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TWO DISTINCT MECHANISMS FOR REGULATION OF γ-GLUTAMYL TRANSPEPTIDASE IN CULTURED RAT HEPATOCYTES BY GLUCOCORTICOID-LIKE STEROIDS

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Abstract—Adult rat hepatocytes maintained in primary monolayer culture with defined medium were used to characterise two effects of glucocorticoid-like steroids in regulating γ-glutamyltranspeptidase (GGT). Low concentrations of glucocorticoids alone had little effect on GGT but synergistically enhanced induction of the enzyme by liver tumor-promoting xenobiotics such as 1,1,1-trichloro-2,2-bis-(4-chlorophenyl)-ethane (DDT) and hexachlorocyclohexane. The enhancing effect appears to be mediated by the classical glucocorticoid hormone receptor since structural requirements and concentration-dependence for enhancement were similar to those for induction of tyrosine aminotransferase in parallel cultures. Higher concentrations (1–100 μM) of various glucocorticoids alone increased GGT activity. Most glucocorticoids induced GGT but their order of potency did not parallel that for induction of tyrosine aminotransferase under similar culture conditions. Among the most potent glucocorticoids, triamcinolone was a weak GGT inducer and cortivazol appeared to act as an antagonist of GGT induction by steroids. Some non-glucocorticoids including pregnenolone 16α-carbonitrile, and some progestins, also induced but required addition of 30 nM dexamethasone for maximal effect. Some specific steroid structural features were identified which increased (presence of a 16α methyl group) or impaired GGT-inducing activity. Although interpretation is complicated by differential metabolism of individual steroids in culture, the results suggest that GGT induction by pharmacological levels of steroids may be mediated, directly or indirectly, by one or more relatively specific receptors distinct from the classical glucocorticoid receptor.

γ-Glutamyltranspeptidase (GGT†) catalyses transfer of a γ-glutamyl group from glutathione to acceptors such as water, amino acids or peptides [1]. It has been suggested that GGT favours detoxification of various xenobiotics via conjugation with glutathione, either by providing the cell with amino acids necessary for glutathione synthesis [2] or by removing glutamate from glutathione conjugates, thus facilitating subsequent conjugate excretion as mercapturic acids [3]. GGT in liver cells is induced *in vivo* [4–10] and in primary hepatocyte cultures [11–15] by a variety of steroids and xenobiotics, many of which also induce a group of other enzymes involved in drug metabolism and detoxification [16, 17]. Induction of GGT may thus be part of a broad adaptive response to potential toxins and provides a system for investigating mechanisms of metabolic adaptation to drug and steroid exposure.

A high level of expression of multiple enzymes involved in detoxification reactions including GGT is also a feature of the "preneoplastic phenotype" in liver. This phenotype is observed very frequently in foci of hepatocytes resulting from a combination of initiation and promotion in liver carcinogenesis [18, 19]. Schulte-Hermann [19] has suggested that the "preneoplastic phenotype" may result from aberrant or over-expression in initiated cells, of an adaptive programme favoured in all hepatocytes by phenobarbital and other liver tumor promoters. Most of the xenobiotics and steroids known to be liver promoters have been shown to induce GGT in normal hepatocytes [13, 14]. Elucidation of the mechanism(s) by which such compounds induce GGT should help, in conjunction with other studies on promotion, to establish whether changes in GGT are directly linked with tumor promotion or unrelated to carcinogenesis. This study represents one phase of efforts to characterise the mechanism(s) of GGT induction using rat hepatocytes in primary culture.

In previous work to define the range of drugs and steroids capable of elevating GGT activity in cultured hepatocytes [11, 13, 14], it became apparent that dexamethasone, a glucocorticoid which has tumor-promoting activity in liver [20], has two types of effect on GGT. Relatively low (nM) concentrations of dexamethasone alone have little effect on GGT but synergistically enhance the inducing action of phenobarbital and other xenobiotics [13, 14]. Higher (μM) concentrations of dexamethasone [11, 13, 14] or hydrocortisone [15] directly induce GGT. In the present study a range of glucocorticoids and structurally-related compounds has been tested to define structural and other requirements for the enhancing and inducing effects observed with dexamethasone.

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† Abbreviations: DDT, 1,1,1-trichloro-2,2-bis-(4-chlorophenyl)-ethane; GGT, γ-glutamyltranspeptidase (EC 2.3.2.2); HCH, α-hexachlorocyclohexane; PCN, pregnenolone 16α-carbonitrile; TAT, tyrosine aminotransferase (EC 2.6.1.5); RU25055, 11β-thienyl-17α-ethynyl-19-nor-androsta-4,9-diene-17β-ol-3-one; and RU38486, 11β(*p*-dimethylamino)phenyl - 17α - ethynylmethyl - 19 - nor-androsta-4,9(10)diene-17β-ol-3-one.

