Steroid inhibition of calcitriol-induced prolactin production in GH₄C₁ cells

Specificity and sensitivity

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The induction of prolactin (PRL)-gene expression by calcitriol (1,25-dihydroxyvitamin D₃, 1,25-dihydroxycholecalciferol) in clonal rat pituitary tumour (GH₄C₁) cells was selectively inhibited by cortisol [IC₅₀ (concentration causing 50% inhibition) = 3.2–4.1 nM]. The steroid specificity of this effect was investigated and various steroids were found to inhibit calcitriol-stimulated PRL production with the following relative potencies: cortisol, 1; dexamethasone, 8; 11-deoxycortisol, 0.5; corticosterone, 0.4; aldosterone, 0.07; testosterone and oestradiol, < 0.003. The steroid antagonist RU 38486 did not affect basal or calcitriol-stimulated PRL production, but antagonized the effect of 10 nM-cortisol in a concentration-dependent manner. Neither progesterone nor 11-deoxycortisol antagonized the effect of 10 nM-cortisol. Calcitriol-induced PRL production was 14 times more sensitive to dexamethasone inhibition than was non-stimulated PRL production. Growth-hormone production was stimulated by dexamethasone, in the presence or absence of calcitriol, with a concentration-dependence similar to that of dexamethasone inhibition of basal PRL production. These data indicate that steroid inhibition of calcitriol-stimulated PRL production is a specific glucocorticoid effect. The sensitivity of calcitriol-stimulated PRL production to dexamethasone was 14–26-fold greater than that of other measured responses in the same cells. Two of the possible explanations for this selectively increased sensitivity to glucocorticoids are: (1) amplification of the glucocorticoid effect via an induced mediator; and (2) the presence of very-high-affinity glucocorticoid-receptor-binding sites on DNA.

INTRODUCTION

Calcitriol, a major Ca²⁺-regulating hormone, has been found to potently and selectively enhance PRL-gene expression in a clonal strain of rat pituitary tumour cells in serum-free culture (Wark & Tashjian, 1982, 1983). Distinctive features of this effect were its slow onset and dependence on extracellular Ca²⁺. Further investigation (Wark & Gurtler, 1986) revealed that calcitriol-induction of PRL production and PRL mRNA was highly sensitive to inhibition by cortisol (IC₅₀ = 3.2 nM). Inhibition of basal PRL production by cortisol, which has been reported previously (Tashjian *et al.*, 1970) was observed only at considerable higher glucocorticoid concentrations. Enhancement of PRL production by other stimuli (e.g. thyroliberin, epidermal growth factor and phorbol 12-myristate 13-acetate) was little affected by 10 nM-cortisol, which abolished the effect of calcitriol. These data indicated that cortisol inhibited calcitriol action in GH₄C₁ cells with considerable selectivity. Moreover, they suggested that (i) the GH-cell model may prove useful for studying vitamin D–glucocorticoid interactions and (ii) glucocorticoids may be helpful

experimentally in efforts to understand the mechanism of action of calcitriol in the present system and in target cells generally. The interaction between calcitriol and various steroids has now been characterized further and is the subject of the present paper.

MATERIALS AND METHODS

Materials

Radioimmunoassay-kit reagents for measuring rat PRL and rat growth hormone were gifts from the National Hormone and Pituitary Program, Baltimore, MD, U.S.A. For the growth-hormone assay, reference preparation no. 2 and antiserum no. 5 were used and, for PRL, reference preparation RP-3 and antiserum no. 9. Radiations were performed using the Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril) method (Fraker & Speck, 1978). Calcitriol was a gift from Dr. M. Uskokovic, Hoffman–La Roche, Nutley, NJ, U.S.A.; other steroid hormones were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; RU 38486 was a gift from Dr. R. Deraedt, Roussel Uclaf, Romainville, France.

