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DEMONSTRATION AND CHARACTERIZATION OF A 1α,25-$(OH)_2D_3$ RECEPTOR-LIKE MACROMOLECULE IN CULTURED RAT PITUITARY CELLS

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Summary—Recent studies have demonstrated the importance of prolactin (PRL) and growth hormone (GH) in the regulation of 25-hydroxycholecalciferol-1α-hydroxylase activity. We have previously shown that 1α,25-dihydroxycholecalciferol (1α,25-$(OH)_2D_3$) reduces PRL and GH production by a clonal strain of rat pituitary tumour cells (GH_3) [1]. The biologically active form of vitamin D_3, 1α,25-$(OH)_2D_3$, acts via an initial binding to cytoplasmic receptor proteins in target cells, and we demonstrate in this study the presence of specific receptors for 1α,25-$(OH)_2D_3$ in the GH_3 cells. GH_3 cell cytosol was incubated with [^{3}H]1α,25-$(OH)_2D_3$ at 0–4°C. Maximal binding was obtained between 2 and 6 h, and Scatchard analysis showed one single class of binding sites with K_d of 0.33 ± 0.05 nM (mean + SD) and a B_{max} of 103 ± 26 fmol/mg cytosol protein. Competitive binding experiments revealed the following potency order: 1α,25-$(OH)_2D_3$ > 25-OHD_3 > 1α-OHD_3, 24,25-$(OH)_2D_3$. In contrast, corticosterone, testosterone, progesterone and oestradiol showed negligible ability to displace [^{3}H]1α,25-$(OH)_2D_3$ from its receptor. Sucrose gradient ultracentrifugation in high salt concentration revealed that GH_3 cell cytosol possessed at 3.7S [^{3}H]1α,25-$(OH)_2D_3$ receptor protein which was inactivated by heating and protease treatment, but not after incubation with DNase or RNase. The receptor protein aggregated in salt-free sucrose gradients since the 3.7S complex was shifted reversibly to a ~6S form. Isoelectric focussing localized most of the [^{3}H]1α,25-$(OH)_2D_3$ to a protein peak with an isoelectric point of ~6 (pI 5.8–6.2). Since this 1α,25-$(OH)_2D_3$ receptor protein has similar properties as the corresponding 1α,25-$(OH)_2D_3$ receptors found in normal rat tissues, we suggest that lactotropes and somatotropes represent true target cells for 1α,25-$(OH)_2D_3$.

INTRODUCTION

Renal 25-hydroxyvitamin D_3 (25-OHD_3)-1α-hydroxylase is responsible for converting 25-OHD_3 into 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), the hormonally active form of vitamin D_3. Parathyroid hormone (PTH) is the dominant hormonal regulator of this key enzyme [2]. There is, however, evidence indicating that prolactin (PRL) and growth hormone (GH) may increase the activity of 25-OHD_3-1α-hydroxylase, and thus increase circulating levels of 1α,25-$(OH)_2D_3$ in situations with increased calcium and phosphate demands [3–10].

If, indeed, PRL and GH are involved in the regulation of 25-OHD_3-1α-hydroxylase activity, then one might expect feedback effects of 1α,25-$(OH)_2D_3$ at the level of the anterior pituitary gland. The recent discovery that 1α,25-$(OH)_2D_3$ inhibits PRL and GH hormone production by rat pituitary tumour cells (GH_3) in culture [11, 1], supports the suggestion that a feedback loop exists between the renal tubular cells and the anterior pituitary. As a first step in elucidating the mechanism of action of 1α,25-$(OH)_2D_3$ we have examined the GH_3 cells for the presence of specific 1α,25-$(OH)_2D_3$ receptors. Although the GH_3 cells are of tumour origin, they have retained the ability to synthesize PRL and GH that are biologically active and immunologically indistinguishable from the corresponding authentic rat hormones [12, 13], and they respond to physiological signals in a manner analogous to normal pituitary cells [14, 12, 13]. This study shows that GH_3 cells possess a 1α,25-$(OH)_2D_3$ binding protein which sediments at 3.7S in high salt sucrose gradients and binds the steroid specifically and with high affinity (K_d = 10^{-11}–10^{-10} M).

A preliminary account has been reported in abstract form at the 14th Acta Endocrinologica Congress, Stockholm, 1983.

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EXPERIMENTAL

Chemicals

1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol (sp. act. 130–180 Ci/mmol) and 25-hydroxy[26,27-methyl-^{3}H]cholecalciferol (sp. act. 130–180 Ci/mmol) were obtained from Amersham International plc (Buckinghamshire, England). Crystalline 1α,25-$(OH)_2D_3$ was a gift from F. Hoffmann-La Roche and Co. A. G. (Basle, Switzerland), 25-$(OH)D_3$ from Phillips-Duphar B.V. (Veenendal, The Netherlands), and 24,25-$(OH)_2D_3$ and 1α-$(OH)D_3$ from Leo Pharmaceutical (Copenhagen, Denmark). Testosterone, 17β-oestradiol, cortisol and progesterone were obtained from Steraloids Inc. (Pawling, N.Y.).

Tris(hydroxymethyl)aminomethane (Tris–HCl), sodium molybdate, mersalyl, DNase, RNase, bacterial protease (type IV), ovalbumin, bovine serum albumin (BSA) and human immunoglobulin G (IgG) were purchased from Sigma. Ethylene-diaminetetraacetic acid (EDTA) was obtained from Fluka A. G. (Buchs S. G, Switzerland), 2-mercaptoethanol from Bio-Rad Laboratories (Richmond, California), charcoal (Norit A Neutral) from Ahmend Drug and Chemical Co. (Irvington, NJ) and Dextran T-70 from Pharmacia Fine chemicals (Uppsala, Sweden). The non-ionic detergent P-40 was donated by Shell Oil Co. Ham's F-10 medium, amphotericin B, sterile horse serum and foetal calf serum were obtained from Flow Laboratories (Irvine, U.K.), and the penicillin–streptomycin solution and anti-PPLO agent, Tylocine, from Grand Island Biological Co. (Grand Island, N.Y.).

Cell culture

The establishment [15] and the propagation of the GH_3 cell strain have been described previously [16, 14]. Briefly, the GH_3 cells were routinely passed in culture in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in Ham's F-10 medium supplemented with 7.5% horse serum and 2.5% calf serum. Penicillin (50 U/ml medium), streptomycin (50 μg/ml medium) and amphotericin B (2.5 μg/ml medium) were always added to the culture medium, and did not influence cell growth or hormone production at these concentrations.

