dose–response curve.

The above reports of factors/conditions that reduce the EC_{50} for gene induction by agonists have also shown that the same factors increase the partial agonist activity of receptor-antisteroid complexes. We have not tried to model this response because so little is known about the determinants of partial agonist activity. However, past results have shown an excellent correlation between changes in both parameters [15]. This leads us to suspect that our model, or a refinement thereof, may eventually be able to predict how those modifications that affect the EC_{50} of GR-agonists can also modify the partial agonist activity of GR-antagonists.

It should be stressed that while the model does predict that changing an equilibrium constant, or reactant concentration, in Fig. 1 can perturb the EC_{50} of the dose–response curve, not every change will produce a noticeable alteration [10–12,16,47]. The presence of a rate-limiting step will prevent downstream reactions from influencing the EC_{50}. For example, the ability of increasing concentrations of GR to alter the EC_{50} appears to reach a plateau value, presumably due to the titration of some factor "X" [10]. However, the capacity of Ubc9 to modify the EC_{50} only at saturating GR concentrations is postulated to result from a step beyond "X" becoming limiting and sensitive to Ubc9 [12,18]. Similarly, it is expected that the EC_{50} will be unresponsive to specific inhibitors under many conditions.

Given the predictions of our model that steps downstream from steroid binding might be able to modify the EC_{50} of GR-agonist complexes (and the partial agonist activity of antagonists), we compared the effects of inhibitors of a presumed relatively upstream step (protein deactylation) to those of inhibitors of more downstream steps (RNA pol II CTD phosphorylation and DNA unwinding). In most cases, we utilized two inhibitors. While it is rare that any inhibitor is completely specific like CPT [43], it is highly likely that two inhibitors are each acting as proposed if they both give the same results. In every case, we examined the capacity of inhibitor to reverse the effects of two different processes for modulating the EC_{50} and partial agonist activity: (1) changing concentrations of GR and (2) changing concentrations of Ubc9 with low and high concentrations of GR.

3.2. *Effects of inhibitors of histone deacetylation*

Many enzymes that cause histone acetylation also attach acetyl groups to other transcription factors and modify their activity [48–50]. Two well-known inhibitors of deacetylation are TSA and VPA. To see if histone or protein acetylation is involved in regulating the EC_{50} of GR-agonist complexes, and the partial agonist activity of GR-antagonist complexes, we asked if TSA or VPA can either: (1) mimic the responses seen upon increasing the concentration of GRs or (2) reverse the effects of elevated concentrations of GR [6,7]. The results of Fig. 2 show the usual decrease in EC_{50} when the amount of GR plasmid in transiently transfected CV-1 cells is raised. The addition of sufficient amounts of VPA (Fig. 2A) or TSA (Fig. 2B) to increase the total amount of GR-mediated transactivation has negligible effects on the EC_{50}, or partial agonist activity, with low concentrations (4 ng) of GR complexes (Fig. 2; Table 1). Furthermore, neither TSA nor VPA are able to reverse the effects of elevated GRs (100 ng) and give the higher EC_{50} and lower partial agonist activity associated with low (4 ng) GR concentrations (Table 1). It is curious that

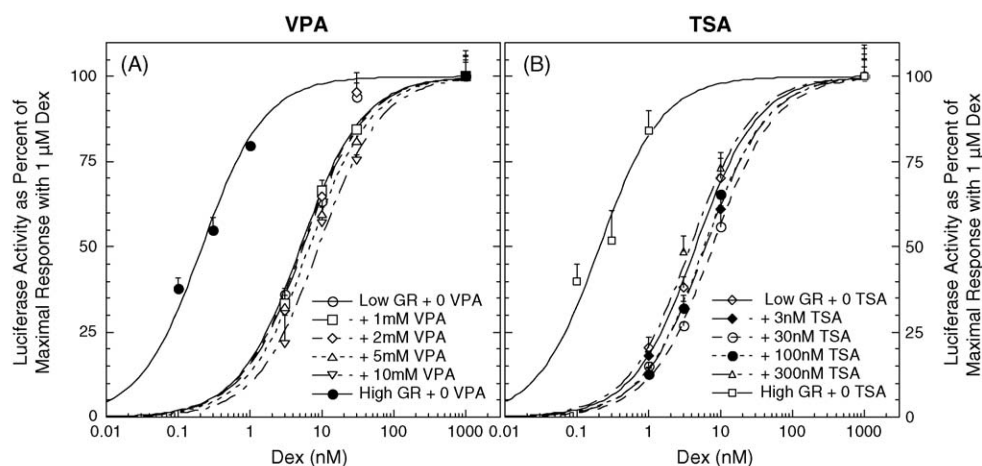

Fig. 2. Effect of HDAC inhibitors on dose–response curve with low GR concentrations. Cells were transfected with 4 ng (low GR) or 100 ng (high GR) of GR plasmid, incubated with the indicated concentrations of Dex and VPA (A) or TSA (B), and plotted as described in Section 2.

TSA and VPA further increase the amount of partial agonist activity displayed in the presence of high (100 ng) GR concentrations. Nevertheless, we conclude that the changes in EC_{50} and partial agonist activity that accompany increased amounts of transfected GRs in cells does not involve deacetylation processes that can be blocked by TSA or VPA at high GR concentrations (100 ng) or mimicked by TSA or VPA at low GR concentrations (4 ng).

