Glucocorticoids antagonize induction of prolactin-gene expression by calcitriol in rat pituitary tumour cells

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Clonal strains of rat pituitary tumour (GH₄C₁) cells are known to possess specific intracellular binding sites for calcitriol (1,25-dihydroxycholecalciferol, 1,25-dihydroxyvitamin D₃). GH₄C₁ cells respond to calcitriol by a selective increase in prolactin(PRL)-gene expression. The interaction between calcitriol and glucocorticoids was studied by using this cultured-cell model. It was found that cortisol potently antagonized the induction of PRL mRNA and PRL production by calcitriol. The effects were concentration-dependent and were evident at glucocorticoid concentrations that did not alter basal PRL production. Inhibition was half-maximal at 3.2 nM-cortisol and 0.4 nM-dexamethasone. Calcitriol-induced PRL mRNA fell by more than 50% at 25 h and reached the control level 50 h after treatment with cortisol. The inhibition by cortisol of calcitriol induction of PRL production was selective when compared with effects on other inducers of PRL-gene expression [thyroliberin, epidermal growth factor and phorbol myristate acetate ('12-O-tetradecanoylphorbol 13-acetate')]. Potent antagonism by glucocorticoids of vitamin D action on specific gene expression has been demonstrated. Further studies with this cultured-cell model may help to explain the mechanism of this hormonal interaction, which assumes particular importance at major sites of vitamin D action such as the intestine.

INTRODUCTION

An imposing body of evidence indicates that calcitriol (1,25-dihydroxycholecalciferol, 1,25-dihydroxyvitamin D₃) acts in target cells by a nuclear mechanism analogous to that of the 'classical' steroid hormones (Norman *et al.*, 1982). Thus the presence of a specific intracellular binding macromolecule, the calcitriol 'receptor', has come to be regarded as a marker of putative sites of action of calcitriol. After its discovery in the rat pituitary gland (Haussler *et al.*, 1980), the calcitriol receptor was also detected in clonal strains of rat pituitary tumour (GH) cells (Murdoch & Rosenfeld, 1981; Haussler *et al.*, 1981). Subsequently, the effects of calcitriol (cholecalciferol, vitamin D₃) metabolites on hormone production by GH cells have been examined in several laboratories (Murdoch & Rosenfeld, 1981; Wark & Tashjian, 1982; Haug *et al.*, 1982; Wark & Tashjian, 1983). Because serum-supplemented culture media contain potentially confounding factors such as vitamin D metabolites, their serum binding protein and a complex mixture of hormones and other humoral agents, it seemed appropriate to study the effects of vitamin D metabolites in cells incubated in serum-free, chemically defined medium. When such conditions were used, calcitriol potently and selectively increased PRL production and PRL mRNA in GH cells of the GH₄C₁ strain (Wark & Tashjian, 1982, 1983). Half-maximal effects were observed at 0.1–0.2 nM-calcitriol. Effects on growth-hormone production were minimal, attesting to the selectivity of the effect on PRL-gene expression. The relative potencies of vitamin

D metabolites and analogues were similar to those which have been observed for other vitamin D-responsive processes *in vitro* (Corradino, 1979; Franceschi & DeLuca, 1981). The effect of calcitriol on PRL production and PRL mRNA was delayed in onset (requiring at least 24 h to become evident), and depended on extracellular Ca²⁺ for its full expression.

Although studies of this type could not prove a physiological role for vitamin D in the regulation of pituitary-cell function, they did support that possibility. Moreover, the findings clearly demonstrated the usefulness of GH₄C₁ cells as a model in which to investigate the cellular and molecular mechanisms involved in calcitriol action. These studies have now been extended. In the experiments reported here, glucocorticoids were found to antagonize potently and selectively calcitriol induction of PRL-gene expression. Further studies with the present experimental model may lead to an increased understanding of the cellular and molecular mechanisms by which glucocorticoids antagonize vitamin D action.

MATERIALS AND METHODS

Materials

[α -³²P]dCTP (2000–3000 Ci/mmol) and nick-translation reagents were obtained from Amersham Australia Pty. Ltd. Recombinant plasmid pPRL-1 (Gubbins *et al.*, 1979) was a gift from Dr. D. K. Biswas, Harvard School of Dental Medicine, Boston, MA, U.S.A. Zetapor membrane (0.45 μ m pore size) was obtained from

Abbreviations used: CS, corticosterone (11 β ,21-dihydroxypregn-4-ene-3,20-dione); DXM, dexamethasone (9 α -fluoro-11 β ,17,21-trihydroxy-16 α -methylpregna-1,4-diene-3,20-dione); EGF, β -epidermal growth factor; F10⁺, Ham's F10 medium supplemented with 15% (v/v) foetal bovine serum; IC₅₀, concentration causing 50% inhibition; MEM, Minimum Essential Medium; PRL, prolactin; 15 $\times$ SSC, 2.25 M-NaCl/0.225 M-trisodium citrate; 20 $\times$ SSC, 3.0 M-NaCl/0.3 M-trisodium citrate; PMA, phorbol myristate acetate ('TPA'); TRH, thyroliberin ('thyrotropin-releasing hormone').