In experiments designed to study hormone production, the GH_3 cells were grown in tissue-culture plates (Costar, 24 wells) containing 0.5–1 ml F-10 medium per well. The culture medium was changed every 2 days throughout the study. At the end of the treatment period the culture medium was collected, and the floating cells were pelleted by centrifugation (300 ***g***, 5–10 min) and washed 3 times with 2 ml of ice-cold 0.15 M NaCl. The cells still adhesive to the bottom of the wells were also washed 3 times with 2 ml of ice-cold saline. Both the culture medium and the cells were stored at −20°C until assayed for hormone and protein content. The cell cultures were free of mycoplasma contamination (National Institute of Public Health, Oslo).

Measurement of hormone production and cell growth

Culture medium concentrations of PRL and GH were measured by radioimmunoassays as previously described [12, 13], using rPRL and rGH donated by NIAMDD, Bethesda, Maryland 20014.

Hormone production was measured as the amount of hormone that accumulated in the medium during 48 h. The intracellular stores of PRL and GH are very limited and vary in parallel with the extracellular levels [14]. The hormones are stable in the culture medium for as long as 48 h under culture conditions [17, 18]. Therefore, the amount of PRL and GH that accumulated in the medium during 48 h represents >97% of the total amount of hormone produced during the same time [14].

Because cell size does not vary markedly with the age of a culture after plating [14], the total protein content per well (floating plus adherent cells) was used to measure cell mass. Cell protein was measured by the Coomassie blue dye method with BSA as standard, using the Bio-Rad Protein Assay.

Cell preparation and cytosol binding assay

Monolayer cultures of GH_3 cells were scraped into ice-cold 0.15 M NaCl, pelleted, washed with ice-cold 0.15 M NaCl (10 ml × 3), and disrupted by sonication on ice in hypertonic TEKMM buffer (10 mM Tris–HCl, 1.5 mM EDTA, 300 mM KCl, 10 mM Na_2MoO_4, 5 mM 2-mercaptoethanol, pH 7.4 at 2°C). Cytosol was prepared by centrifugation at 105,000 ***g*** (60 min at 4°C) and stored at −70°C until used in binding experiments.

Cytosol (1–2 mg cytosol protein/ml) in 200 μl aliquots was incubated with either [^{3}H]1α,25-$(OH)_2D_3$ or [^{3}H]25-$(OH)D_3$ for 3 h at 0°C. Apart from the controls, the cytosol samples were submitted to one of the following treatments: (1) protease, DNase or RNase (100 μg/ml cytosol) were added to the incubate together with [^{3}H]1α,25-$(OH)_2D_3$; (2) heat treatment (30 min at 45°C) following incubation; (3) the sulfhydryl-blocking reagent, mersalyl (1 mM), was added either before or after completion of hormone binding in buffer with (TEKMM) or without 2-mercaptoethanol (TEKM); (4) unlabelled vitamin D_3 metabolites 25-$(OH)D_3$, 1α-$(OH)D_3$, 1α-25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ or unlabelled steroids (17β-oestradiol, testosterone, progesterone or corticosterone) were added to the incubate together with 1 nM [^{3}H]1α,25-$(OH)_2D_3$.

The separation of bound and free steroid was accomplished by charcoal adsorption. The charcoal solution (5% (w/v) Norit A and 0.5% (w/v) Dextran T-70) was prepared in TEKMM buffer. The charcoal solution (4.75 ml) was treated with 0.25 ml of horse serum for 10 min at 0°C and centrifuged at 2,500 ***g*** for 10 min at 4°C [19]. The serum-coated charcoal pellet was resuspended in 20 ml TEKMM buffer (1.25% (w/v) Norit A and 0.12% (w/v) Dextran T-70), and aliquots (1.5 ml) were added to the cytosol fractions which had been incubated with the appropriate steroids. The samples were blended on a Vortex mixer, kept on ice-water for 10 min and centrifuged. A 1 ml aliquot of the supernatant was mixed with 5 ml Insta-Gel II and assayed for radioactivity in a Packard Tri-Carb liquid scintillation counter at ~50% efficiency.

Sucrose density gradient ultracentrifugation

At the end of the incubation period the unbound ligand was removed by charcoal pellets prepared by pelleting 1.5 ml of the serum-coated charcoal (1.25%), dextran T-70 (0.125%) solution (see above).

Linear 5–20% (w/v) sucrose gradients (16.5 ml) were prepared in TEKMM buffer in 17 ml polyallomer tubes (Sorvall Instruments) and kept at 4°C for 1–2 h before use. A 500 μl aliquot of each charcoal-treated cytosol was carefully layered over the gradients. Tubes were centrifuged at 400,000 *g* max (3 h at 4°C) in a Sorvall OTD-65B ultracentrifuge using a Dupont 8 × 17 ml TV-865 vertical tube rotor (acceleration rate 3). An ω^2t integrator was used to operate the centrifuge (setting: 4.86×10^{11}, time on "*t*"). Following termination of each run, tubes were punctured at the bottom, and 22-drop fractions were collected into 6 ml Insta-Gel II and radioassayed as described above.

Portions (500 μl) of dye-labelled marker proteins of known sedimentation constant were run on separate gradients in each experiment. The following standard proteins: ovalbumin (3.7S, BSA (4.5S) and human IgG (6.8S), were labelled with Coomassie Blue as described by Freedman and Hawkins [20]. Portions of the dye–protein complex were stored at −20°C until used.

Isolation of nuclei

Monolayer cultures of GH_3 cells, harvested as described above, were resuspended in hypotonic TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.0), lysed at 0°C in the presence of 0.5% (v/v) P-40 (5 min), and the nuclei pelleted by centrifugation. The supernatant was removed, and the P-40 treatment was repeated with the resuspended nuclei. Finally, the nuclear pellet was resuspended in TEKMM buffer (2.5–3.0 mg nuclear protein/ml) and extracted for 30 min at 0°C. The nuclear extracts thus obtained were used in the vitamin D_3 binding assay.