Glucocorticoid activities of test compounds under the conditions of the study have been monitored by their abilities to induce tyrosine aminotransferase (TAT), since induction of this enzyme by steroids is considered to involve the classical glucocorticoid receptor [21, 22].

MATERIALS AND METHODS

Animals and materials. Male Wistar Rats (Porton Strain, 200–300 g) given laboratory chow and water *ad lib.* were used for all hepatocyte preparations. Media formulations and sources were as previously described [11]. Some steroids were provided as follows: RU25055, RU38486 and cortivazol (Roussel Uclaf, Romainville, France); cloprednol, paramethasone acetate and fluclorolone acetonide (Syntex, Palo Alto, CA, U.S.A.); cyproterone acetate (Schering AG, Berlin, Federal Republic of Germany); and pregnenolone-16α-carbonitrile (The Upjohn Co., Kalamazoo, MI, U.S.A.). Other steroids were purchased from Sigma. DDT and α-hexachlorocyclohexane were from Serva Feinbiochemica (Heidelberg, Federal Republic of Germany), and all other biochemicals from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Hepatocyte isolation and culture. Hepatocytes were isolated by a two-step collagenase perfusion procedure, plated into collagen-coated culture dishes to produce confluent hepatocyte monolayers, and maintained (after attachment) in defined medium for up to 5 days, essentially as described previously [11, 14]. For these studies approximately 2.5×10^6 hepatocytes were plated into 50-mm plastic culture dishes (Disposable Products, Adelaide, South Australia) coated with rat rail collagen. After allowing 3 hr for cell attachment, the medium was changed to modified Waymouth medium with insulin and antibiotics as defined in Ref. 11 or, where specified, to a "selective" version of this medium (containing 1 mM ornithine in place of arginine) designed to prevent outgrowth of fibroblast-like cells [12, 23].

Assays of GGT, TAT and protein. After experimental treatments, hepatocyte monolayers were washed twice with 0.15 M NaCl, and for GGT assays were scraped from dishes into 2 ml of 0.1 M Tris–HCl, pH 7.4. Cell lysates were rapidly frozen in liquid N_2 and stored at −20°. Thawed samples were homogenised for 10 sec with an Ultraturrax homogeniser at two-thirds maximum speed and then were assayed for GGT activity as previously described [11]. A unit of GGT activity is defined as the amount of enzyme catalysing formation of 1 μmole *p*-nitroaniline/min at 37°. For TAT assays, saline-washed monolayers were harvested into 2 ml buffer containing 0.1 M potassium phosphate, pH 7.5, 1 mM EDTA, and 0.1% Triton X-100. Samples were briefly homogenised as for GGT assays, and TAT activity was determined by the method of Diamondstone [24]. Proteins were determined by the procedure of Lowry *et al.* [25]. The statistical significance of differences between sets of data was assessed using Student's *t*-test, and values of $P < 0.05$ were considered significant. Unless otherwise indicated, the results shown in each figure or table were obtained with a single hepatocyte preparation. In every case, comparable results were obtained in replicate experiments with independent cell preparations.

Measurements of protein synthesis. To initiate measurements of total protein synthesis, fresh modified Waymouth medium containing 1 μCi L-[4,5(n)-^{3}H]leucine (New England Nuclear, Boston, MA, U.S.A.; specific activity in medium 1.8 μCi/μmole) was added to monolayers. After incubation for 2 hr at 37°, dishes were washed with 0.15 M NaCl containing 0.1 mM leucine, harvested into 1 M KOH, and homogenised briefly with an Ultraturrax homogeniser. Aliquots of homogenate were taken for protein assay [25] and transferred to squares of 3 MM paper for determination of [^{3}H]leucine incorporation into trichloroacetic acid-precipitable material by the method of Mans and Novelli [26].

RESULTS

Enhancing effects of low glucocorticoid concentrations. Culture conditions and experimental protocols for this section of the study were based on previous observations that phenobarbital [13] and a variety of other drugs and non-glucocorticoid steroids [14] cause GGT induction, clearly detectable after 4–5 days of exposure to the inducers, which is largely dependent on the presence of a low concentration (20–30 nM) of dexamethasone. In addition to its enhancing action, this dexamethasone level also suppresses the outgrowth of GGT-positive fibroblast-like cells which occurs in some cultures [12] maintained with the serum-free modified Waymouth medium. Thus, the enhancing action of glucocorticoid-like steroids was tested in 5-day culture experiments and, when glucocorticoid was omitted from some dishes, the experiment was conducted with selective medium (see Materials and Methods), and/or cultures were checked for contamination by fibroblast-like cells.