AMF-Cuno, Meriden, CT, U.S.A. Radioimmunoassay-kit reagents for measuring rat PRL were gifts from the National Hormone and Pituitary Program, Baltimore, MD, U.S.A. Calcitriol was a gift from Dr. M. Uskokovic, Hoffmann-La Roche, Nutley, NJ, U.S.A.; other steroid hormones, PMA and Nonidet P40 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; TRH was obtained from Peninsula Laboratories, Belmont, CA, U.S.A.; β -EGF from mouse salivary glands was kindly provided by the Ludwig Institute for Cancer Research, The Royal Melbourne Hospital, Melbourne, Vic., Australia.

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) × 10⁵/60 mm 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 constituted as previously described (Wark & Tashjian, 1982), or was based on Ham's F12 medium to which supplementary amino acids and 10% serum substitute (Bauer *et al.*, 1976) were added. In either case, the medium was hormone-free; the total Ca²⁺ concentration was adjusted to 0.4 mM by 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. Peptides were added in 155 mM-NaCl. PMA was stored at a concentration of 6.17 mg/ml (10 mM) in dimethyl sulphoxide and diluted with phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.9 mM-NaHPO₄ · 7H₂O/1.5 mM-KH₂PO₄, pH 7.2) before use.

Measurement of specific mRNA

PRL mRNA was measured by cytoplasmic dot hybridization (White & Bancroft, 1982), with minor modifications as previously described (Wark & Tashjian, 1983). The cells were mobilized mechanically, washed twice in Hanks balanced salt solution, suspended in 45 μ l of ice-cold 10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA and lysed by the addition of 5 μ l of 5% (v/v) Nonidet P40 twice over 7.5 min during gentle mixing on ice. After centrifugation of the samples (15000g, 4 °C, 2.5 min) in an Eppendorf model 5414S centrifuge, 50 μ l of supernatant were added to 210 μ l of 20 × SSC and 65 μ l of formaldehyde solution (37.0–40.0%, w/v). Samples were incubated at 60 °C for 15 min, rapidly cooled and stored at –70 °C. Denatured cytoplasmic samples were serially diluted in 15 × SSC and applied to Zetapor membrane using a Minifold filtration manifold (Schleicher and Schuell, Keene, NH, U.S.A.). The filters were baked *in vacuo* at 80 °C for 90 min. Prehybridization, hybridization and washing conditions were as described by Wark & Tashjian (1983). Plasmid pPRL-1 was prepared by established methods (Clewett & Helinski, 1969) and labelled with [α -³²P]dCTP by nick translation (Benz *et al.*, 1979) to a specific radioactivity of approximately 1.0 × 10⁸ d.p.m./ μ g. All procedures involving recombinant DNA-containing micro-organisms were performed in accordance with the guidelines of the Australian Recombinant DNA Monitoring Committee. Auto-

Fig. 1. Effect of cortisol and cortisol-plus-calcitriol on PRL production by GH₄C₁ cells

Cell cultures were prepared as described in the Materials and methods section. After equilibration for 24 h in serum-free Eagle's MEM-based medium 96 h treatment was commenced with 1 nM-calcitriol (●) or ethanol-containing vehicle (○), plus various concentrations of cortisol or vehicle. The cultures received fresh medium and treatment after 48 h. The results shown are the rates of PRL accumulation in the medium in the final 48 h of treatment. Medium PRL was measured by radioimmunoassay. Each point represents the mean (± S.E.M.) value for six replicate dishes. The mean final cell protein content per culture was 1.2 mg and did not vary with treatment.

radiography was performed for 24 h at –70 °C using X-Omat XAR-5 film (Eastman Kodak Co.) and two image-intensifying screens (Du Pont Chromex Lightning Plus). After autoradiography, the filters were cut into small squares and hybridization quantified by liquid-scintillation counting.