Isoelectric focussing

Cylindrical polyacrylamide gels (3 × 100 mm) were prepared as described by Righetti and Drysdale [21]. Each gel contained 4.25% (w/v) acrylamide monomer (T = 4.4% (w/v)), 0.17% (w/v) *bis*-acrylamide cross-linker (C = 3.9% (w/v)), and 2% (w/v) ampholytes (Ampholine pH 5–7 (1.5%) and pH 7–9 (0.5%)). Glycerol was added to a final concentration of 12.5% (w/v). After polymerization the gels were overlaid with a solution containing 1% ampholytes and stored overnight at 4°C. Focussing was performed with a disc-gel electrophoresis apparatus (modified Canalco Model 1200) and a LKB 2179 power supply. The instruments were set in a cold room (~4°C), and ice-water was continuously circulated through the apparatus. Anolyte and catolyte solutions were 0.1 N H_3PO_4 and 0.2 N NaOH, respectively. To establish the gradient, gels were pre-run without sample at 0.5 mA/gel constant current until 300 V was attained. Then the power supply was switched to constant voltage until the current level had dropped to 0.1 mA/gel.

Aliquots (100 μl) of cytosol, preincubated with [^{3}H]1α,25-$(OH)_2D_3$ ± radioinert 1α,25-$(OH)_2D_3$ for 3 h at 0°C, were loaded onto the cathodic end of each pre-run gel. Isoelectric focussing was initiated at a constant current of 0.5 mA/gel. When 300 V was attained, the power supply was switched to constant voltage until the current level had dropped to 0.1 mA/gel. The total focussing time from the application of sample was about 4 h.

After termination of the run, the gels were removed and stored at −20°C, and subsequently the frozen gels were sliced at 2 mm intervals. In each run one gel served as control, and the slices were incubated overnight in 300 μl of deionized water to extract the ampholytes. The pH of each slice was measured at room temperature using a microelectrode (Bio-Rad Gel Pro-pHiler). Each slice from the gels used to resolve vitamin D_3 binding was transferred to plastic vials, and the radioactive vitamin D_3 was extracted into 5 ml of Instafluor II containing 5% Soluene 350 and radioassayed as described above.

RESULTS

Effects of 1α,25-dihydroxyvitamin D_3, calcitonin (CT) and parathyroid hormone (PTH) on production of PRL and GH

Figure 1 shows the time-course of the effects of 1α,25-$(OH)_2D_3$ (10^{-6} M) on the production of PRL and GH. Groups of treated and untreated control cultures were harvested at the different times indicated, and hormone production expressed as per cent of controls. The inhibitory effects of 1α,25-$(OH)_2D_3$ on PRL and GH production were significant ($P < 0.05$) after 2 days, and the maximal effects were obtained after 6 days of treatment. When cells which had been treated for 6 days, received culture medium without additions, the production of PRL and GH returned to control levels in about 7 days (Fig. 1). The inhibitory effects of 1α,25-$(OH)_2D_3$ on hormone production were not accompanied by a change in cell growth (data not shown).

Tables 1 and 2 show that CT (1–100 ng/ml) and PTH (0.002–20 ng/ml) had no effect on hormone production and cell growth.

Time-course of the binding of [^{3}H]1α,25-$(OH)_2D_3$ to GH_3 cell cytosol

We first had to determine the time-course of binding of [^{3}H]1α,25-$(OH)_2D_3$ to GH_3 cell cytosol prepared in hypertonic TEKMM buffer. Cytosol fractions were incubated (20 min to 18 h) with [^{3}H]1α,25-$(OH)_2D_3$ (2 nM) in the absence (total binding) or presence of a 200-fold molar excess of unlabelled 1α,25-$(OH)_2D_3$ (non-specific binding) at 0°C. At the end of the incubation period bound and free steroid were separated by charcoal adsorption of the unbound steroid (see Experimental). Figure 2 (left panel) shows that non-specific binding increased rapidly during the first 20 min of the incubation and thereafter only very slowly. Total binding also in-

Table 1. Effects of calcitonin (CT) on prolactin (PRL) and growth hormone (GH) production in GH_3 cells (mean ± SEM, $n = 4$–8)

CT (ng/ml)	Cell protein (μg/well)	PRL (ng/μg protein per 48 h)	GH (ng/μg protein per 48 h)
0	68.7 ± 3.6	3.3 ± 0.2	137.2 ± 6.9
1	80.3 ± 1.7	3.2 ± 0.3	135.1 ± 12.1
5	80.5 ± 1.5	3.2 ± 0.4	117.4 ± 4.8
10	83.3 ± 0.9	3.2 ± 0.2	143.2 ± 5.7
50	84.7 ± 0.3	3.0 ± 0.2	162.7 ± 7.8
100	74.0 ± 5.0	3.2 ± 0.3	140.7 ± 9.9

Table 2. Effects of parathyroid hormone (PTH) on prolactin (PRL) and growth hormone (GH) production in GH_3 cells (mean ± SEM, $n = 4$)

PTH (ng/ml)	Cell protein (μg/well)	PRL (ng/μg protein per 48 h)	GH (ng/μg protein per 48 h)
0	126.6 ± 5.2	1.6 ± 0.1	84.6 ± 2.3
0.002	142.5 ± 2.5	1.6 ± 0.2	88.9 ± 5.5
0.02	143.1 ± 6.1	1.7 ± 0.2	86.1 ± 2.5
0.2	141.2 ± 3.3	1.7 ± 0.2	86.2 ± 1.0
2.0	141.2 ± 3.1	1.5 ± 0.2	83.1 ± 2.2
20.0	136.3 ± 3.8	1.9 ± 0.1	90.0 ± 5.5

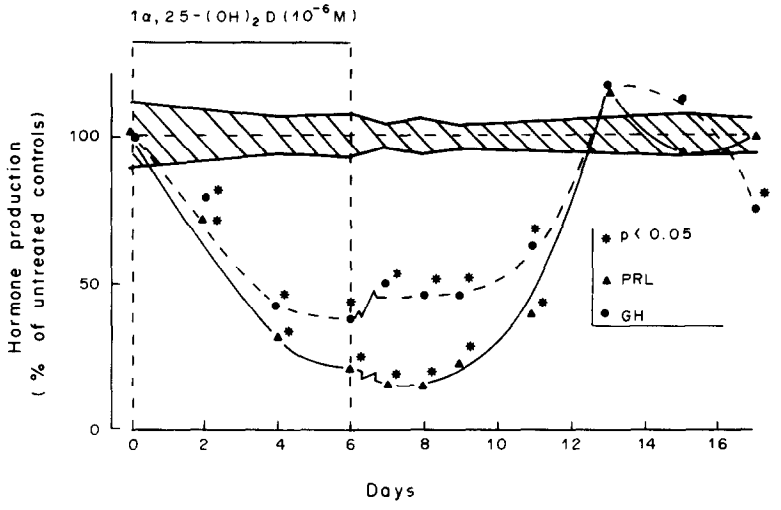

Fig. 1. Time-course of the effects of 1α,25-$(OH)_2D_3$ on PRL and GH production. Quadruplicate cultures were incubated in complete F-10 medium in the presence of 1α,25-$(OH)_2D_3$ (10^{-6} M) for 2, 4 and 6 days. Control cultures received vehicle only. The values (means) for hormone production represent extracellular PRL and GH expressed as a percentage of control values. The shaded area represents the hormone production (mean ± SEM) in the control cultures, and the asterisks indicate values significantly ($P < 0.05$) different from control values.