Table 1 shows the relative induction of GGT by three xenobiotics with dissimilar structures (HCH, PCN and DDT) in the absence or presence of various steroids. The added concentrations of the known glucocorticoids, dexamethasone, prednisolone, triamcinolone acetonide and corticosterone, were sufficient to cause maximal or near-maximal induction of TAT in similar hepatocyte cultures (see below, Table 3), whereas the anti-glucocorticoid fluoxymesterone [27] had no effect on TAT activity at the concentration shown. At these levels, none of the added steroids alone had very marked effects on GGT activity. The three xenobiotic inducers tested differed somewhat in their relative dependence on added glucocorticoid as previously reported [14], but in each case all of the glucocorticoids synergistically enhanced induction of GGT, whereas the anti-glucocorticoid fluoxymesterone did not.

In Fig. 1, the effects on GGT of a single xenobiotic, DDT, in combination with various concentrations of four glucocorticoids, are compared with the concentration-dependence of TAT induction by the glucocorticoids in parallel cultures. The conditions for TAT and GGT inductions were identical except

Table 1. Effects of steroids in combination with various xenobiotic inducers on GGT activity in hepatocytes maintained in culture for 5 days*

Additions	GGT activity (mUnits/mg protein)			
	None	HCH (50 μM)	PCN (50 μM)	DDT (50 μM)
None	4.8 ± 0.5	5.8 ± 0.4†	13.2 ± 0.4§	8.7 ± 0.6†
Dexamethasone, 30 nM	4.4 ± 0.6	15.6 ± 1.1§	23.2 ± 1.8§	12.5 ± 0.4§
Prednisolone, 1 μM	5.3 ± 1.0	15.1 ± 1.4§	20.1 ± 2.4§	13.1 ± 1.6§
Triamcinolone acetonide, 3 μM	5.9 ± 0.5†	13.8 ± 1.4§	ND‖	15.8 ± 1.0§
Corticosterone, 10 μM	6.9 ± 0.8‡	15.5 ± 0.6§	24.1 ± 1.5§	ND
Fluoxymesterone, 50 μM	7.7 ± 0.7‡	6.9 ± 0.7	10.3 ± 1.8	ND

* Hepatocyte cultures were maintained as described in Materials and Methods. Each of the additions shown was readded at each daily medium change, i.e. was present throughout the 5-day culture period. Values are means ± SD (N = 4).

†–§ Significances of increases:

Over dishes with no additions: † $P < 0.02$ and ‡ $P < 0.001$.

Over corresponding controls either without xenobiotic (i.e. values in left-hand column) or without steroid (i.e. values in top row of table): § $P < 0.001$.

‖ Not determined.

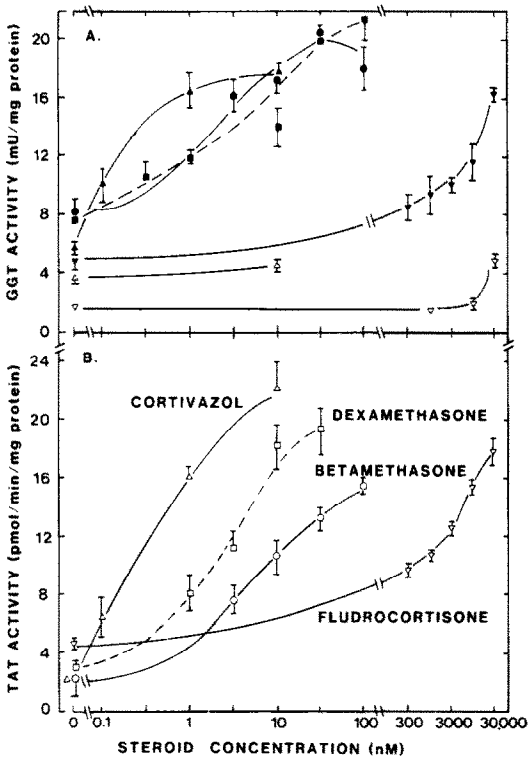

Fig. 1. Comparison of the concentration-dependence of the ability of glucocorticoids to enhance GGT induction by DDT and induce TAT. Hepatocyte monolayers were maintained in modified Waymouth medium without (open symbols) or with 50 μM DDT (closed symbols) and steroids as shown. For GGT, test compounds were added after attachment and with each daily medium change, and activity was assayed after 5 days (panel A). For TAT, steroids were added after 24 hr, and TAT was assayed after a further 24 hr (panel B). Curves for each steroid were obtained with separate hepatocyte preparations. Values are means ± SD (N = 4).

