DEMONSTRATION OF A RECEPTOR-LIKE BINDING PROTEIN FOR 1,25-$(OH)_2$-D_3 IN CULTURED INTESTINAL EPITHELIAL CELLS FROM THE ADULT RAT

John S. ADAMS, Thomas L. CLEMENS, Noboru HORIUCHI, Andrea QUARONI* and Michael F. HOLICK

*Vitamin D Laboratory, Endocrine Unit and *Gastrointestinal Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114* and *Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, MA, USA*

Received 12 March 1982

1. Introduction

Intracellular binding proteins for the active metabolite of vitamin D_3, 1,25-$(OH)_2$-D_3, have been identified in a variety of avian and mammalian tissues and cultured cells [1]. The most thoroughly characterized and, perhaps, most physiologically important hormone–'receptor' interaction has been that of intestinal mucosa, where 1,25-$(OH)_2$-D_3 acts as a potent stimulator of intestinal calcium absorption [1]. Until recently, however, attempts to maintain a well-characterized line of intestinal epithelial cells in long-term cultures have failed. In [2], a technique was described for establishing pure cultures of epitheloid cells derived from the duodenal crypt of germ free adult rats. Here, we describe the isolation and initial characterization of an intracellular binding protein for 1,25-$(OH)_2$-D_3 from this morphologically and functionally stable line of intestinal epithelial (IEC) cells.

2. Materials and methods

2.1. *Seco-sterols*

We used 1,25-$(OH)_2$-[26,27-^{3}H]D_3 (160 Ci/mmol; New England Nuclear, Boston MA) and crystalline 1,25-$(OH)_2$-D_3 (a gift from Dr Milan Uskokovic, Hoffmann-La Roche, Nutley NJ) for all binding studies. Both compounds were purified over Silica Sep-Pak (Waters Assoc., Milford MA) prior to use.

2.2. *Cell cultures*

Cultures of rat intestinal epithelial cells were established as in [3]. Cells were routinely grown in 60 mm plastic petri dishes (Costar, Cambridge MA) in Delbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FCS), 10 μg/ml insulin and 4 mM glutamine at 37°C in an atmosphere of 95% air, 5% CO_2.

2.3. *Extraction and characterization of an intracellular binding protein for 1,25-$(OH)_2$-D_3*

After pre-incubation of confluent monolayer cultures for 48 h in DMEM without FCS (to remove serum vitamin D-binding protein from the incubate), cells were harvested by gentle scraping with a rubber-tipped spatula following incubation of cultures with 4 ml 0.02% EDTA in phosphate-buffered saline (PBS; 16 mM NaH_2PO_4–7 H_2O, 3 mM KCl, 2 mM KH_2PO_4, 154 mM NaCl (pH 7.2)) for 45 min at 37°C. The cells were pelleted at 500 × *g* for 3 min and resuspended in 1 ml TKM buffer (4°C) consisting of 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 5 mM dithiothreitol, 300 mM KCl, and 10 mM sodium molybdate. The cells were disrupted by sonication and the homogenate was centrifuged at 100 000 × *g* for 1 h. The supernatant (designated cytosol) was collected, and 0.25 ml aliquots were incubated at 4°C for 4 h with 4 nM 1,25-$(OH)_2$[^{3}H]D_3 alone or in the presence of 40 nM unlabeled 1,25-$(OH)_2$-D_3. The incubation was terminated by absorption of unbound sterol with dextran-coated charcoal. The cytosol was then layered on sucrose gradients (4–20%) prepared in TKM buffer and centrifuged for 18 h at 257 000 × *g*. Parallel gradients of 0.25 ml cytosol prepared from intestinal mucosa of vitamin D-dependent chicks [4] were similarly processed. Sedimentation coefficients were estimated by comparison with protein markers (bovine albumin, 4.4 S; ovalbumin, 3.7 S). Gradients were

Abbreviation: 1,25-$(OH)_2$-D_3, 1,25-dihydroxyvitamin D_3

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

fractionated from bottom-to-top and radioactivity determined by scintillation counting.