TSA and VPA each decrease the amount of basal transcription from the internal control gene (Renilla). This does not appear to be due to massive cell toxicity and death. Instead, it suggests that constitutive (Renilla) and inducible (GR with 1 μM Dex) transcription are unequally affected (see also below). This might be expected as numerous factors have different effects on basal versus induced transcription [51,52].

3.3. *Effect of inhibitors of RNA pol II CTD phosphorylation*

Steroid-induced gene expression is repressed by a lower concentration (20 mM) of the two inhibitors of pol II CTD phosphorylation (DRB and H8) than is the constitutive gene expression from the internal control (Renilla) (Table 2). With both low and high concentrations of GR, each inhibitor causes a slight increase in the EC_{50} for Dex induction and little or no decrease in the amount of partial agonist activity for Dex-Mes (Table 2). However, even at the highest concentration of either inhibitor, the EC_{50} and partial agonist activity values with 4 and 100 ng of GR are still very different from those seen at either GR concentration without inhibitor. Therefore, we conclude that CTD phosphorylation inhibitors cannot reverse

Table 1
Effect of deacetylase inhibitors VPA and TSA on GR induction properties

	Percent of control activity							
	4 ng GR				100 ng GR			
	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)
VPA (mM)								
0	100	100	**<u>27</u>**	**<u>5.1</u>**	100	100	**<u>45</u>**	**<u>0.28</u>**
1	114	169	33	6.1	110	137	66	0.13
2	172	117	35	5.0	178	93	72	0.19
10	190	13	27	8.7	376	22	74	0.33
TSA (mM)								
0	100	100	**<u>30</u>**	**<u>4.9</u>**	100	100	**<u>43</u>**	**<u>0.22</u>**
10	106	145	34	5.4	91	120	65	0.09
100	381	9.7	39	5.1	288	15	77	0.27
300	603	5.4	36	5.3	348	10	83	0.38

The averages from two experiments as depicted in Fig. 2 are given for different concentrations of VPA (top) and TSA (bottom) with low (4 ng) and high (100 ng) amounts of GR plasmid. The changes in total activity with saturating concentrations Dex (1 μM) and with Renilla are given as percent of the value without inhibitor. The actual values of the partial agonist activity of Dex-Mes (DM activity) and the EC_{50} of the dose–response curve with Dex under each condition are listed, with the values of the untreated samples being underlined and in bold type for ease of reference. The average S.E.M. ($n = 2$), as percent of the listed value, is 9.8%.

Table 2
Effect of CTD phosphorylation inhibitors DRB and H8 on GR induction properties

	Percent of control activity							
	4 ng GR				100 ng GR			
	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)
DRB (μM)								
0	100	100	<u>**29**</u>	<u>**6.4**</u>	100	100	<u>**45**</u>	<u>**0.30**</u>
20	39	122	17	11	52	109	39	0.91
60	42	8.6	9.2	15	51	9.7	52	1.6
100	37	6.2	13	10	31	7.0	41	1.1
H8 (μM)								
0	100	100	<u>**27**</u>	<u>**8.2**</u>	100	100	<u>**45**</u>	<u>**0.35**</u>
20	54	89	22	8.2	73	94	36	0.95
40	34	106	18	7.7	53	104	29	1.39
100	18	10	14	17	37	16	37	0.89

The averages from two to four experiments for different concentrations of DRB (top) and H8 (bottom) with low (4 ng) and high (100 ng) amounts of GR plasmid are presented as for Table 1. The average S.E.M. (n = 2–4) is 14% of the listed values.

the effects of elevated GR and do not mimic the changes seen with increased GR.

3.4. Effects of an inhibitor of Topo I

CPT is widely used as a specific inhibitor of Topo I and is thought to act by stabilizing the covalent complex of Topo I and strand-broken DNA [40,41]. As expected, CPT reduces the amount of Dex-induced gene activation with both low and high concentrations of GR by 90% (Table 3). In neither case, however, is CPT able to reproduce the effects on EC_{50} and partial agonist activity that are seen with changing GR concentrations. Thus, we conclude that Topo I is not involved in the modulation of EC_{50} and partial agonist activity by different GR concentrations. It is interesting that CPT has negligible effects on the levels of Renilla gene expression, which is the internal control (Table 3).

3.5. Effects of inhibitors with high GR concentrations plus Ubc9

The mechanism by which altered GR concentrations modify the EC_{50} and partial agonist activity appears to be different from that employed by Ubc9. In particular, the effects of varying concentrations of GR and the cis-acting element GME are thought to be mediated by a rate-limiting step or intermediate “X” [10] while the modulatory activity of Ubc9 is seen only at high or saturating, concentrations of GR and is proposed to act downstream of “X” only after “X” is saturated [12,18]. Therefore, we reinvestigated the responses to each inhibitor with Ubc9 and high concentrations of GR (100 ng), where “X” is saturated and other downstream steps are now rate-limiting.