that GGT was assayed after 5 days whereas the more rapid response in TAT levels was assayed after a 1-day exposure to added steroid. Similar results were obtained when TAT was assayed after 3–5 days (results not shown), indicating that variations in the extent or routes of steroid metabolism or the accumulation of metabolites with time in culture did not change significantly the relative response of TAT to a number of added steroids. Figure 1B shows that the steroids tested differed by 3–4 orders of magnitude in their relative potency as glucocorticoids, as indicated by relative TAT induction. At the concentrations used in Fig. 1 the glucocorticoids alone had negligible or small effects on GGT activity (shown in Fig. 1A for cortivazol and fludrocortisone, see also Fig. 2). Each steroid enhanced induction by the xenobiotic, DDT, with a concentration-dependence similar to that for TAT induction, although optimal concentrations for GGT induction were slightly lower than required for maximal elevation of TAT. In experiments related to those in Fig. 1, some additional steroids were tested (results not shown). The potent glucocorticoids, triamcinolone, flumethasone and paramethasone acetate, at nanomolar concentrations all enhanced induction of GGT by DDT, while at concentrations up to 30 μM the weak glucocorticoid agonist/antagonist [27, 28], 11-deoxycortisol, and the non-glucocorticoids [27], tetrahydrocortisol and 20β-hydroxycortisol, had little effect on either TAT or GGT.

To provide some guide to the mechanism by which glucocorticoids enhanced the GGT-inducing actions of xenobiotics, the relative effects of adding dexamethasone and/or DDT during the early or late stages of 5-day culture experiments were examined. Previous studies established that relatively small changes in GGT occcur during the first 2–3 days with more marked increases on days 4 and 5 [13, 14]. To obtain larger differences in GGT for this study, a low-glucose medium was used, resulting in higher

Table 2. Relative effects of early and late additions of DDT and dexamethasone on hepatocyte GGT activity measured after 5 days in culture*

	GGT activity (m Units/mg protein)			
		Additions on days 4,5		
	None	Dexamethasone (30 nM)	DDT (50 μM)	Dexamethasone + DDT
Additions on days 1–3:				
None	5.2 ± 0.5	6.8 ± 1.1†	11.3 ± 0.7‡	9.0 ± 0.8‡
Dexamethasone 30 nM	6.5 ± 1.0	8.8 ± 0.8†	9.5 ± 1.3†	10.3 ± 1.9†
DDT, 50 μM	18.8 ± 1.8§	25.5 ± 1.8‡§	19.0 ± 2.0§	22.2 ± 1.4†§
DDT + dexamethasone	22.0 ± 0.8‖	29.6 ± 2.3‡‖	26.5 ± 1.4‡‖	27.6 ± 1.5‡‖

* Cultures were maintained with selective modified Waymouth medium as defined in Materials and Methods except that the glucose concentration was 2.8 mM. Values are means ± SD (N = 4).
†–‖ Significance of increases:
Over corresponding dishes with no additions on days 4,5: † $P < 0.02$, ‡ $P < 0.001$.
Over corresponding dishes with no additions on days 1–3: § $P < 0.001$.
Over corresponding dishes with DDT on days 1–3: ‖ $P < 0.01$.

control and induced GGT activities, as previously reported [11]. Table 2 shows that marked elevations of GGT after 5 days in culture were observed only when DDT was present on days 1–3; the continued presence of DDT after day 3 or the addition of DDT for days 4 and 5 only had small, or no effects on 5-day activities. Although the effects of glucocorticoid were less clearly divided between early and late phases of induction, dexamethasone appeared to increase GGT activity more when present with DDT or after DDT exposure rather than prior to DDT treatment.

Induction of GGT by high concentrations of glucocorticoid-like steroids. Figure 2 shows the concentration-dependent induction of GGT by a range of glucocorticoids added to cultured hepatocytes at relatively high concentrations for 5 days. Dexamethasone, flumethasone and paramethasone acetate caused marked elevation of GGT at 0.03 to 0.1 μM with a maximal induction of 7-times the control value. Fluclorolone acetonide, cloprednol and prednisolone caused a similar maximal induction but over a 10- to 100-times higher concentration range. Triamcinolone and cortivazol are both potent glucocorticoids but triamcinolone caused marked induction only at concentrations above 10 μM; cortivazol had little effect up to 3 μM and caused cells to detach from dishes at higher concentrations.