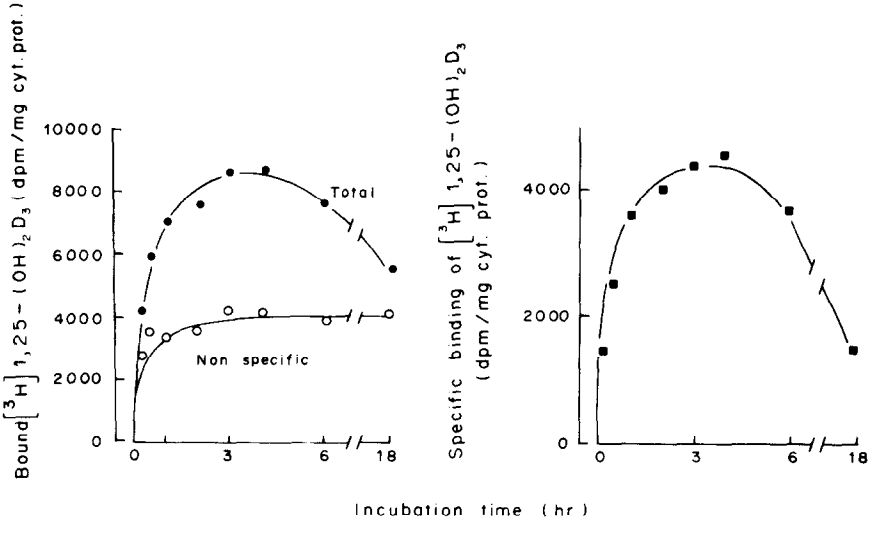

Fig. 2. Time-course of [^{3}H]1α,25-$(OH)_2D_3$ binding in GH_3 cell cytosol. Aliquots (200 μl) of cytosol were incubated with [^{3}H]1α,25-$(OH)_2D_3$ (4 nM) in the absence (Total, ●) or presence of a 200-fold molar excess of unlabelled 1α,25-$(OH)_2D_3$ (Non-specific, ○) at 0°C for the various times indicated. Bound and free steroid was separated by dextran-charcoal adsorption. Specific binding (■) was calculated by subtracting the non-specific from the total binding. Mean values of duplicates are shown.

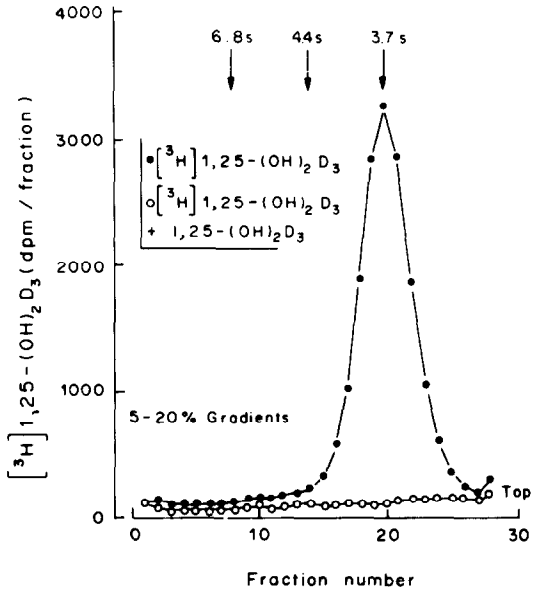

Fig. 3. Sucrose density gradient analysis of 1α,25-$(OH)_2D_3$ binding components in GH_3 cell cytosol. Aliquots of cytosol, preincubated with [^{3}H]1α,25-$(OH)_2D_3$ (4 nM) alone or in the presence of a 200-fold molar excess of unlabelled steroid, were treated with dextran-coated charcoal to remove free hormone. Cytosol (500 μl) was then layered on gradients prepared in hypertonic buffer and centrifuged for 3 h at 400,000 *g* max using a vertical tube rotor. Fractions were collected through the bottom of the tubes. The arrows indicate the sedimentation positions of the internal markers, immunoglobulin G (6.8S), bovine serum albumin (4.4S) and ovalbumin (3.7S).

creased rapidly during the first 60 min and reached its maximum value after 3–4 h, and then showed a decrease at 6 and 18 h. Specific binding (total minus non-specific) was half-maximal already after 30 min (Fig. 2, right panel), and reached its maximum at 2–4 h. Thereafter specific binding decreased and was only 30% of maximal binding at 18 h. For the subsequent binding studies an incubation time of 3 h was therefore chosen.

Sucrose density gradient ultracentrifugation

When GH_3 cell cytosol was labelled with [^{3}H]-1α,25-$(OH)_2D_3$ and analyzed by sucrose density gradient centrifugation in TEKMM buffer, the radioactivity profile shown in Fig. 3 was obtained. At high ionic strength a single binding peak sedimenting in the 3.7S region was seen. When cytosol was labelled in the presence of a 200-fold molar excess of radioinert steroid, [^{3}H]1α,25-$(OH)_2D_3$ was completely displaced from the 3.7S peak.

GH_3 cell cytosol was then subjected to enzymatic treatment. Protease treatment eliminated the 3.7S radioactive peak, whereas RNase and DNase had no effect on binding (Fig. 4, left panel). Heating (30 min at 45°C) of cytosol previously labelled with [^{3}H]-1α,25-$(OH)_2D_3$ totally abolished the binding (Fig. 4, left panel).