Saturation analysis of binding of 1,25-$(OH)_2$-[^{3}H]D_3 to cytosol from IEC cells were performed, and separation of bound from free hormone was achieved on DEAE-cellulose filters (DE81; Whatman, England) as in [5].

In other experiments, cytosol was prepared in TKM buffer as above. Cytosol (1 ml) was incubated with 4 nM 1,25-$(OH)_2$-[^{3}H]D_3 for 4 h at 4°C. The cytosol was then diluted to 4 ml with TM buffer (TKM buffer without KCl) and applied to a 2 ml DNA–cellulose column (Millipore, Freehold NJ) slurried in TM buffer for affinity chromatography. Protein concentrations in cytosol preparations were determined as in [6].

3. Results

The sedimentation characteristics over sucrose gradients of cytosol prepared from cultured IEC cells and incubated with 1,25-$(OH)_2$-[^{3}H]D_3 are shown in fig.1A. A single peak of hormone binding sedimented at 3.7 S and was totally abolished when cytosol was incubated in the presence of excess unlabeled 1,25-$(OH)_2$-D_3. For comparison to a well-established intestinal cytoplasmic-binding protein, fig.1B shows the sedimentation properties of cytosol similarly prepared from intestinal mucose of vitamin-D-deficient chicks. Chick cytosol also demonstrated a peak of 1,25-$(OH)_2$-[^{3}H]D_3 binding that sedimented at 3.7 S and was abolished when cytosol was labeled in the presence of 40 nM 1,25-$(OH)_2$-D_3.

A saturation analysis of the binding of increasing concentrations of 1,25-$(OH)_2$-[^{3}H]D_3 to cytosol from IEC cells revealed a component of saturable binding (fig.2). Regression analysis of data, according to [7], disclosed an app. $K_d = 1.2 \times 10^{-10}$ M and 7.14 fmol specific binding protein/mg protein. Further characterization of the binding protein was obtained by demonstrating the ability of the receptor-1,25-$(OH)_2$-[^{3}H]D_3 complex to bind to DNA. Fig.3 shows affinity chromatography over DNA–cellulose of cytosol from IEC cells incubated with 4 nM 1,25-$(OH)_2$-[^{3}H]D_3 and eluted with a linear KCl gradient. Radioactivity was retained on DNA–cellulose at low KCl concentrations and was eluted as a single, symmetrical peak at 2.3 M KCl.