PRL production

PRL accumulation in the medium was determined by rPRL radioimmunoassay and was used as a measure of PRL synthesis, since GH cells store only a minimal amount of PRL and degradation of PRL by the cells is negligible (Haug *et al.*, 1982). Total protein was measured by using the Coomassie Brilliant Blue dye method (Bradford, 1976).

RESULTS

As previously reported (Wark & Tashjian, 1982), 10 nM-calcitriol caused highly significant stimulation of PRL production by GH₄C₁ cells ($P < 0.0001$; Fig. 1). Cortisol caused concentration-dependent inhibition of this effect; the inhibition was half-maximal at 3.2 nM-cortisol (Figs. 1 and 5). By contrast, only a modest

Fig. 2. Effects of cortisol and calcitriol on PRL mRNA in GH₄C₁ cells

Cell cultures were prepared as described in the Materials and methods section. Immediately after the culture medium was changed to serum-free Eagle's MEM-based medium, treatment was commenced with 1 nM-calcitriol with (■) or without (●) 0.1 μ M-cortisol, or with ethanol-containing vehicles only (□). After an induction period of 3 days, i.e., at 0 h, fresh medium was added and treatment of some calcitriol-induced cultures with 0.1 μ M-cortisol was commenced (●). Some calcitriol-induced cultures had calcitriol withdrawn and vehicle substituted, whereas in others the sterol was continued. PRL and mRNA, measured by cytoplasmic dot hybridization, was plotted as a percentage of the level in the last-described cultures at 0, 5, 10, 25 and 50 h. The broken lines indicate the limits of the S.E.M. for calcitriol-induced PRL mRNA at each time point. Treatments were continued unchanged in the cultures that received vehicles only, or calcitriol plus cortisol during the induction period. Each point represents the mean ($\pm$ S.E.M.) value for four replicate cultures.

decrease in basal PRL production was observed, and this was evident only at the highest cortisol (11 β ,17,21-trihydroxypregn-4-ene-3,20-dione) concentration tested, 0.1 μ M ($P < 0.01$). The inhibition of calcitriol-induced PRL production by cortisol was a reflection of a similar decrease in PRL mRNA (Fig. 2). In cells treated with calcitriol and cortisol throughout the experimental period, induction of PRL mRNA was prevented. Moreover, when PRL mRNA was induced by calcitriol treatment (for 72 h before '0 h') the addition of cortisol led to a prompt decrease in PRL mRNA. PRL mRNA fell by more than 50% at 25 h and reached the control level by 50 h. If calcitriol treatment was withdrawn at 0 h (i.e., after 72 h prior induction) and cortisol not administered, PRL mRNA was maintained at or above the induced level at 50 h (separate results not shown), indicating that the effect of calcitriol on PRL-gene expression has a long biological half-life. This phenomenon was confirmed by an experiment in which cultures were treated with 1 nM-calcitriol for 4 h, 24 h or 96 h (Table 1). The stimulation of PRL production in the final 48 h period was the same regardless of the duration of calcitriol treatment.

The selectivity of the effect of cortisol on calcitriol induction of PRL-gene expression was examined by using

Table 1. Effect of various periods of treatment with calcitriol on later response of PRL production by GH₄C₁ cells

Cell cultures were prepared as described in the Materials and methods section. After equilibration for 24 h in serum-free Eagle's MEM-based medium, treatment with 1 nM-calcitriol or ethanol-containing vehicle was commenced. Treatment with calcitriol was continued for 96 h, or for 4 h or 24 h, after which times the medium was removed from a set of treated and control cultures and replaced with medium containing vehicle only. After 48 h, all cultures received fresh medium with calcitriol or vehicle, as appropriate. The results shown are the mean ($\pm$ S.E.M.) rates of PRL accumulation in the medium between 48 and 96 h; each value was determined from four replicate cultures. Medium PRL was measured by radioimmunoassay. The mean final cell protein per culture was 0.6 mg for those receiving calcitriol and 0.5 mg for those receiving vehicle.

Period of treatment (h)	Addition	PRL production between 48 and 96 h (μ g/24 h per mg of protein)
4	Calcitriol (1 nM)	7.0 $\pm$ 0.44
	Vehicle	2.8 $\pm$ 0.23
24	Calcitriol (1 nM)	6.9 $\pm$ 0.92
	Vehicle	2.6 $\pm$ 0.36
96	Calcitriol (1 nM)	6.3 $\pm$ 1.12
	Vehicle	3.0 $\pm$ 0.38

maximally effective concentrations of several well-documented potent stimuli: TRH (Dannies *et al.*, 1976), EGF (Schonbrunn *et al.*, 1980) and PMA (Osborne & Tashjian, 1981). Whereas 10 nM-cortisol abolished the effect of 10 nM-calcitriol, it had insignificant or minor effects on the other agonists (Fig. 3). TRH induction of PRL production was studied in detail. Cortisol did not alter the level of PRL production stimulated by TRH in concentrations ranging from 0.3 nM to 0.1 μ M (Fig. 4). By contrast, cortisol abolished the effect of 0.1 μ M-calcitriol regardless of how the data were analysed.