We first used both high concentrations of GR and a reporter containing the GME element (GMEGREtkLUC), conditions which are known to saturate “X” [10]. The addition of Ubc9 has the same effects as previously seen with the GREtkLUC reporter either with or without the GME [12,18]: the EC_{50} is decreased by a factor of about 5 and the amount of partial agonist activity of Dex-Mes approaches that of a full agonist (Table 4). The HDAC inhibitor VPA decreases the amount of gene expression of a reporter both from the GR-regulated reporter and the internal Renilla control but has insignificant effects on the EC_{50} and partial agonist activity. Thus, even with high GR concentrations when steps downstream of “X” are limiting, an HDAC inhibitor does not alter the EC_{50} and partial agonist activity.

In contrast to the results of Table 3, where CPT has minimal effects upon the partial agonist activity or the EC_{50}, 20 μM CPT in the presence of high GR concentrations and Ubc9 markedly reduces the partial agonist activity of Dex-Mes and increases the EC_{50} of Dex by a factor of 10 (Fig. 3; Table 5). Thus, CPT reverses the effects of the added Ubc9 on GR partial agonist activity and EC_{50} while there is no

Table 3
Effect of Topo I inhibitor CPT on GR induction properties

CPT (μM)	Percent of control activity							
	4 ng GR				100 ng GR			
	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)
0	100	100	<u>**18**</u>	<u>**15**</u>	100	100	<u>**49**</u>	<u>**0.4**</u>
20	11	130	17	7.2	11	89	35	1.0

The averages from three experiments for different concentrations of CPT with low (4 ng) and high (100 ng) amounts of GR plasmid are presented as for Table 1. The average S.E.M. (n = 3) is 19% of the listed values.

Table 4
Effect of deacetylase inhibitor VPA on GR induction properties in presence of Ubc9

VPA (mM)	Percent of control activity							
	100 ng GR w/o Ubc9				100 ng GR with Ubc9			
	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)
0	100	100	**34**	**0.46**	100	100	**88**	**0.10**
1					34	160	84	0.06
2					36	115	70	0.05
10					47	75	81	0.17

The averages from two experiments with 100 ng of GMEGREtkLUC reporter and different concentrations of VPA with high (100 ng) amounts of GR plasmid ± 150 ng of Ubc9 plasmid are presented as for Table 1. The average S.E.M. ($n = 2$) is 21% of the listed values.

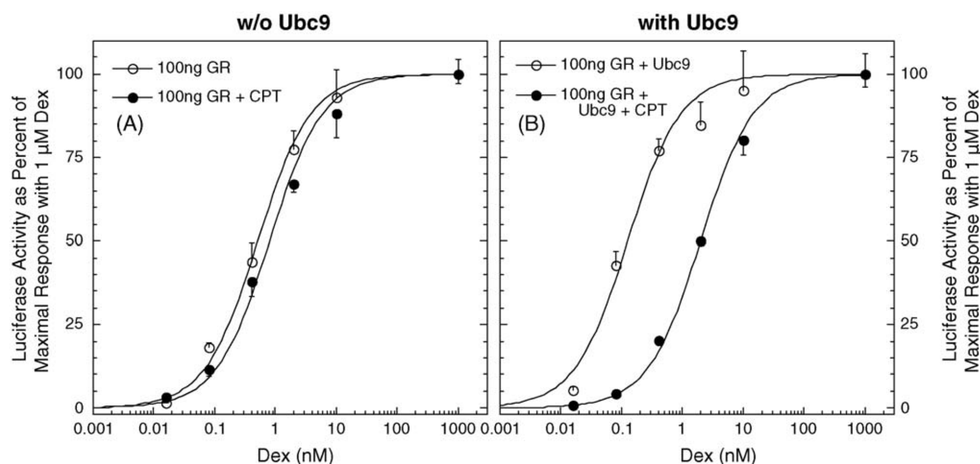

Fig. 3. Effect of CPT on dose–response curve with high GR concentrations ±Ubc9. Cells were transfected with 100 ng of GR plasmid without (A) or with (B) 150 ng of Ubc9 plasmid, incubated with the indicated concentrations of Dex ± 20 μM CPT, and plotted as described in Section 2.

change in the total Dex induced activity and Renilla expression ± Ubc9 (c.f. Table 5 versus Table 3). Ubc9 is a key component in the covalent modification of proteins by the attachment of SUMO, which has been reported to alter the transcriptional activity of GRs [53] in a promoter-dependent manner [18,54,55]. By using both a mutant Ubc9 (C93S) that is defective in sumoylation [56,57] and a GR in which the sumoylation site has been mutated [55], we found that sumoylation of GR is not required for the modulatory effects of Ubc9 [12,18]. Similarly, the ability of CPT to reverse the effects of Ubc9 is retained with the Ubc9(C93S) mutant (Table 5). We therefore conclude that the inhibitory effects of CPT in this assay are not due to any possible ability of CPT to inhibit protein sumoylation.