Abbreviations used: aldosterone, 11 β ,21-dihydroxy-3,20-dioxopregn-4-en-18-al; corticosterone, 11 β ,21-dihydroxypregn-4-ene-3,20-dione; 11-deoxycortisol, 17 α ,21-dihydroxypregn-4-ene-3,20-dione; dexamethasone, 9-fluoro-11 β ,17,21-trihydroxy-16 α -methylpregna-1,4-diene-3,20-dione; oestradiol, oestra-1,3,5(10)-triene-3,17 β -diol; EC₅₀, concentration causing 50% stimulation; F10⁺, Ham's F10 medium supplemented with 15% (v/v) fetal bovine serum; cortisol, 11 β ,17,21-trihydroxypregn-4-ene-3,20-dione; HTC, hepatoma tissue culture; IC₅₀, concentration causing 50% inhibition; progesterone, pregn-4-ene-3,20-dione; PRL, prolactin; RU 38486, 17 β -hydroxy-11 β -(4-dimethylaminophenyl)-17 α -(1-propynyl)-oestra-4,9-dien-3-one; testosterone, 17 β -hydroxyandrost-4-ene-3-one; calcitriol, 1,25-dihydroxycholecalciferol ('1,25-dihydroxyvitamin D₃').

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Fig. 1. Effects of aldosterone, testosterone, oestradiol and 11-deoxycortisol on basal and calcitriol-stimulated PRL production

Cell cultures were prepared as described in the Materials and methods section. After equilibration for 24 h in chemically defined medium, 96 h treatment with 1 nM-calcitriol (■) or ethanol-containing vehicle (□) was commenced. Calcitriol- and vehicle-treated cultures concurrently received 1 μ M-aldosterone (A), 1 μ M-testosterone (T), 1 μ M-oestradiol (E), 1 μ M-11-deoxycortisol (D) or appropriate vehicle. The cultures received fresh medium and treatment after 48 h. Shown above are the rates of PRL accumulation in the medium in the final 48 h of treatment. Each bar represents the mean ($\pm$ S.E.M.) value for three replicate cultures.

Cell culture

GH₄C₁ cells were a gift from Dr. A. H. Tashjian, Jr., Harvard School of Public Health, Boston, MA, U.S.A. Media and medium components for cell culture generally were obtained from Gibco Laboratories, Grand Island, NY, U.S.A. The cells were plated [(2.5–5.0) $\times 10^5$ /60 mm-diameter dish] and grown for 4–5 days in F10⁺. Before an experiment, F10⁺ was replaced by chemically defined medium after gently washing the cultures with the same solution. This synthetic medium was based on Ham's F12 medium to which supplementary amino acids and 10% serum substitute (Bauer *et al.*, 1976; modified by the omission of all hormones) were added (Wark & Gurtler, 1986). The medium was hormone-free, and the total Ca²⁺ concentration was adjusted to 0.4 mM by the addition of CaCl₂. Steroid hormones in concentrated ethanolic solutions were diluted in medium before addition to cultures. The final concentration of ethanol did not exceed 0.06% (v/v) and was the same in all dishes.

PRL and growth-hormone production

PRL and growth-hormone accumulation in the medium were determined by radioimmunoassay and used as a measure of hormone synthesis, since intracellular stores of PRL and growth hormone in GH cells are very small and hormone degradation by the cells is negligible (Dannies & Tashjian, 1973; Dannies *et al.*, 1976; Haug

Fig. 2. Concentration-dependent effects of progesterone and 11-deoxycortisol on PRL production by GH₄C₁ cells treated with calcitriol, calcitriol plus cortisol, cortisol or vehicle

Cell cultures were prepared as described in the Materials and methods section. After equilibration for 24 h in chemically defined medium, 96 h treatment with various concentrations of progesterone (a) or 11-deoxycortisol (b) was commenced. Cultures were also treated with 1 nM-calcitriol (□), 1 nM-calcitriol plus 10 nM-cortisol (■), 10 nM-cortisol (●) or vehicle (○). The cultures received fresh medium and treatment after 48 h. Shown above are the rates of PRL accumulation in the medium in the final 48 h of treatment. Each point represents the mean ($\pm$ S.E.M.) value for three replicate cultures.

et al. 1982). Total protein was measured by using the Coomassie Brilliant Blue dye method (Bradford, 1976). Statistical analysis was performed using Student's *t* test for unpaired observations.