In further experiments, we compared the relative potencies of several glucocorticoids as antagonists of calcitriol action in the present system. CS was ineffective at the concentrations of 1 nM and 10 nM (results not shown). Further study will be required to determine whether it possesses antagonist activity. DXM was appreciably more potent than cortisol (Fig. 5). On the basis of comparative concentration-response curves, DXM ($IC_{50} = 0.4$ nM) was 8-fold more effective than cortisol as an antagonist of calcitriol. Inhibition of basal PRL production was evident only at 10 nM-DXM.

DISCUSSION

The major finding in these experiments was that glucocorticoids potently antagonize the inductive effect of calcitriol on specific mRNA levels in a cultured-cell model of vitamin D action. The ability of cortisol at high concentrations to inhibit PRL production by GH cells has been reported previously (Tashjian *et al.*, 1970) and was confirmed in the present studies. However, the antagonistic effect of cortisol and DXM on induction of PRL-gene expression, seen at relatively low glucocorticoid

Fig. 3. Effect of cortisol on PRL production by non-stimulated GH₄C₁ cell cultures and by cultures treated with calcitriol, TRH, EGF and PMA

Cell cultures were prepared as described in the Materials and methods section. After equilibration for 24 h in serum-free Ham's F12-based medium, 96 h treatment with 10 nM-cortisol (▨) or ethanol-containing vehicle (□) was commenced. Cortisol and vehicle-treated cultures concurrently received calcitriol (10 nM), TRH (100 nM), EGF (100 nM, 10 nM) or PMA (100 ng/ml) and appropriate vehicles. The cultures received fresh medium and treatment after 48 h. The results shown are the rates of PRL accumulation in the medium in the final 48 h of treatment. Medium PRL was measured by radioimmunoassay. Each bar represents the mean ($\pm$ S.E.M.) value for three replicate cultures. The mean final cell protein per culture was 1.0 mg and did not vary with treatment.

concentrations that did not alter basal PRL production, is a novel finding. This observation may indicate a more subtle, but nonetheless important, site of glucocorticoid action. The relative potencies of DXM, cortisol and CS may be best explained by mediation of the effect through a 'classical' glucocorticoid receptor with moderately high affinity for DXM.

Cortisol quite selectively opposed the action of calcitriol. From previously published data, the PRL inducers we compared (calcitriol, TRH, EGF and PMA) were present at maximally effective concentrations (Wark & Tashjian, 1982; Dannies *et al.*, 1976; Schonbrunn *et al.*, 1980; Osborne & Tashjian, 1981). Yet only the effect of calcitriol was abolished. There was no effect on EGF-evoked stimulation. Although we found a decrease from 3.8-fold to 2.7-fold induction by PMA (100 ng/ml) in the presence of 10 nM-cortisol, others have found no change in the fold induction of PRL production by PMA in closely related GH₃ cells, even in the presence of 5 μ M-cortisol (Osborne & Tashjian, 1981). In our concentration-response study, the sensitivity of the cells to TRH was not diminished by cortisol; rather, it might have been increased. However, the latter interpretation is

Fig. 4. Effect of cortisol on the stimulation of PRL production by treatment of GH₄C₁ cells with various concentrations of TRH and with calcitriol

Cell cultures were prepared as described in the Materials and methods section. After equilibration for 24 h in serum-free Ham's F12-based medium, 96 h treatment with various concentrations of TRH either alone (○) or plus 10 nM-cortisol (●), and with 0.1 μ M-calcitriol alone (△) or plus 10 nM-cortisol (▲) was commenced. The cultures received fresh medium and treatment after 48 h. The results shown are the rates of PRL accumulation in the medium in the final 48 h of treatment. Medium PRL was measured by radioimmunoassay. Each point represents the mean ($\pm$ S.E.M.) value for three or four replicate cultures. The mean final cell protein per culture was 0.9 mg and did not vary with treatment.

based on the small significant difference between the zero values and so must be made cautiously. An increase in the response to TRH might be explained by the observation by Tashjian *et al.* (1977) that cortisol augmented TRH-specific binding in GH₄C₁ cells. However, a comparison of the effective cortisol concentrations tested in the two series of experiments is not possible, because Tashjian *et al.* (1977) used a culture medium supplemented with 17.5% serum, whereas our experimental medium was serum-free.