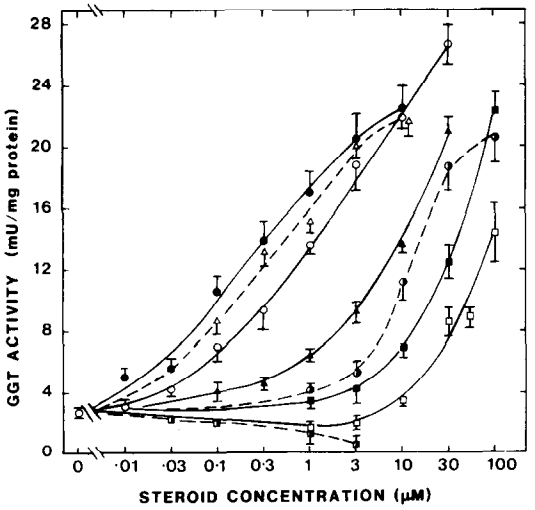

Fig. 2. Concentration-dependence of GGT induction by glucocorticoids. Hepatocytes were incubated for 5 days with the steroid concentrations shown: (●) flumethasone; (△) dexamethasone; (○) paramethasone acetate; (▲) fluclorolone acetonide; (◑) cloprednol; (■) prednisolone; (□) triamcinolone; and (◨) cortivazol. The figure combines data from six different liver cell preparations. Values are means ± SD (N = 4).

Data for a large number of experiments of the type shown in Fig. 2 are summarised in Table 3. A range of known glucocorticoids are ranked as GGT inducers on the basis of concentrations required to produce half-maximal GGT induction, where maximal GGT induction was defined as the increase over control caused by 3 μM dexamethasone in each experiment. A comparative measure of glucocorticoid potency, the steroid concentration required for half-maximal TAT induction in comparable cultures, is also shown for most of the steroids. With the exception of cortivazol already noted in Fig. 2, all of the glucocorticoids tested induced GGT, although at concentrations from 30- to 8000-times higher than required for TAT induction. In general, the rank order as a GGT inducer did not parallel relative activity in TAT induction.

To supplement the data in Table 3 on the relationship between glucocorticoid structure and GGT-inducing activity, a further group of non-glucocorticoids with some structural features in common with active glucocorticosteroids was tested. Consistent with observations above and in previous reports [13, 14] optimal induction by non-glucocorticoid steroids was shown in preliminary experiments to require the presence of a low level of dexamethasone, so to obtain data for Table 4 all media contained 30 nM dexamethasone. PCN induced GGT at relatively low concentrations as previously reported [14], as did the synthetic progestins cyproterone acetate and chlormadinone acetate. While progesterone caused significant induction only at levels above 10 μM, its 6α,16α-dimethyl- and

Table 3. Comparison of potencies of glucocorticoids as inducers of GGT and TAT in cultured hepatocytes

Steroid						Steroid concentration for half-maximal enzyme induction	
Structure*					Common name		
1	6	9	16	17		GGT† (μM)	TAT‡ (nM)
ene	αF	αF	αCH_3	αOH	Flumethasone	0.1	3
ene	H	αF	αCH_3	αOH	Dexamethasone	0.2	4
ene	αF	H	αCH_3	αOH	Paramethasone acetate*	0.6	2
ene	H	αF	βCH_3	αOH	Betamethasone	2.1	5
ene	αCH_3	αF	H	αOH	Fluorometholone	4.0	90
ene	αF	αCl	Acetonide		Fluclorolone acetonide*	8	80
ene	αF	αF	Acetonide		Fluocinolone acetonide	8	0.1
ene	Cl,ene	H	H	αOH	Cloprednol	9	ND§
ene	H	H	H	αOH	Prednisolone	23	15,000
H	H	H	H	H	Corticosterone	33	ND
H	H	αF	H	αOH	Fludrocortisone	35	1,000
H	H	H	H	αOH	Hydrocortisone	43	20,000
ene	H	αF	Acetonide		Triamcinolone acetonide	50	0.3
ene	H	αF	αOH	αOH	Triamcinolone	70	3
H	CH_3,ene	H	αCH_3	αOH	Cortivazol*	‖	0.2

* All steroids tested had a common 11β,21-dihydroxy-4-pregnene-3,20-dione structure with further substituents shown except fluclorolone acetonide in which 11βOH is replaced by 11βCl; paramethasone acetate which is a 21-acetoxyderivative; and cortivazol which is c3,2-pyrazole-2'-phenyl-6,16α-dimethyl-21-acetoxy-pregna-11β,17α,21-triol-2,4,6-trien-20-one.

† Average value from at least two experiments, as in Fig. 2. Maximal induction was defined as the increase in GGT compared with control cultures, caused by 3 μM dexamethasone.

‡ Average value from at least two experiments, as in Fig. 1B. Maximal induction was defined as the increase in TAT compared with control cultures, caused by 100 nM dexamethasone.

§ Not determined.

‖ No induction at concentrations up to 3 μM.