RESULTS

Various steroid hormones were tested to determine whether the inhibition of calcitriol action in GH₄C₁ cells was specifically a glucocorticoid effect. Neither oestradiol-17 β nor testosterone modified the effect of calcitriol on PRL production (Fig. 1). Progesterone was a weak inhibitor of calcitriol (IC₅₀ = 0.22 μ M) and was inactive as a glucocorticoid antagonist (Fig. 2a). The relative potencies of several adrenal steroids and the synthetic glucocorticoid dexamethasone were compared in a series of experiments (Fig. 3). In each experiment, cortisol concentration-response curves for basal and calcitriol-stimulated PRL production were performed and compared with curves for one other steroid. The curves for cortisol were highly reproducible between experiments (IC₅₀ = 4.1 $\pm$ 0.22 nM; mean $\pm$ S.E.M.) and have been used as reference values to determine the relative potencies of the other steroids tested. From these data, the relative activities of these steroids as inhibitors of calcitriol-stimulated PRL production were: dexamethasone, 8; cortisol, 1; corticosterone, 0.4; aldosterone, 0.07. 11-Deoxycortisol also inhibited calcitriol action appreciably

Fig. 3. Relative potencies of cortisol, dexamethasone, corticosterone and aldosterone as inhibitors of calcitriol-stimulated PRL production by GH₄C₁ cells

Cell cultures were prepared as described in the Materials and methods section. The potencies of dexamethasone, cortisol and aldosterone were compared with that of cortisol in separate experiments. After equilibration for 24 h in chemically defined medium, 96 h treatment with 1 nM-calcitriol or ethanol-containing vehicle was commenced, together with various concentrations of cortisol (●), dexamethasone (■), corticosterone (▲) or aldosterone (○). The cultures received fresh medium and treatment after 48 h. Shown above are the relative rates of PRL accumulation in the medium in the final 48 h of treatment. Each point represents the mean value for three replicate cultures. The points on the cortisol curve are mean values for four separate experiments. The mean and range of values for basal PRL production in these experiments is shown (◆) and half-maximal inhibition of calcitriol-stimulated PRL production is indicated by the broken line.

(Fig. 2b). Like progesterone, it had no glucocorticoid antagonist activity in the present system. The potent steroid antagonist RU 38486 also was tested (Fig. 4). Although it showed no cortisol-like activity when used in concentrations up to 1 μ M, RU 38486 reversed the calcitriol-inhibitory effect of 10 nM-cortisol. This concentration-dependent effect was significant at 10 nM ($P < 0.01$) and complete at 0.1 μ M-RU 38486. The interactive effects of dexamethasone and calcitriol on PRL and growth-hormone production were compared (Figs. 5a and 5b). Dexamethasone (0.1 μ M) caused an approx. 10-fold enhancement of growth-hormone production, and this effect was not altered by 1 nM-calcitriol, which had no effect on growth hormone. In the absence of dexamethasone, calcitriol stimulated PRL production more than 3-fold. Dexamethasone inhibited both calcitriol-induced ($IC_{50} = 0.5$ nM) and non-stimulated PRL production ($IC_{50} = 7.2$ nM), but the latter was 14 times less sensitive to dexamethasone inhibition. The potency of dexamethasone as an inhibitor of basal PRL production and as a stimulator of growth-hormone production ($IC_{50} = 13$ nM) was similar.

None of the agents tested caused any change in the final cell protein content of the cultures. This finding, taken with the speed and selectivity of the inhibitory effect of cortisol, provides strong evidence against glucocorticoids acting via a toxic or anti-proliferative mechanism.