In the presence of high levels of GR and the GREtkLUC reporter, the CTD phosphorylation inhibitors DRB and H8 are also able to reverse the effects of Ubc9 on GR partial agonist activity and EC_{50} (Table 6). However, the changes in total

Table 5
Effect of Topo I inhibitor CPT on GR induction properties ± wild type and mutant Ubc9

CPT (μM)	Percent of control activity							
	100 ng GR w/o Ubc9				100 ng GR with Ubc9			
	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)
0	100	100	**42**	**0.42**	100	100	**92**	**0.12**
20	12	125	40	0.79	21	82	47	1.6
CPT (μM)					Percent of control activity			
					100 ng GR with Ubc9(C93S)			
					1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)
0					100	100	**90**	**0.13**
20					20	91	50	1.2

The averages from four to eight experiments with different concentrations of CPT and high (100 ng) amounts of GR plasmid ± 150 ng of Ubc9 plasmid are presented as for Table 1. The average S.E.M. ($n = 4–8$) is 17% of the listed values.

Table 6
Effect of CTD phosphorylation inhibitors DRB and H8 on GR induction properties in presence of Ubc9

	Percent of control activity							
	100 ng GR w/o Ubc9				100 ng GR with Ubc9			
	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)
DRB (μM)								
0	100	100	**30**	**0.62**	100	100	**96**	**0.062**
20	48	109	27	0.58	80	111	62	0.61
60	26	37	30	0.74	50	39	52	1.04
H8 (μM)								
0	100	100	**29**	**1.1**	100	100	**81**	**0.14**
20	78	104	29	1.6	108	109	63	0.68
60	53	97	28	1.4	81	111	46	1.3

The averages from two experiments with different concentrations of DRB (top) and H8 (bottom) and high (100 ng) amounts of GR plasmid ± 150 ng of Ubc9 plasmid are presented as for Table 1. The average S.E.M. ($n = 2$) is 9.9% of the listed values.

Dex induced activity and Renilla expression with increasing DRB or H8 are very similar ±Ubc9. This suggests that the ability of DRB and H8 to modulate a pathway that determines the EC_{50} and partial agonist activity of high concentrations of GRs can be seen only when exogenous Ubc9 is present.

3.6. Possible role of XPD in modulation of GR properties

A well-known target of DRB is TFIIH [58]. P-TEFb is another target of DRB but the greater sensitivity of P-TEFb to DRB ($IC_{50} \approx 1$–3 μM versus ≈10 μM) [35–37] suggests that TFIIH may be the target in the experiments of Table 6. TFIIH contains nine subunits [59,60] and the precise target of DRB is not known. XPD is a critical component of TFIIH that holds the two multi-subunit components of TFIIH together [59,60]. If TFIIH is a common target of DRB and Ubc9, then Ubc9 should be unable to affect a left shift in the dose–response curve or increase the partial agonist activity of Dex-Mes in cells containing a disabling mutation of XPD. Coin et al. [59] have described cells (HD2 cells) containing a mutant XPD that causes a weakened association between the two TFIIH subunits and has decreased cdk7 activity. These cells are hybrids between HeLa cells and fibroblasts of a sub-population of humans suffering from *Xeroderma pigmentosum* (XP), who carry a R683W point mutation in the XPD gene that inactivates the XPD protein.

We first determined whether elevated GR concentrations cause a change in GR partial agonist activity and EC_{50} in HD2 cells. As seen in Table 7, greater amounts of transfected GR plasmid increase the partial agonist activity of Dex-Mes, and decrease the EC_{50} of Dex, just as has been reported in other cells (reviewed in refs. [15,61]). Next, we examined the response to Ubc9. As seen in other cells [12,18], there is little effect of Ubc9 with low concentrations of GR in HD2 cells (Table 7). With high GR concentrations, Ubc9 does alter the partial agonist activity and EC_{50} of GR complexes. This ability of HD2 cells to respond to added Ubc9 even though they contain an inactive XPD mutant suggests that XPD is not a target of Ubc9. It should be noted that DRB is a much less effective inhibitor of the actions of Ubc9 in HD2 cells than in CV-1 cells (Table 7 versus Table 6).

As an alternative approach to examining the involvement of XPD, we asked if the addition of XPD to the XPD-deficient HD2 cells could make them both more sensitive to added Ubc9 and more resistant to DRB. In the absence of Ubc9, ectopic XPD has little effect on the partial agonist activity and EC_{50} (Table 8). The addition of Ubc9 affords much less change in these experiments than in those of Table 7. Unex-

Table 7
Effect of CTD phosphorylation inhibitor DRB on GR induction properties ± Ubc9 in HD2 cells

DRB (μM)	Percent of control activity							
	≤5 ng GR w/o Ubc9				≤5 ng GR with Ubc9			
	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)
0	100	100	**4.6**	**12**	100	100	**10**	**7.2**
	100 ng GR w/o Ubc9				100 ng GR with Ubc9			
	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)
0	100	100	**22**	**1.0**	100	100	**61**	**0.36**
20	37	93	23	1.0	56	23	56	0.30
60	24	25	28	1.2	19	12	51	0.95

The averages from two to seven experiments with different concentrations of DRB and low (≤5 ng; top) or high (100 ng) amounts of GR plasmid ± 150 ng of Ubc9 plasmid are presented as for Table 1. The average S.E.M. ($n = 2$–7) is 15% of the listed values.