16α-methyl-derivatives induced at lower concentrations. Spironolactone, a minerolocorticoid antagonist, caused significant induction at 10 μM or higher levels, but the agonist aldosterone had no effect at levels up to 100 μM (not shown). Pregnenolone (Table 4) and several other C-21 pregnene or pregnane derivatives (not shown), including 11-deoxycortisol, tetrahydrocortisol, 5β-pregnandione and 5α-pregnandiol, caused small but significant GGT inductions when added at 50–100 μM.

The effects of two compounds, RU38486 and RU25055, known to be potent antagonists of the classical glucocorticoid receptor [28, 29] were examined. Preliminary experiments (not shown) established that RU38486 indeed exhibited anti-glucocorticoid activity in hepatocyte primary cultures since RU38486 prevented TAT induction by 30 nM dexamethasone. The anti-glucocorticoid was effective only at relatively high concentrations (3–10 μM) in agreement with Ref. 29. Figure 3 shows that both of the anti-glucocorticoids were weak inducers of GGT. When tested in combination with a concentration of

Table 4. Comparison of the potencies of some non-glucocorticoid steroids as inducers of GGT in cultured hepatocytes

Steroid	Steroid concentration for half-maximal induction of GGT* (μM)
Pregnenolone 16α-carbonitrile	3
Cyproterone acetate	8
Chlormadinone acetate	11
6α,16α-Dimethyl progesterone	16
Spironolactone	23
16α-Methyl progesterone	25
Progesterone	45
Pregnenolone	100

* Average value from at least two experiments conducted as in Fig. 2 except that dexamethasone, 30 nM, was also present in the medium throughout the 5-day culture period. Maximal induction was defined as the increase in GGT, compared to control cultures with 30 nM dexamethasone, caused by 3 μM dexamethasone.

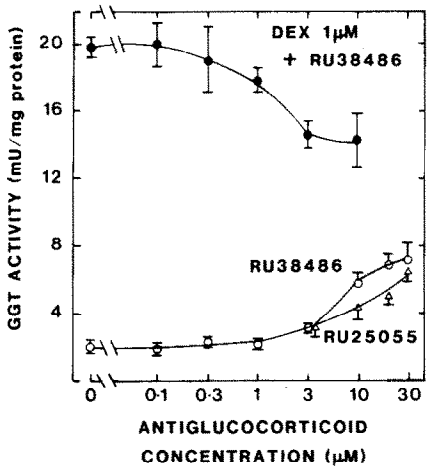

Fig. 3. Effects of antiglucocorticoids on GGT activity in hepatocyte cultures maintained for 5 days. Cultures were incubated in the absence (open symbols) or presence of 1 μM dexamethasone (closed symbols) and the concentrations of antiglucocorticoids shown: (○) RU38486; and (△) RU25055. Values are means ± SD (N = 4).

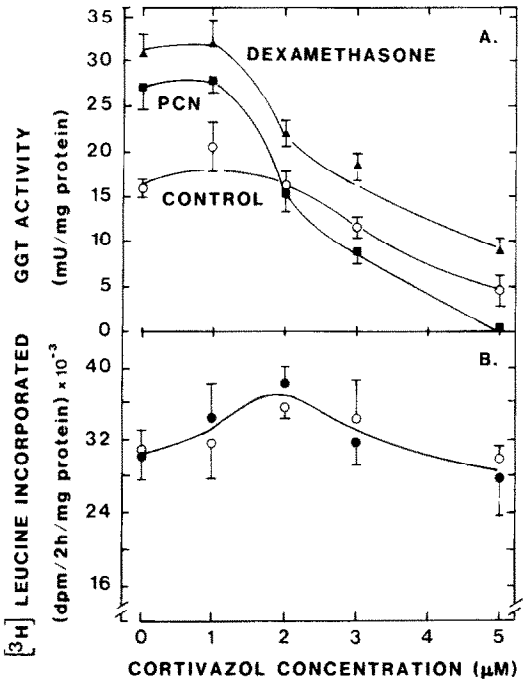

Fig. 4. Effects of cortivazol concentration on GGT and protein synthesis. In panel A, GGT was assayed after maintaining hepatocytes in L-15 medium with insulin (10^{-7} M) and dexamethasone (30 nM) plus the further additions shown for 5 days: (○) no addition; (■) PCN, 50 μM; and (▲) dexamethasone, 1 μM. In panel B, hepatocytes were incubated in modified Waymouth medium. After 24 hr, cells were maintained without (○) or with (●) 30 nM dexamethasone and the cortivazol concentrations shown. After a further 2 days the rate of protein synthesis was measured as described in Material and Methods in the presence of the added steroids. Values are means ± SD (N = 4).

dexamethasone which alone caused near-maximal elevation of GGT, increasing concentrations of RU38486 above 1 μM caused a small progressive decrease in the GGT activity observed (Fig. 3).