To examine whether binding of [^{3}H]1α,25-$(OH)_2D_3$ requires the presence of active sulfhydryl groups, cytosol was exposed to the sulfhydryl-blocking reagent, mersalyl. The addition of mersalyl to GH_3 cell cytosol prepared in hypertonic buffer without mercaptoethanol (TEKM) eliminated the radioactive 3.7S peak (Fig. 5) The effect of mersalyl was similar whether the reagent was added before or after completion of the incubation. In complete TEKMM buffer with mercaptoethanol, however, mersalyl had no effect.

Figure 6 shows that when GH_3 cell cytosol preincubated with [^{3}H]1α,25-$(OH)_2D_3$ (4 nM) was analyzed on sucrose gradients containing reduced concentrations of KCl, the 3.7S binding component was gradually lost. When analyzed in gradients containing only 25 mM KCl a small peak was found in the

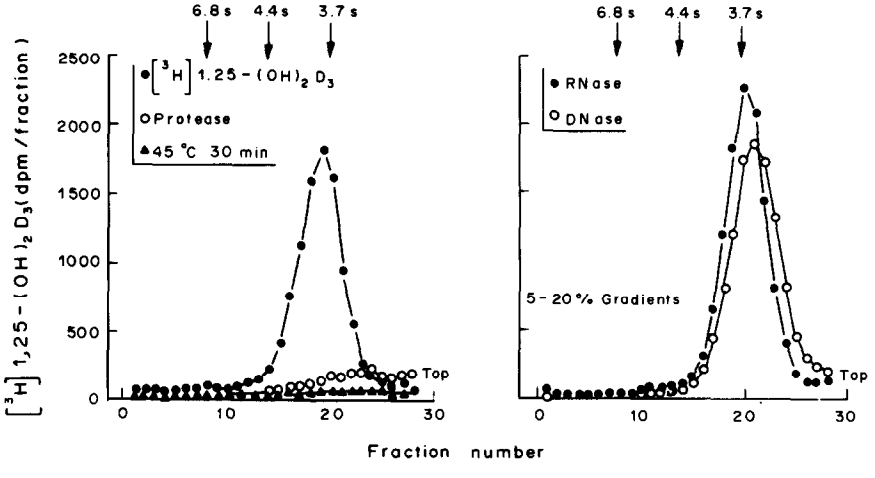

Fig. 4. Sucrose density gradient analysis of [^{3}H]1α,25-$(OH)_2D_3$ binding components in GH_3 cell cytosol. Aliquots of cytosol were preincubated with [^{3}H]1α,25-$(OH)_2D_3$ (4 nM) alone (control) or in the presence of one of the following enzymes (100 μg/ml): protease, DNase and RNase. One aliquot of cytosol was heated at 45°C for 30 min following the incubation with radiolabelled steroid. After dextran-charcoal treatment cytosol was examined by sucrose gradient analysis. For details, see legend to Fig. 3.

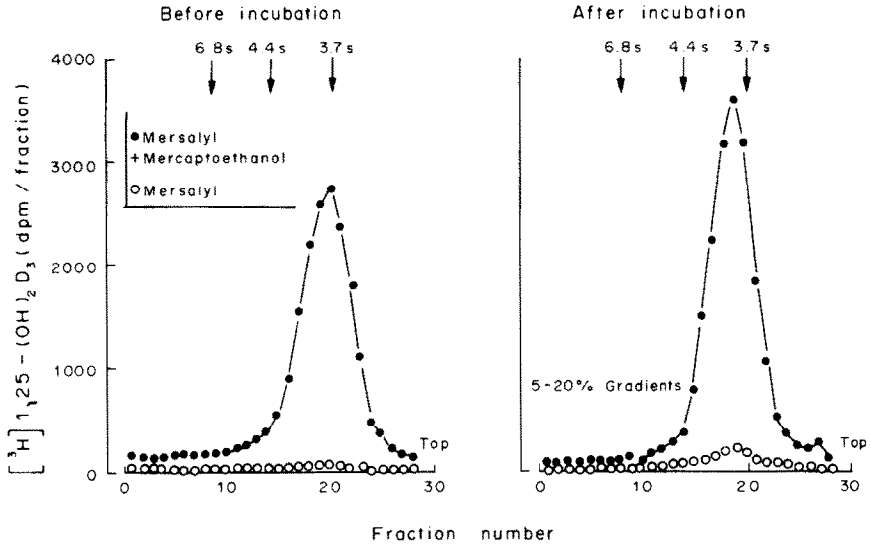

Fig. 5. Sucrose density gradient analysis of [^{3}H]1α,25-$(OH)_2D_3$ binding components in GH_3 cell cytosol. Aliquots of cytosol prepared in hypertonic buffer with or without mercaptoethanol (5 nM) were preincubated with [^{3}H]1α,25-$(OH)_2D_3$ (4 nM). The sulfhydryl-blocking agent mersalyl (1 mM) was either added to the incubates simultaneously with the radiolabelled steroid (left) or after completion of the hormone binding (right). After dextran-charcoal treatment cytosol was examined by sucrose gradient analysis, as described in the legend to Fig. 3.

4.4–6.8S region in addition to the dominant 3.7S peak. In gradients prepared with no KCl the 3.7S binding component was almost completely lost, and a concomitant increase in the binding of 1α,25-$(OH)_2D_3$ to components with higher sedimentation coefficients was seen. These radioactive peaks were totally abolished by a 200-fold molar excess of 1α,25-$(OH)_2D_3$ (data not shown).

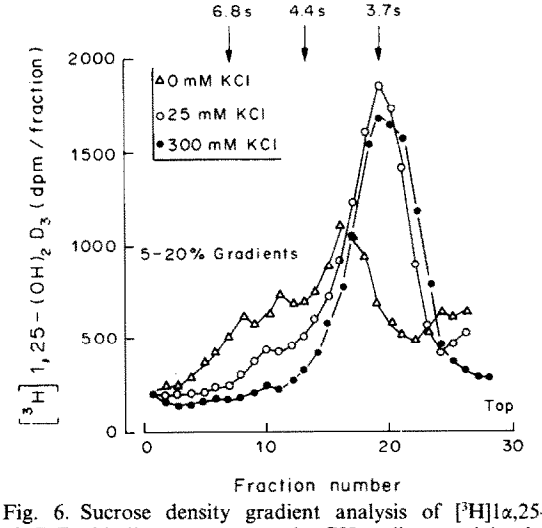

Fig. 6. Sucrose density gradient analysis of [^{3}H]1α,25-$(OH)_2D_3$ binding components in GH_3 cell cytosol in the presence of decreasing concentrations of KCl. Cytosol was prepared in hypertonic TEKMM buffer containing 300 mM KCl and preincubated with [^{3}H]1α,25-$(OH)_2D_3$. After dextran-charcoal treatment aliquots (500 μl) of cytosol were layered on gradients prepared in buffer containing either 300 nM KCl, 25 mM KCl or no KCl, and analyzed as described in the legend to Fig. 3.