Table 8
Effect of CTD phosphorylation inhibitor DRB on GR induction properties ±Ubc9 and ±XPD in HD2 cells

DRB (μM)	Percent of control activity							
	100 ng GR w/o Ubc9 w/o XPD				100 ng GR with Ubc9 w/o XPD			
	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)
0	100	100	**18**	**2.0**	100	100	**44**	**1.4**
20					28	99	35	1.6
60					18	31	35	1.8
	100 ng GR w/o Ubc9 with XPD				100 ng GR with Ubc9 with XPD			
	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)	1 μM Dex	Renilla (EtOH)	% DM activity	EC_{50} (nM)
0	100	100	**29**	**1.9**	100	100	**32**	**2.8**
20					27	161	26	2.7
60					17	58	23	2.7

The averages from two to four experiments with different concentrations of DRB and high (100 ng) amounts of GR plasmid ± 150 ng of Ubc9 plasmid without (top) or with (bottom) 110 ng of XPD plasmid are presented as for Table 1. The average S.E.M. (n = 2–4) is 12% of the listed values.

pectedly, added XPD blunts even the limited responses to Ubc9 (bottom two charts versus top two charts). XPD also does not rescue the ability of DRB to alter the partial agonist activity and EC_{50} of GR complexes with Ubc9 in HD2 cells (Tables 7 and 8 versus Table 6). Because added XPD does not improve either the activity of Ubc9 or the inhibition by DRB in HD2 cells, we conclude that XPD is not a major component in the steps that are affected by Ubc9 and DRB and that some other feature of the XPD-deficient HD2 cells is responsible for attenuating the responses to Ubc9 and DRB.

4. Discussion

The ability of various factors to modulate the position of the dose–response curve of GR-agonist complexes and the amount of partial agonist activity of GR-antagonist complexes is well documented and appears to be general for the classical steroid receptors ([16–18,62,63] and reviewed in refs. [15,61]). We have now constructed a simple mathematical model for GR-regulated gene induction that predicts that the shape of the dose–response curve for this multi-step process can be the same as that for a one-step reaction following Michaelis–Menten kinetics. This model, albeit unsophisticated, also predicts that changes in the equilibrium constant or factor concentration of individual steps can alter the position of the dose–response curve, even when the affected step is well downstream from steroid binding to receptors. This prediction led us to ask whether the addition of inhibitors of several possible steps downstream of GR binding of steroid could prevent the changes in EC_{50} and partial agonist activity of GR complexes that are seen: (i) with different concentrations of GRs and (ii) with Ubc9 in the presence of high but not low concentrations of GRs. Previous results indicated that different rate-limiting steps are operative under conditions (i) and (ii) [10,12], in which case the ability of specific inhibitors to block any changes in EC_{50} and partial agonist activity would also be expected to be different. Our finding that only DRB, H8, and CPT prevent the effects of added factors under conditions (ii) but not (i) supports aspects of both our model (Fig. 1) and its predictions.

The ability of DRB, H8, and CPT to alter GR EC_{50} and partial agonist activity only with high amounts of GR and Ubc9 also reinforces our earlier conclusion that the determination of these parameters by elevated levels of GR versus high GR and Ubc9 occurs via different mechanisms [10,12]. The effects of CPT with high GR and Ubc9 concentrations are most likely mediated by Topo I in view of the very high specificity of CPT for Topo I [43].

CTD phosphorylation of RNA pol II is inhibited by DRB and H8 and participates in the transition from initiation to elongation of mRNA transcripts [3]. All of the targets of DRB and H8 are not yet known but two documented targets are TFIIH and P-TEFb [35–37]. Cdk7/cyclin H, a component of the general transcription factor TFIIH, is the kinase that phosphorylates the CTD after the pre-initiation complex is formed [64]. Cdk9/cyclin T1, a component of P-TEFb, is critical for the transition of RNA pol II to a productive elongating complex [65]. TFIIH is a multi-subunit complex, consisting of a core of five proteins (XPB, p62, p52, p44, and p34) that is linked to a Cdk-activating kinase complex (cdk7, cyclin H, and MAT1) by XPD [59,60], which increases the transcriptional activity of several steroid receptors [66]. The fact that both DRB and H8 produce the same response strongly suggests that their effects are both due to their primary activity of inhibiting CTD phosphorylation. A further indication of the selectivity of DRB and H8 (and CPT) is that their effects on EC_{50} and partial agonist activity are seen at high GR concentrations but only with Ubc9. The absence of any effect at high GR concentrations without added Ubc9 argues against non-specific targets of these inhibitors under a variety of cellular conditions.

The molecule(s) that mediate the inhibitory actions of DRB and H8 in our study is currently unknown. XPD, which is closely associated with a Cdk-activating kinase complex of TFIIH, does not appear to be the target of DRB and H8

because of the similar modulatory activity of Ubc9 in cells with (Table 4) and without (Table 7) functional XPD. Conversely, the data of Table 6 do not uniquely implicate P-TEFb as the target because: (1) the DRB concentrations needed for maximal inhibition here are much higher than the 1–3 μM that normally inhibits P-TEFb [35–37] and (2) H8 is usually more active than DRB in preventing P-TEFb actions [36], while H8 and DRB are about equally active in Table 6. We cannot completely rule out the involvement of XPD, though, in light of the observation that overexpression of XPD in HD2 cells, containing an inactivated XPD, prevents Ubc9 from exerting a weak modulatory activity (Table 8), perhaps due to squelching and the sequestration of TFIIH components. This explanation would be consistent with the recently reported binding of Ubc9 to RAD52, which also binds to XPD [67].