Fig. 4. Concentration-dependent effect of RU 38486 on PRL production by GH₄C₁ cells treated with calcitriol, cortisol, calcitriol plus cortisol, or vehicle

Cell cultures were prepared as described in the Materials and methods section. After equilibration for 24 h in a chemically defined medium, 96 h treatment with various concentrations of RU 38486 was commenced. Cultures were treated concurrently with 1 nM-calcitriol (□), 10 nM-cortisol (●), 1 nM-calcitriol plus 10 nM-cortisol (■), or vehicle (○). The cultures received fresh medium and treatment after 48 h. Shown above are the rates of PRL accumulation in the medium in the final 48 h of treatment. Each point represents the mean ($\pm$ S.E.M.) value for three or four replicate cultures.

DISCUSSION

The present studies of experiments provides further strong evidence that the inhibition of calcitriol induction of PRL gene expression by cortisol is a specific glucocorticoid effect. The only hormonally active agents that we found to inhibit the effect of calcitriol have also been reported to have glucocorticoid effects in other systems, e.g. induction of tyrosine aminotransferase activity in HTC cells (Samuels & Tomkins, 1970). Although progesterone is known generally as a glucocorticoid antagonist (Rousseau & Schmit, 1977), weak glucocorticoid activity has also been described (Samuels & Tomkins, 1970). 11-Deoxycortisol has been classified as a partial glucocorticoid agonist. In the present studies, both progesterone and 11-deoxycortisol were inhibitors of calcitriol-induced PRL production, with the former being an order of magnitude less potent than the latter. Neither steroid modified the inhibitory effect of 10 nM-cortisol. Their lack of glucocorticoid antagonism was unexpected, and contrasts with the pure anti-glucocorticoid effect observed when the potent steroid antagonist RU 38486 was used. RU 38486 has been well characterized, as both an anti-glucocorticoid and an anti-progestagen; it appears to be devoid of any steroid agonist activity (Chrousos *et al.*, 1983). The ability of RU 38486 to abolish the effect of cortisol, taken with the relative potencies of various steroids in the present system, suggests strongly that the observed inhibition of calcitriol action was mediated via an interaction with glucocorticoid receptors. The mechanisms of these hormonal effects on PRL gene expression are yet to be determined.

On the basis of our previous findings (Wark &

Fig. 5. Concentration-dependent effects of dexamethasone on PRL and growth-hormone production by GH₄C₁ cells treated with calcitriol or vehicle

Cell cultures were prepared as described in the Materials and methods section. After equilibration for 24 h in a chemically defined medium, 96 h treatment with various concentrations of dexamethasone was commenced. Cultures were treated concurrently with 1 nM-calcitriol (■, ●) or vehicle (□, ○). The cultures received fresh medium and treatment after 48 h. Shown above (a) are the rates of PRL (●, ○) and growth-hormone accumulation (■, □) in the medium in the final 48 h of treatment. Each point represents the mean ($\pm$ S.E.M.) value for four replicate cultures. The same data have been plotted as the change in hormone production expressed as a percentage of the maximal effect of each treatment (b) to illustrate the sensitivity of each parameter to dexamethasone.

Tashjian, 1982, 1983; Wark & Gurtler, 1986), we hypothesized that calcitriol stimulated the expression of a stable regulator of PRL-gene expression which acted by changing cellular Ca²⁺ homeostasis. Glucocorticoids could act by stimulating the degradation, or opposing the action, of the putative regulator at a variety of levels, including the PRL gene itself, since the gene is thought to possess glucocorticoid regulatory sequences (Somasekhar & Gorski, 1986).

The growth-hormone gene in GH cells has been used extensively as an experimental model of glucocorticoid-hormone action. The work of several laboratories has established that glucocorticoids rapidly and directly stimulate growth-hormone-gene transcription in clonal rat pituitary tumour cells closely related to the GH₄C₁ strain used in the present studies (Spindler *et al.*, 1982;