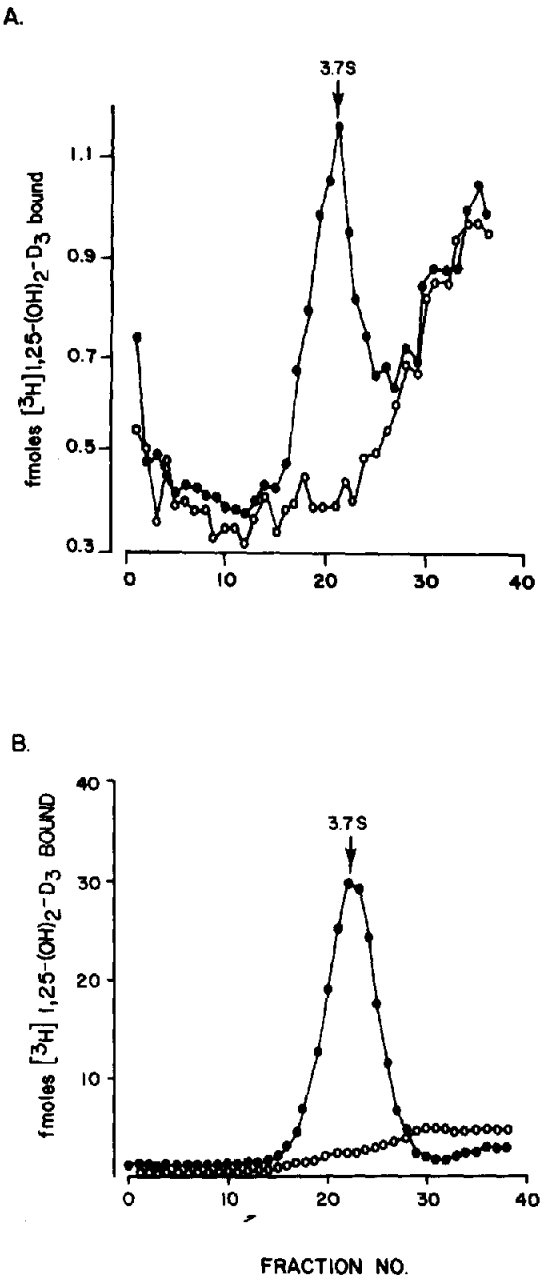

Fig.1. Analysis by sucrose density-gradient centrifugation (0.12 ml fractions) of the binding of 1,25-$(OH)_2$-[^{3}H]D_3 to cytosol prepared in TKM buffer from cultured IECs cells and intestinal mucosa from vitamin-D-deficient chicks: (A) cytosol (0.9 mg) extracted from 10×10^6 IEC cells, incubated for 4 h at 4°C with 4 nM 1,25-$(OH)_2$-[^{3}H]D_3 in the presence (–o–) or absence (–•–) of 40 nm 1,25-$(OH)_2$-D_3 and centrifuged for 18 h at 257 000 × *g* through a 4–20% sucrose gradient; (B) cytosol (5.0 mg) extracted from intestinal mucosa scraped from duodenum of rachitic chicks, similarly incubated, and centrifuged in a sucrose density-gradient.

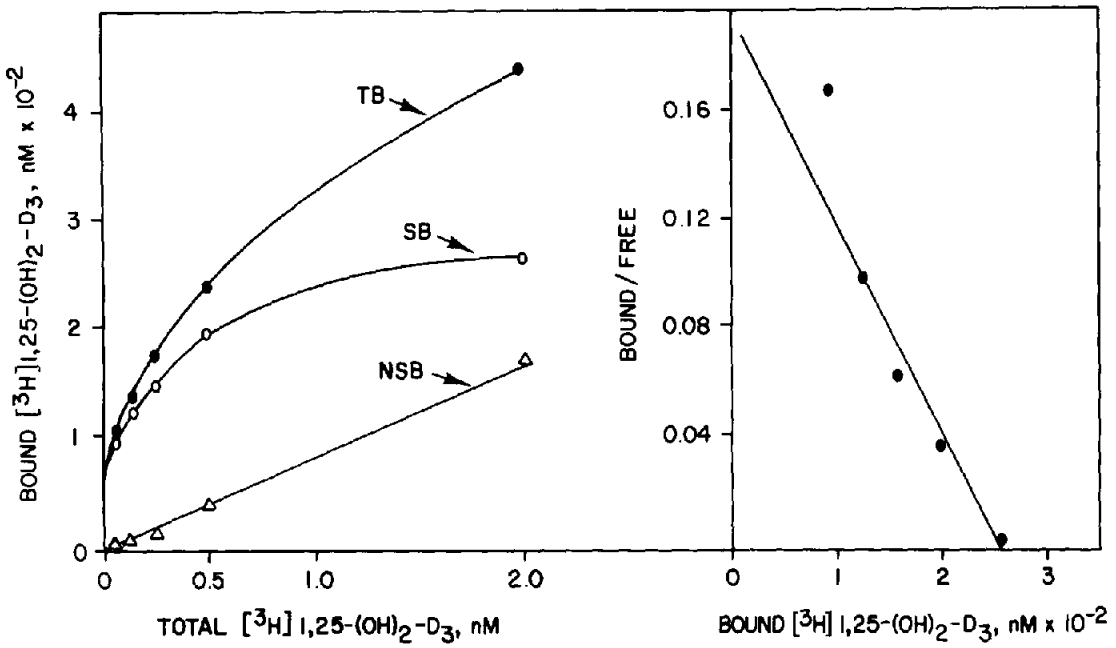

Fig.2. Saturation of 1,25-$(OH)_2$-[^{3}H]D_3 binding to cytosol prepared from cultured IEC cells. Cytosol was extracted from 10 × 10^6 IEC cells in 2.5 ml TKM buffer and 0.2 ml aliquots were incubated for 4 h at 4°C with concentrations of 1,25-$(OH)_2$-[^{3}H]D_3 ranging from 0.06–2.0 nM (solubilized in 10 μl ethanol) in the presence (NSB) or absence (TB) of 40 nM 1,25-$(OH)_2$-D_3. Bound hormone was collected on DEAE–cellulose filters and washed thrice with 5 ml Triton X-100 in 10 mM Tris–HCl (pH 7.4) containing 1 mM EDTA. Right: Scatchard plot of specific binding.