Since cortivazol failed to induce GGT despite its structural similarity to other active inducers, it was tested for ability to antagonise GGT induction. Figure 4a shows the effects of increasing cortivazol concentrations in cultures maintained for 5 days in the glucose-free medium, L-15. Under these conditions, GGT increased more markedly in the presence or absence of added inducers than in high-glucose modified Waymouth medium, as previously reported [11]. At concentrations up to 2 μM, cortivazol had little effect on GGT activity in the absence of inducer but largely prevented the further increase in GGT caused by PCN or dexamethasone. Since cortivazol caused some hepatocytes to detach from culture dishes at concentrations above 3 μM, it was necessary to distinguish general toxicity from any more specific effects in antagonising GGT induction. Figure 4B shows that 2 μM cortivazol caused a small but significant *stimulation* of general protein synthesis in hepatocytes in the absence ($P < 0.05$) or presence ($P < 0.01$) of dexamethasone. At higher concentrations, cortivazol caused a parallel decline in GGT activity of cultures with or without inducers (Fig. 4A), although there was little effect on total protein synthesis (Fig. 4B).

DISCUSSION

The results extend previous observations [11, 13, 14] that glucocorticoid-like steroids regulate GGT in two ways. The first type of effect, previously reported for low concentrations of dexamethasone [13, 14], was a synergistic enhancement of the ability of xenobiotics to induce GGT. This effect, demonstrated here for three structurally-varied xenobiotic inducers (Table 1) and in more detail with DDT as inducer (Fig. 1, Table 2), was exhibited by all the known glucocorticoids tested (at steroid concentrations which alone had little effect on GGT) but not by structurally-related compounds considered to be suboptimal-, non-, or anti-glucocorticoid. This structural specificity and the similar steroid concentration-dependence for synergism in GGT induction and for induction of TAT (Fig. 1) strongly suggest that the ability of glucocorticoids to enhance GGT induction by xenobiotics is mediated by the classical glucocorticoid receptor or at least via binding at a site with very similar properties to this receptor.

Synergism between low concentrations of dexamethasone and xenobiotics such as PCN has been reported previously for induction of monooxygenases in cultured liver cells [30, 31]. Dexamethasone is known to influence a wide range of activities in hepatocytes (e.g. Refs. 30–37) as well as favouring survival of cells in culture [12, 33, 38], and its effect in combination with xenobiotics on GGT expression may be a relatively non-specific one such as an overall stimulation of protein synthesis [33]. The results in Table 2 indicated that dexamethasone was more stimulatory when added with or after DDT rather than prior to DDT exposure. Induction of

GGT by xenobiotics was shown previously to be sensitive to inhibition of RNA synthesis [13, 14], and it is possible that glucocorticoids act cooperatively with xenobiotics to stimulate transcription or favour processing and/or translation of mRNAs resulting from xenobiotic-stimulated transcription.

In a second type of effect on GGT, glucocorticoid-like steroids alone at relatively high concentrations increased GGT activity in hepatocytes. Some characteristics of reversible GGT induction by dexamethasone were described and discussed previously [11]. There is no evidence on whether steroids control GGT gene expression directly or via some indirect mechanism, or on whether GGT synthesis, degradation or both are altered by steroids. Two groups of steroids were tested in this study. Various known glucocorticoids (Fig. 2, Table 3), at concentrations, in general, 2–4 orders of magnitude higher than required for TAT induction, induced GGT. This group presumably satisfied any glucocorticoid receptor-mediated permissive or enhancing requirement of the type discussed above. A second group of non-glucocorticoid steroids also exhibited GGT-inducing activity (Table 4) but required added glucocorticoid (30 nM, dexamethasone) for maximal effect. It is not yet clear whether the inducing action of these two groups involves a common mechanism (such as binding to a common receptor), and the structural requirements for GGT induction by non-glucocorticoid steroids are under further investigation.

From the results summarised in Table 3 some specific steroid structural features which favour GGT induction can be identified. The results suggest that a 16α-methyl group, a Δ^1-double bond and 6α- and 9α-halogen (or possible 6α-methyl) substituents all increase inducer potency, whereas a substantial modification of the steroid A-ring as in cortivazol abolishes activity. The significance of the 16α-methyl group, for instance, is illustrated by the potent activity of flumethasone, dexamethasone and paramethasone acetate (16α-methyl) compared with the somewhat reduced activity of betamethasone (16β-methyl), the lower potency of compounds with H (fluorometholone, cloprednol) or acetonide groups (fluocinolone acetonide) at C-16, and the very low activity of triamcinolone with a 16α-hydroxyl group. Although it is still unclear whether glucocorticoid (Table 3) and non-glucocorticoid (Table 4) steroids fall into one or multiple groups as GGT inducers, in Table 4 strong induction by PCN with a 16α-carbonitrile group and the greater inducing potencies of 6α,16α-dimethyl- and 16α-methyl-progesterone compared with progesterone are consistent with the significance of C-16 substituents. The most active compounds in Table 3 had halide (or methyl) groups at C-6 and/or C-9 as did cyproterone acetate and chlormadinone acetate in Table 4.