Determination of binding constants

The binding affinity and capacity of the 1α,25-$(OH)_2D_3$ binding components were examined by preincubating aliquots of GH_3 cell cytosol with increasing concentrations of [^{3}H]1α,25-$(OH)_2D_3$ at 0°C for 3 h. Figure 7 shows that the binding component was almost saturated at 2 nM [^{3}H]1α,25-$(OH)_2D_3$. By Scatchard analysis of the binding data a straight line indicating one single class of high-affinity binding sites was obtained with the apparent K_d 0.8 nM and the B_{max} 29.5 fmol/mg of cytosol protein. Several binding experiments performed by the dextran-charcoal method (see Experimental) all yielded linear Scatchard plots. The mean ± SD of eight such experiments performed during 18 months with cytosol prepared from different GH_3 cell cultures showed the apparent K_d to be 0.33 ± 0.05 nM and the B_{max} to be 103 ± 26 fmol/mg of cytosol protein.

Specificity of binding to GH_3 cell cytosol

The specificity of the vitamin D_3 receptor binding was examined by incubating aliquots of GH_3 cell cytosol with [^{3}H]1α,25-$(OH)_2D_3$ (1 nM) in the absence or presence of increasing concentrations of various vitamin D_3 analogues. In Fig. 8 the control binding in the absence of competing steroid (89 ± 7 fmol/mg protein, mean ± SD, $n = 7$) is set equal to 100%. The relative affinity of the various vitamin D_3 analogues for receptor binding was defined as the concentration required to decrease [^{3}H]1α,25-$(OH)_2D_3$ binding by 50% (IC_{50}). As shown in Fig. 8, 1α,25-$(OH)_2D_3$ is the most potent competitor of the vitamin D_3 analogues tested, reducing the binding to 50% on an equimolar basis. The Relative Competitive Indices of the

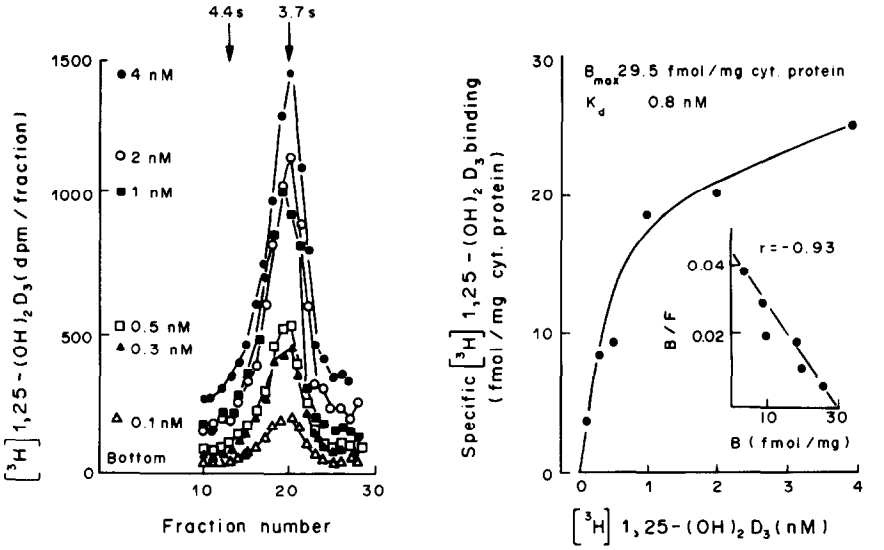

Fig. 7. Sucrose density gradient analysis of [^{3}H]1α,25-$(OH)_2D_3$ binding by GH_3 cell cytosol. Aliquots of cytosol prepared in hypertonic buffer were preincubated with [^{3}H]1α,25-$(OH)_2D_3$ at various concentrations from 0.1 to 4 nM. After dextrancharcoal treatment cytosol was examined by sucrose gradient analysis (left panel). Specific binding was obtained by calculating the area of the individual 3.7S peaks (right panel). Scatchard analysis of the binding data is shown in the inset. The regression line was calculated using the least squares method.

compounds tested were found to be: 1α,25-$(OH)_2D_3$ 100; 25-OHD_3 2; 24,25-$(OH)_2D_3$ and 1α-OHD_3 0.5 [22]. Other compounds tested for their ability to compete with [^{3}H]1α,25-$(OH)_2D_3$ for binding were testosterone, corticosterone, progesterone and 17β-oestradiol. At a 5000-fold molar excess these steroids failed to reduce the binding of [^{3}H]1α,25-$(OH)_2D_3$.

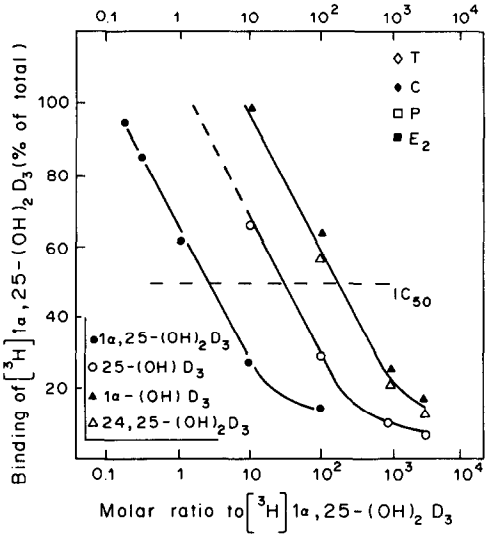

Fig. 8. Effects of unlabelled steroids on the binding of [^{3}H]1α,25-$(OH)_2D_3$ to the GH_3 cell cytosol. Aliquots (200 μl) of cytosol were incubated with [^{3}H]1α,25-$(OH)_2D_3$ (1 mM) in the absence or presence of increasing concentrations of various vitamin D_3 metabolites and testosterone (T), corticosterone (C), progesterone (P) and 17β-estradiol (E_2). Bound and free steroid was separated by charcoal adsorption. The values are expressed as percentage of [^{3}H]1α,25-$(OH)_2D_3$ binding equalling 100% in the absence of competition.