The CTD phosphorylation inhibitors DRB and H8 reduce the levels of transactivation by GR (Tables 2 and 6–8), just as described for androgen receptors [68]. We find that low concentrations of inhibitor preferentially decrease the levels of receptor-regulated gene expression, compared to constitutive expression of the internal Renilla control gene, in all cases except for high levels of GR with Ubc9 in the XPD mutant cells, HD2. This supports the conclusion that enhancer-driven gene transcription is particularly sensitive to the effects of CTD phosphorylation of RNA polymerase II [68,69].

Inhibitors of protein deacetylation (TSA and VPA), Topo I (CPT), and RNA pol II CTD phosphorylation (DRB and H8) are all unable to mimic the effects of increasing the concentration of GR plasmid from 4 to 100 ng (Fig. 2; Tables 1–3). These inactivities are not due to insufficient amounts of inhibitor because the amounts used always affect the total levels of GR-induced or control plasmid-directed gene expression. The inhibitors occasionally induce changes in the EC_{50} or partial agonist activity but, in each of these cases, the magnitude of the change is either much less than, or is in the opposite direction from, that seen when changing the GR concentration. Therefore, we conclude that the reactions that are prevented are not critical for the modulation of EC_{50} and partial agonist activity when varying GR concentrations in the absence of Ubc9.

The current model of steroid hormone action is that HRE-bound receptor-steroid complexes recruit coactivators and corepressors that, by virtue of their associated histone acetyltransferase (HAT) [70–73], and histone deacetylase (HDAC) activity [74–76], respectively, alter histone acetylation patterns and thus modify chromatin reorganization and eventually the levels of gene expression [26–28]. Recently, HATs and HDACs have also been found to influence the activities of a variety of non-histone proteins (reviewed in ref. [77]). TSA is believed to be a more specific inhibitor of histone acetylation than VPA [29] but the effects of TSA can be variable [78–80]. TSA is reported to both increase and decrease GR transactivation of the MMTV gene [78–80]. Whether we see an increase (Table 1 and Wang et al. [16]) or decrease (Table 4) in total activity with the agonist Dex depends upon the specific conditions. However, there is little change with added HDAC inhibitor in either the EC_{50} or the partial agonist activity. Similarly, we recently reported that TSA does not alter the ability of the corepressor SMRT to modulate either the dose–response curve or the partial agonist activity of GR complexes [16]. This is in marked contrast with the general notion that histone acetylation causes increased gene transcription due to chromatin remodeling [26–28]. These responses to HDAC inhibitors are yet another example of how the total amount of gene induction is influenced by factors, and appears to proceed by steps, that are different from those that control the position of the dose–response curve and the amount of partial agonist activity [5–7,10,12,13,15,16,47,61].

The results with 100 ng of GR and TSA or VPA are particularly intriguing because the partial agonist activity and EC_{50} both increase (Table 1). Up until now, an increase in partial agonist activity has always been associated with a decrease in EC_{50} (reviewed in refs. [15,61]). Thus, for the first time, we have uncoupled the responses of these two parameters. These observations may provide a new approach for investigating how these two parameters are altered.

We previously concluded that modulation of these properties of GR is independent of the histone acetyltransferase activity of CBP [10]. Because TSA and VPA have been found to also prevent the deacetylation of some non-histone proteins [77], we now can expand our conclusion to say that acetylation/deacetylation of some non-histone proteins is also not important. However, we cannot yet conclude that protein acetylation/deacetylation in general is not important.

Little has been reported regarding CPT and steroid receptors. Pretreatment of human HL 60 leukemia cells with CPT is reported to enhance vitamin D_3-responsive CD14 expression two-fold [81]. In contrast, CPT in our system with GRs causes a 5–10-fold decrease in the steroid receptor-mediated gene transcription (Tables 3 and 5), which is similar to the decrease in Dex induction of TAT mRNA caused by CPT in FTO-2B cells [82]. Unfortunately, there are not enough data to speculate on the causes of these different responses. Interestingly, it has been reported that treatment of mammalian cells or yeast cells expressing human Topo I with CPT induces covalent modification of Topo I by SUMO-1 [83]. Ubc9 is key intermediate in the sumoylation of proteins [56]. Therefore, it was possible that the modulatory effects of Ubc9 seen in Tables 4–8 could reflect Ubc9's ability to add SUMO-1 to Topo I. However, the similar activities in Table 5 of wild type Ubc9 and Ubc9 containing the C93S mutation, which eliminates the sumoylation activity of Ubc9 [56,57], proves that the observed behavior is independent of any sumoylation of Topo I by Ubc9.