Evans *et al.*, 1982). Hence a comparison of the glucocorticoid sensitivity of growth-hormone- and PRL-gene expression was of considerable interest. The finding that basal PRL production and growth-hormone production were similarly sensitive to dexamethasone confirmed our impression that calcitriol-induced PRL production was unusually sensitive to glucocorticoid inhibition. Our observation of differential glucocorticoid sensitivity of specific gene expression bears some similarity to the report of Mercier *et al.* (1983), who found a 9-fold greater dexamethasone-sensitivity of tyrosine aminotransferase compared with glutamine synthetase induction and whole-cell specific steroid binding in FUS-5 rat hepatoma cells. Those authors discussed many possible explanations for differential steroid sensitivity, but favoured the involvement of a second messenger or different nuclear processes. Although our findings differ in the major respect that the induction of a gene by one of several agents was exceptionally sensitive to glucocorticoid inhibition, many of the mechanistic considerations are similar.

To explain the selective inhibition of calcitriol-induced PRL-gene expression at low glucocorticoid concentrations, and the non-selective inhibition at high glucocorticoid concentrations, one could postulate that, of several glucocorticoid-receptor-binding sites on the PRL gene, one or more with higher affinity for the glucocorticoid receptor selectively prevent(s) the effect of calcitriol. Thus the striking sensitivity of calcitriol-induced PRL production to glucocorticoid inhibition ($IC_{50} \text{ dexamethasone} = 0.5 \text{ nM}$) could be explained by variation in the affinities of glucocorticoid-receptor-binding sites. In an alternative model the heightened sensitivity of calcitriol-induced PRL production to glucocorticoid inhibition would be due to amplification of the glucocorticoid effect via an induced mediator, whereas the non-selective inhibitory effect on PRL production would involve interaction of the glucocorticoid receptor with regulatory sequences of the PRL gene. Many aspects of these hypothetical models could be readily tested.

A less likely explanation for the interactive effects of calcitriol and glucocorticoids on PRL-gene expression was that calcitriol increased the sensitivity of GH cells to glucocorticoid, for example by induction of glucocorticoid receptors. However, the finding that stimulation of growth-hormone production by dexamethasone was unaffected by calcitriol effectively excludes that postulate.

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REFERENCES

- Bauer, R. F., Arthur, L. O. & Fine, D. L. (1976) *In Vitro* **12**, 558–563
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Chrousos, G. P., Cutler, G. B., Sauer, M., Simons, S. S., Jr. & Loriaux, D. L. (1983) *Pharmacol. Ther.* **20**, 263–281
- Dannies, P. S. & Tashjian, A. H., Jr. (1973) *J. Biol. Chem.* **248**, 6174–6179
- Dannies, P. S., Gautvik, K. M. & Tashjian, A. H., Jr. (1976) *Endocrinology* (Baltimore) **98**, 1147–1159

- Evans, R. M., Birnberg, N. C. & Rosenfeld, M. G. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7659–7663
- Fraker, P. J. & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849–857
- Haug, E., Pedersen, J. I. & Gautvik, K. M. (1982) *Mol. Cell. Endocrinol.* **28**, 65–79
- Mercier, L., Thompson, E. B. & Simons, S. S., Jr. (1983) *Endocrinology (Baltimore)* **112**, 601–609
- Rousseau, G. G. & Schmit, J. P. (1977) *J. Steroid Biochem.* **8**, 911–919
- Samuels, H. H. & Tomkins, G. M. (1970) *J. Mol. Biol.* **52**, 57–74
- Somasekhar, M. B. & Gorski, J. (1986) *Abstr. Annu. Meet. Endocr. Soc.* 68th 433
- Spindler, S. R., Mellon, S. H. & Baxter, J. D. (1982) *J. Biol. Chem.* **257**, 11627–11632
- Tashjian, A. H., Jr., Bancroft, F. C. & Levine, L. (1970) *J. Cell Biol.* **47**, 61–70
- Wark, J. D. & Gurtler, V. (1986) *Biochem. J.* **233**, 513–518
- Wark, J. D. & Tashjian, A. H., Jr. (1982) *Endocrinology (Baltimore)* **111**, 1755–1757
- Wark, J. D. & Tashjian, A. H., Jr. (1983) *J. Biol. Chem.* **258**, 12118–12121

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