Nuclear binding of [^{3}*H*]1α,25-$(OH)_2D_3$

The nucleus is thought to be the specific site of action of 1α,25-$(OH)_2D_3$. Specific, high affinity binding sites for [^{3}H]1α,25-$(OH)_2D_3$ have been observed in nuclear fractions of GH_3 cells [22]. Thus, it was of interest to characterize the nuclear binding component. Figure 9 shows that when nuclear extract was incubated with [^{3}H]1α,25-$(OH)_2D_3$ and analyzed by

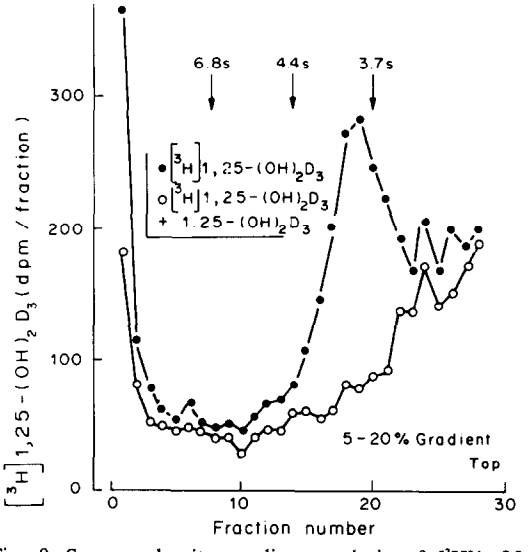

Fig. 9. Sucrose density gradient analysis of [^{3}H]1α,25-$(OH)_2D_3$ binding components in GH_3 cell nuclear extract prepared in hypertonic TEKMM buffer. Aliquots of the extract were preincubated with [^{3}H]1α,25-$(OH)_2D_3$ (4 nM) alone or in the presence of a 200-fold molar excess of unlabelled steroid. The mixture was treated with dextran-charcoal to remove free hormone, and layered on sucrose gradients and ultracentrifuged as described in the legend to Fig. 3.

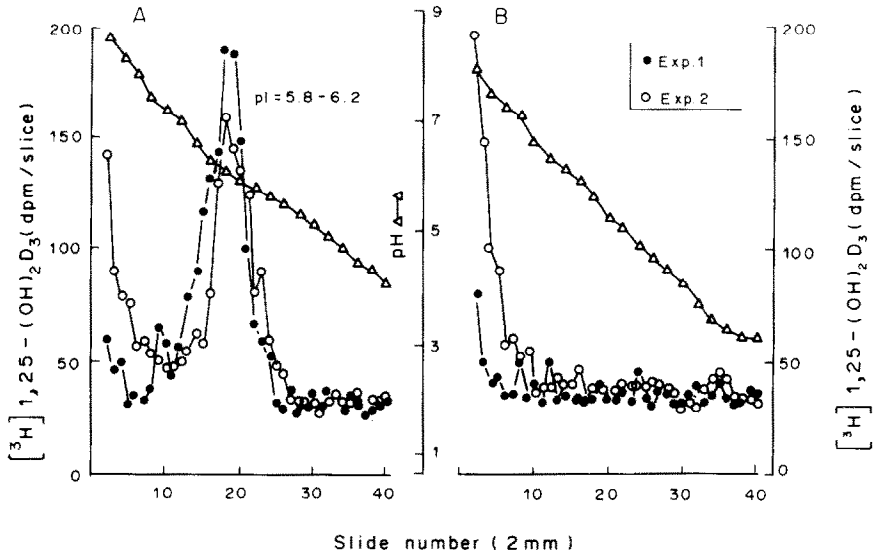

Fig. 10. Isoelectric focussing of [^{3}H]1α,25-$(OH)_2D_3$ binding components in GH_3 cell cytosol. Aliquots (100 μl) of cytosol, preincubated with [^{3}H]1α,25-$(OH)_2D_3$ (4 nM) alone or in the presence of a 200-fold molar excess of unlabelled steroid, were subjected to isoelectric focusing in 4.25% (w/v) acrylamide gels containing 2% (w/v) ampholines. After electrofocussing the gels were sliced at 2 mm intervals, and pH (△) and radioactivity (●, ○) were measured. The results from two identical experiments are shown.

sucrose density gradient centrifugation at high ionic strength, a single peak in the 3.7S region was seen, that was completely eliminated when the incubation was carried out in the presence of a 200-fold molar excess of 1α,25-$(OH)_2D_3$.

Isoelectric focussing of 1α,25-$(OH)_2D_3$ binding components from GH_3

The results of isoelectric focussing of GH_3 cell cytosol labelled *in vitro* [^{3}H]1α,25-$(OH)_2D_3$ (4 nM) in the absence or presence of a 200-fold molar excess of radioinert hormone are shown in Fig. 10. The vitamin D_3 binding component focussed between pH 5.8–6.2 and the radioactive peak was completely eliminated in the presence of excess 1α,25-$(OH)_2D_3$.

DISCUSSION

Functional cell lines are ideal model systems for studying hormone action, since they provide a homogeneous cell population in which the components of an endocrine response can be identified and characterized. Admittedly, the GH_3 cells used in this study are of tumour origin [15], but they spontaneously synthesize and secrete both PRL and GH which are biologically active [18] and immunologically indistinguishable from the corresponding authentic rat hormones [12,13]. The GH_3 cells also respond to oestradiol-17β [23], progesterone [24], testosterone [23] and corticosterone [25] in a manner analogous to normal, freshly explanted pituitary tissue. The GH_3 cells are obviously capable of carrying out several differentiated functions and respond normally to a variety of physiological signals. We therefore consider the GH_3 cell system a suitable model for studying the hormonal control of PRL and GH production, although the results need validation in a more physiological system.

It has recently been demonstrated that PTH can stimulate PRL release in normal man [26, 27], and that calcitonin (CT) can influence PRL secretion in rats [28–30] as well as in man [31–33]. The anatomical site of action of these two hormones is not known. We found no effect of PTH and CT on the production of PRL and GH by GH_3 cells, suggesting that PTH and CT influence PRL and GH secretion through an effect in the central nervous system.