In summary, the effects of elevated GR and the GME are proposed to be mediated by a common step "X" [10] that eventually influences the levels of mRNA transcripts [4]. The inability of any of the inhibitors of this study to reverse the effects of changing GR concentrations on the EC_{50} of agonists, or the partial agonist activity of antagonists, suggests that the critical rate-limiting step or intermediate "X" is not

affected by a deacetylation that can be blocked by TSA or VPA, by CTD phosphorylation of RNA polymerase II, or by Topo I. Conversely, the observations that inhibitors of CTD phosphorylation of RNA polymerase II and Topo I, but not HDAC inhibitors, do reverse the effects of added Ubc9 with high concentrations of GR indicates that the affected steps are downstream of the hypothetical step or intermediate "X". These results appear to restrict the possible steps that are modulated by added GR to those that occur before transcription initiation/elongation and DNA unwinding. Finally, the ability of two different classes of inhibitors to reverse the modulatory activity of Ubc9 with high concentrations of GR indicate that more than one step is involved. The next task is to identify these sensitive steps.

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Appendix A

Strickland and Loeb [86] showed in a mathematical model of hormone binding and biological response that a sequence of two or more second-order reactions could be constructed so that standard Michaelis–Menten kinetics is preserved and the half-maximal biological response would be evoked at a concentration below half-maximal receptor occupation. Later, they showed that, with a modification, this shift in biological efficacy (a decrease in the EC_{50}) could go in either direction [46]. A critical assumption in their model was that the concentration of a downstream mediator be directly proportional to the end-product of the upstream reaction. Here, we show that this assumption is not realized in a simple cascade of second order reactions. We then give two examples of when this assumption may hold.

We first re-derive the Strickland and Loeb [86] result. Consider the simple reaction

$$\mathrm{H} + \mathrm{R} \leftrightarrow \mathrm{C}$$

where H is the steroid hormone, R the receptor, and C is the end-product of the reaction. At equilibrium, the dissociation constant β is

$$\beta = [\mathrm{H}]\frac{[\mathrm{R}]}{[\mathrm{C}]} \tag{1}$$

For a limited number of receptors, the conservation condition $[\mathrm{R}] + [\mathrm{C}] = \mathrm{R_T}$ holds, where $\mathrm{R_T}$ is the total (initial) concentration of R. Inserting this conservation condition into (1) and rearranging gives

$$[\mathrm{C}] = \frac{\mathrm{R_T}[\mathrm{H}]}{(\beta + [\mathrm{H}])} \tag{2}$$

This is the classic Michaelis–Menton relationship.

Now consider a second reaction

$$\mathrm{C}' + \mathrm{D} \leftrightarrow \mathrm{P}$$

where D is a second mediator and P is a downstream product. For a limited amount of D, we have the condition $[\mathrm{D}] + [\mathrm{P}] = \mathrm{D_T}$ and the equilibrium concentration of P is

$$[\mathrm{P}] = \frac{\mathrm{D_T}[\mathrm{C}']}{(\gamma + [\mathrm{C}'])}$$

where $\gamma = [\mathrm{C}'][\mathrm{D}]/[\mathrm{P}]$. If the two reactions are connected with the relation

$$[\mathrm{C}'] = \alpha[\mathrm{C}], \tag{3}$$

where α is a proportionality constant, then

$$[\mathrm{P}] = \frac{\mathrm{P_{MAX}}[\mathrm{H}]}{(\mathrm{P_{50}} + [\mathrm{H}])} \tag{4}$$

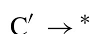
where $\mathrm{P_{MAX}} = \alpha\mathrm{R_T}\mathrm{D_T}/(\gamma + \alpha\mathrm{R_T})$ and $\mathrm{P_{50}} = \beta\gamma/(\gamma + \alpha\mathrm{R_T})$. Thus, the maximum of [P] ($\mathrm{P_{MAX}}$) and the EC_{50} of [P] ($\mathrm{P_{50}}$) can be manipulated by changing the total concentration of R and D, as well as by changing the dissociation constants β and γ. $\mathrm{P_{MAX}}$ is a linear function of $\mathrm{D_T}$ but a Michaelis–Menton function of $\mathrm{R_T}$ with the half-maximum at γ. The roles of $\mathrm{R_T}$ and γ are reversed for $\mathrm{P_{50}}$. Additionally, $\mathrm{P_{50}}$ is a linear function of β. The biological relevance of these reactions for steroid biochemistry will be discussed below.

The Michaelis–Menton form in Eq. (4) depends crucially on relation (3). We note that if we simply set $\mathrm{C}' = \mathrm{C}$ in the second reaction, then (4) would not hold because the conservation condition for the reactants would be $[\mathrm{H}] + [\mathrm{R}] + [\mathrm{P}] = \mathrm{R_T}$. However, if the equilibrium concentration of P is very small (i.e., $[\mathrm{P}] \ll [\mathrm{R}],[\mathrm{H}]$), then we can set $\mathrm{C}' = \mathrm{C}$ in the second reaction and obtain Eq. (4) with $\alpha = 1$. The smallness assumption is equivalent to $\mathrm{R_T} \gg \mathrm{D_T}$. This would imply that the concentrations of end-products must be much smaller than the concentration of the constituents which does not seem to hold for steroid biochemistry.