The marked inhibitory effect of glucocorticoids on intestinal Ca²⁺ absorption has been recognized for many years (see Hahn *et al.*, 1981). This effect is associated with a persistently negative Ca²⁺ balance and progressive loss of bone mass, which causes considerable morbidity in glucocorticoid-treated patients. The bulk of experimental evidence suggests that there is acquired resistance of the intestine to calcitriol in this situation, rather than a pathogenically important change in vitamin D metabolism (Klein *et al.*, 1977; Seeman *et al.*, 1980; Hahn *et al.*, 1981). Regulation by glucocorticoids of calcitriol receptors in the intestine has been proposed as a cause of the vitamin D resistance, but the divergent effects of glucocorticoids on intestinal calcitriol receptors and Ca²⁺ absorption in the neonatal and adult rat weigh heavily against this explanation [see Hirst & Feldman (1982) and Chan *et al.* (1984)]. Hence an interaction at the post-receptor level seems likely. Unfortunately, further investigation of calcitriol-glucocorticoid interactions at the post-receptor level in the intestine has been severely restricted by our poor understanding of the cellular and

Fig. 5. Effect of various concentrations of DXM and cortisol on PRL production by GH₄C₁ cells both non-stimulated and treated with calcitriol

Cell cultures were prepared as described in the Materials and methods section. After equilibration for 24 h in serum-free Ham's F12-based medium, 96 h treatment was commenced with 1 nM-calcitriol plus various concentrations of DXM (▲) or cortisol (●), or with ethanol-containing vehicle plus DXM (△) or cortisol (○). Additional sets of cultures received 1 nM-calcitriol (■) or ethanol-containing vehicle only (□). All cultures received fresh medium and treatment after 48 h. The results shown are the rates of PRL accumulation in the medium in the final 48 h of treatment. Medium PRL was measured by radioimmunoassay. Each point represents the mean ($\pm$ S.E.M.) value for three replicate cultures. The mean final cell protein per culture was 0.9 mg and did not vary significantly with treatment.

molecular mechanism by which calcitriol enhances intestinal Ca²⁺ transport. However, application of the present cultured-cell model in which glucocorticoids potently and selectively antagonize calcitriol induction of specific gene expression may yield valuable information concerning this hormonal interaction. To explain the relatively short period of exposure (i.e. 4 h) and the long latent period before its effect becomes evident (i.e. at least 24 h), we have postulated that calcitriol has an initial effect (presumably genomic) which causes a stable change in either PRL-gene transcription or PRL-mRNA half-life. The quite rapid fall in calcitriol-induced PRL mRNA levels after glucocorticoid treatment suggests that the latter agents are active at the level of either PRL-gene transcription or mRNA degradation. Since the stimulatory effect of calcitriol requires Ca²⁺, it is tempting to speculate that the action of glucocorticoids in the present system involves an alteration in cellular Ca²⁺ homeostasis, as has been proposed to explain some other glucocorticoid effects (Rousseau & Baxter, 1979). The aim of the present study was to explore these possible mechanisms.

The finding of the calcitriol-glucocorticoid interaction in GH₄C₁ cells arose from a systematic investigation of hormonal interactions using a serum-free, hormone-free experimental incubation medium. In previous studies using serum-supplemented media, induction of PRL-gene expression by calcitriol was either not observed (Haug *et al.*, 1982) or was minor in magnitude (Murdoch & Rosenfeld, 1981). The medium concentration of horse serum used in those studies ranged from 6 to 12.5% (v/v). Although we have found very low immunoassayable cortisol levels in several batches of foetal-bovine serum, horse sera contained a mean cortisol concentration of 180 nM. Hence the cortisol content of horse serum supplements typically used in the culture of GH cells may be sufficient to diminish or even prevent totally the effect of calcitriol on PRL-gene expression. Although other factors (e.g. vitamin D-binding protein) may contribute to the inhibition by serum of calcitriol action in the present model, our findings provide evidence which also implicates serum-derived glucocorticoids.

These studies were supported by grants from the National Health and Medical Research Council of Australia and the Victor Hurley Medical Research Fund of The Royal Melbourne Hospital. Cortisol radioimmunoassays were kindly performed through Dr. D. G. Campbell, Department of Biochemistry, The Royal Melbourne Hospital.

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Received 15 July 1985/30 August 1985; accepted 18 September 1985