It is well documented that 1α,25-$(OH)_2D_3$ is the hormonally active form of vitamin D_3 [2]. The renal 25-OHD_3-1α-hydroxylase which is responsible for converting 25-OHD_3 into 1α,25-$(OH)_2D_3$, is regulated by various circulating factors such as calcium [34], phosphate [34, 35], PTH [36, 34], insulin [37], PRL [38], GH [9], and oestrogens [39]. If PRL and GH represent hormonal stimulators of 1α,25-$(OH)_2D_3$ production, then one might expect a feedback effect of 1α,25-$(OH)_2D_3$ at the anterior pituitary. The present results complement our previous studies which demonstrated actions of 1α,25-$(OH)_2D_3$ on pituitary function [1]. In addition, these studies demonstrate the presence in GH_3 cell cytosol of a macromolecule of protein nature with all the properties of a typical 1α,25-$(OH)_2D_3$ receptor [22, 40, 41]. The binding of 1α,25-$(OH)_2D_3$ required intact-SH groups as reported for vitamin D_3 receptors in other tissues (Fig. 5) [42, 43].

The 1α,25-$(OH)_2D_3$ binding protein had a sedimentation coefficient of 3.7S when GH_3 cell cytosol was prepared in hypertonic buffer [44, 45] and centrifuged in 5–20% sucrose gradients prepared in the same

buffer (Fig. 3). When cytosol prepared in hypertonic buffer was analyzed on sucrose gradients containing reduced concentrations of KCl, the 3.7S peak was gradually lost and replaced with radioactive peaks sedimenting at 4S and 6.8S (Fig. 6). This aggregation phenomenon has been reported for the chick bone receptor [46], the chick kidney receptor [47] and the rat intestinal receptor [19], supporting the contention that the GH_3 cell cytosol binding protein indeed represented the $1\alpha,25\text{-}(OH)_2D_3$ receptor. Since other steroid hormone receptors also aggregate in buffers of low ionic strength, the present data confirm the similarity of the vitamin D_3 receptor to other steroid hormone receptors.

Scatchard analyses of the $[^3H]1\alpha,25\text{-}(OH)_2D_3$ binding data indicated an apparent K_d of 0.3–0.8 nM. This result is comparable to circulating levels of $1\alpha,25\text{-}(OH)_2D_3$ in the plasma of rats [37], suggesting partial occupancy of the binding sites at physiological hormone concentrations. Moreover, the K_d of the $1\alpha,25\text{-}(OH)_2D_3$ receptor is reasonably close to the ED_{50} (0.5–1 nM) for the inhibiting effects of $1\alpha,25\text{-}(OH)_2D_3$ on PRL and GH production [1]. The Scatchard plots were always linear indicating one single class of non-interacting binding sites. A 6S binding protein with preference for $[^3H]25\text{-}OHD_3$ over $[^3H]1\alpha,25\text{-}(OH)_2D_3$ was found in cytosol prepared from different vitamin D_3 target organs [48, 19, 47]. This binding protein was reported to result from the interaction of a serum 4S $25\text{-}OHD_3$ transport protein with a cytosolic factor [48]. Sucrose gradient analysis of GH_3 cell cytosol preincubated with $[^3H]25\text{-}OHD_3$ (2 nM) revealed a small radioactive peak sedimenting in the 4.4S region, that was only partially eliminated in the presence of 200-fold molar excess of radioinert $1\alpha,25\text{-}(OH)_2D_3$ (data not shown). No binding was found in the 6S region. The finding that the Scatchard plot resulted in a straight line, and the lack of $[^3H]1\alpha,25\text{-}(OH)_2D_3$ binding in the 4–6S region at high ionic strength, supported the view that practically all of the serum proteins were removed during the washing procedure prior to cell homogenization. The concentration of binding sites (30–156 fmol/mg cytosol protein) varied considerably during 16 months. The reason for this variations was unclear but might at least partly reflect differences in endogenous steroid concentrations in the serum batches used in the preparation of the culture medium, since our preliminary data indicated that both oestradiol and corticosterone could induce $1\alpha,25\text{-}(OH)_2D_3$ receptors in the GH_3 cells. Chen and Feldman[49] showed that the $1\alpha,25\text{-}(OH)_2D_3$ receptor concentration was regulated in a way which was related to the rate of cell division. Therefore, varying ages of the cell cultures used in our studies might also contribute to the variation in receptor concentration.

The Relative Competitive Index [22] for $25\text{-}OHD_3$ was approx 2 and for the metabolites, $24,25\text{-}(OH)_2D_3$ and $1\alpha\text{-}OHD_3$, the Relative Competitive Index was even less. Since the serum level of $25\text{-}OHD_3$ is about 1000-fold the level of $1\alpha,25\text{-}(OH)_2D_3$, the affinity would enable $25\text{-}OHD_3$ to occupy $1\alpha,25\text{-}(OH)_2D_3$ receptors. Corticosterone and the sex steroids are supposed to take part in the regulation of calcium homeostasis. Our data showed that at a 5000-fold molar excess these substances did not bind to the receptor, thus eliminating the possibility that they acted through binding to the vitamin D_3 receptor.

The isoelectric focussing patterns of the $1\alpha,25\text{-}(OH)_2D_3$ receptor had a single radioactive peak with pH within 5.8–6.2. There are many reports of isoelectric focussing of other steroid hormone receptors with isoelectric points of about 6, indicating that this may be a feature common to all cell steroid receptors.

In our work, sedimentation analysis was carried out using a vertical-tube rotor. This technique has shortened the time required for sucrose density gradient centrifugation analysis from about 16 h for a swinging bucket rotor to about 3 h, adding greatly to the practicability of the density gradient technique. Moreover, the shortening of the centrifugation time makes the vertical-tube rotor suitable for the investigation of unstable hormone–receptor complexes which may dissociate or be degraded during conventional gradient centrifugation [50, 20].

The $1\alpha,25\text{-}(OH)_2D_3$ receptor of the GH_3 cells therefore has all the properties of the $1\alpha,25\text{-}(OH)_2D_3$ receptor found in normal rat tissues. The GH_3 cells represent a cloned strain of homogeneous PRL and GH secreting cells, and the finding of normal $1\alpha,25\text{-}(OH)_2D_3$ receptors in these cells ought to have implications for the understanding of the action of vitamin D_3 in normal lactotropes and somatotropes.

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