Another scenario for which relation (3) may hold is if the product C is recycled. For example, consider the reactions

$$\mathrm{H} + \mathrm{R} \leftrightarrow \mathrm{C}$$

$$\mathrm{C} \rightarrow \mathrm{C} + \mathrm{C}'$$



$$\mathrm{C}' \rightarrow {}^{*}$$

$$\mathrm{C}' + \mathrm{D} \leftrightarrow \mathrm{P}$$

For this system we can show that the equilibrium concentration of P also satisfies Eq. (1) with $\alpha = [\mathrm{C}']/[\mathrm{C}]$. In this case, in addition to the previous manipulations, increasing α increases $\mathrm{P_{MAX}}$ and decreases $\mathrm{P_{50}}$ (and vice versa).

In this scenario, C' is derived from C; it needs receptor R to be formed but does not contain any R. However, C' does not have to be directly derived from C. Rather several steps, including irreversible steps like activation of glucocorticoid receptors (GRs), can intervene as long as no enzymatic or factor-mediated steps are involved. An example of such steps would be GR-mediated transcription factor binding to GR responsive elements (GRE) of a GR-regulated gene. Once activated and translocated into the nucleus, it is thought that GR can bind to DNA and recruit other transcriptional cofactors without the intervention of other factors. Once the cofactors are localized to the promoter region, it appears that GR can dissociate [84,85] without attenuating the transcriptional activity of the other GRE-bound proteins. Thus, some of the initial GRE-bound GR-steroid complexes dissociate and recycle back into the previous reaction steps, with the remaining C' complex either collapsing into transcriptionally inactive species (i.e., $C' \rightarrow {}^{*}$) or reacting with other species to give the next intermediate (i.e., $C' + D \leftrightarrow P$) in the ordered series of steps that eventually lead to increased transcription of the target gene.

Now, suppose that further downstream is another reaction that depends on P and produces an end-product F:

$$P + E \leftrightarrow F$$

If we assume the equilibrium concentration of F is much smaller than that of C and D (which would occur if F is rapidly consumed) then the equilibrium concentration is

$$[F] = \frac{F_{MAX}[H]}{(F_{50} + [H])}$$

where

$$F_{MAX} = \frac{\nu E_T P_{MAX}}{(\eta + \nu P_{MAX})} = \frac{\nu\alpha E_T D_T R_T}{(\eta(\gamma + \alpha R_T) + \nu\alpha D_T R_T)},$$

$$F_{50} = \frac{\eta P_{50}}{(\eta + \nu P_{MAX})} = \frac{\eta\beta\gamma}{(\eta(\gamma + \alpha R_T) + \nu\alpha D_T R_T)},$$

$E_T = E + F$, $\nu = 1$, and $\eta = [P][E]/[F]$. Alternatively, an intermediate product P' derived from P could combine with E to form F so that $\nu = [P']/[P]$. The parameters that control F_{MAX} and F_{50} are those that control P_{MAX} and P_{50} along with E_T, ν, and η.

The cascading of reactions can continue indefinitely. At each stage, an end-product combines with an auxiliary reactant to produce a new end-product. Michaelis–Menton dependence of the final product on the hormone will hold if: (1) the equilibrium concentrations of the downstream end-product is much smaller than that of the upstream end-product (an equivalent condition is that the total concentration of the downstream auxiliary reactant is less than the upstream auxiliary reactant); (2) the end-products at each stage are recycled; (3) some combination of (1) and (2).

The resulting final V_{MAX} and EC_{50} will depend on all the intervening equilibrium reaction concentrations and the intervening total auxiliary reactant concentrations. They will have either a linear or Michaelis–Menten functional dependence on all of these parameters. Experimentally, it has been found that the introduction of factors can alter the V_{MAX} and EC_{50} of steroid regulation in four ways: (1) V_{MAX} increases (decreases) while the EC_{50} decreases (increases), (2) V_{MAX} stays constant while EC_{50} decreases, (3) V_{MAX} increases while the EC_{50} stays constant, and (4) both decrease.

We consider these four cases in terms of the action of the steroid H on a final product F. Case (1) is the most straightforward to understand. A number of manipulations will do this including increasing the number of receptors or decreasing the dissociation constants of any reaction. In general, by manipulating a single parameter changing F_{50} leads to an opposite effect in F_{MAX}. However, we emphasize that the magnitude of the changes need not be the same. For example, F_{50} could change by a large value and F_{MAX} could change by very little or not at all and vice versa.

The other three cases are much less clear. For case (2), one possibility would be for the dissociation constant η to be very small so that $\alpha D_T R_T \gg \eta(\gamma + \alpha R_T)$. In this way, $F_{MAX} \approx E_T$ and $F_{50} \approx \eta\beta\gamma/\nu D_T R_T$. Thus, F_{50} can be changed while F_{MAX} remains constant. For case (3), suppose that γ is very large or the number of receptors R_T is very low so that $\eta\gamma \gg \alpha R_T(\eta + D_T)$. This then implies that $F_{MAX} \approx \alpha E_T D_T R_T/\eta\gamma$ and $F_{50} \approx \beta$. Then by keeping β constant, F_{50} will remain constant while the other parameters can increase (or decrease) F_{MAX}. Within our cascaded reaction scheme, there does not seem to be a means to account for case (4) if only one parameter is manipulated. However, if a given agent can manipulate two parameters simultaneously then it may be possible. For example, an increase in D_T and a decrease in E_T could decrease both F_{50} and F_{MAX}.

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