Since hepatocytes are active in steroid metabolism [39, 40], it is likely that differences in the metabolism of individual steroids have influenced their apparent relative potency as enzyme inducers in this study. For instance, the presence of a Δ^1-double bond or substituents at C-6, C-9 and C-16 is known to alter routes and rates of steroid metabolism [41–43]. Thus, as an inducer of TAT, dexamethasone appears to be more potent than natural glucocorticoids both because it binds with higher affinity to the glucocorticoid receptor [22] and because it is metabolised more slowly [43]. Because of such differences in metabolism and the possibility of steroid binding to multiple cell proteins (e.g. Ref. 22), the apparent structure: activity relationships for GGT induction cannot be directly equated with *in vitro* binding properties of putative receptor(s).

With these reservations in mind some conclusions, nevertheless, seem reasonable. First, the relative potencies of steroids as GGT and TAT inducers (Table 3) were determined under conditions designed to minimise differences in metabolism between the GGT and TAT studies. Much higher steroid concentrations were required for GGT induction, and the order of potency as inducers was different for the two enzymes. Three of the most potent TAT-inducers (triamcinolone, triamcinolone acetonide, and cortivazol) had weak or negligible effects on GGT. It is thus most unlikely that the glucocorticoid hormone receptor considered to mediate TAT induction [21, 22] also mediates GGT induction by glucocorticoid-like steroids. Second, although the apparent structural specificity for GGT must be influenced by differential steroid metabolism, some important observations on specificity may be relatively uncomplicated by this. For instance, dexamethasone and triamcinolone differ in structure only at C-16, are both metabolised slowly, mainly via 6-hydroxylation [43, 44], and have similar potencies as TAT inducers in hepatocytes; but they differed markedly in ability to induce GGT (Fig. 2, Table 3). In events mediated by the glucocorticoid receptor, the antagonist RU38486 binds to the receptor (and impedes dexamethasone binding) but yields a complex which is unable to undergo activation necessary for binding to chromatin and a subsequent hormone response [45]. This was confirmed for TAT induction but, under similar culture conditions, both RU38486 and RU25055 were weak "agonists" for GGT induction (Fig. 3). Conversely, at about 2 μM, cortivazol was a strong inducer of TAT (results not shown) but under similar conditions was a partial antagonist of GGT induction (Fig. 4). Such observation suggests that relatively specific structure-recognition is involved in GGT induction and that a novel receptor(s), distinct from the glucocorticoid hormone receptor, may mediate induction of GGT by pharmacological levels of steroids.

There is evidence for multiple glucocorticoid-binding proteins in the liver including proteins in cytosol [46, 47], microsomes [48, 49] and nuclei [50] which have been shown to bind dexamethasone with a K_d about 100–200 nM, comparable to the concentration required for half-maximal GGT induction (Fig. 2, Table 3). None of the binding proteins so far isolated [46–50] clearly corresponds in specificity to the apparent requirements for GGT induction although a nuclear envelope protein [50] bound dexamethasone well but triamcinolone acetonide poorly, like the putative receptor mediating GGT induction.

There are reports that inductions of the cytochrome P-450 isozyme P-450$_{PCN}$ [31, 51], 5-aminolevulinate synthase [52] and tryptophan oxygenase [46] require higher concentrations of dexamethasone

than necessary for glucocorticoid hormone action. The novel receptor postulated by Schuetz and Guzelian as a mediator of P-450$_{PCN}$ induction [31] is of particular interest since P-450$_{PCN}$ and GGT inductions by glucocorticoid-like steroids show many similarities but some apparent differences. For instance, the glucocorticoid triamcinolone and the antimineralocorticoid spironolactone were good P-450$_{PCN}$ inducers but relatively weak GGT inducers. Nebert and coworkers [53] have noted the similarities between responses mediated by the "Ah receptor" and the glucocorticoid receptor and have suggested that these proteins may have evolved from a common ancestor. These comments together with findings above and by Schuetz *et al.* [31, 51] raise the interesting possibility that a small family of receptors may have evolved from an ancestral steroid receptor to mediate responses of liver cells to environmental steroids and related compounds.

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