4. Discussion

The active metabolite of vitamin D_3, 1,25-$(OH)_2$-D_3, is now considered to be a steroid hormone in the classical sense. Studies using intestinal mucosa from vitamin-D-deficient chicks have demonstrated cytoplasmic localization of 1,25-$(OH)_2$-[^{3}H]D_3 with a receptor-like binding protein [8–10] and transfer of the ligand-binding protein complex to the nucleus [9,11]. To date, most workers have employed crudely-prepared intestinal epithelium (obtained by mechanical, sonic or enzymatic disruption of the tissue) or the entire organ as a model for investigating the interaction of the hormone with its target cell. However, work with these tissues has been complicated by the cellular heterogeneity of whole intestine and con-

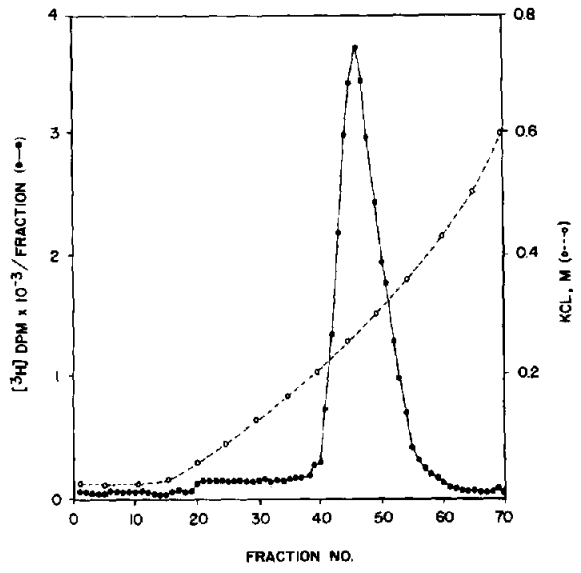

Fig.3. DNA–cellulose chromatography of 1,25-$(OH)_2$-[^{3}H]-D_3-labeled cytosol from IEC cells. Cytosol was extracted from 20 × 10^7 IEC cells in 1 ml TKM buffer, incubated for 4 h at 4°C with 4 nM 1,25-$(OH)_2$-[^{3}H]D_3, diluted to 4 ml TM buffer, and applied to a 2 ml column of DNA–cellulose. The column was washed sequentially with 4 vol. each of 0.5% Triton X-100 in 10 mM Tris–HCl and TM buffer. Bound 1,25-$(OH)_2$-[^{3}H]D_3 was eluted with a 120 ml linear gradient of 0–0.7 M KCl. Fractions (1.5 ml) were collected and analyzed for KCl (conductivity) and radioactivity.

tamination of preparations with the serum vitamin-D-binding protein. In an attempt to circumvent these complicating factors, we sought to develop a convenient model that provided a homogenous population of intestinal epithelial cells from a mammalian source.

Monolayer cultures of intestinal epithelial cells were established by sequential passage of cells isolated from the duodenum of germ-free adult rats [3]. Light- and electron-microscopy of cultured cells showed the cells to be epitheliod in appearance and to possess microvilli, junctional complexes, and extensive basolateral serosal membrane; all characteristics of transporting epithelium. Extensive immunological identification of cell surface markers demonstrated the cells to be of crypt origin [2]. The cells can also transport amino acid and hexose [12]. Moreover, the cell line is very stable in long-term culture, maintaining morphological and functional stability through 40–50 passages [3].

The potential utility of IEC cells in studying the interaction of 1,25-$(OH)_2$-D_3 with intestine is markedly enhanced by the current demonstration of an intracellular binding protein for 1,25-$(OH)_2$-D_3. The binding protein has sedimentation properties (3.7 S) and a binding affinity for 1,25-$(OH)_2$-[^{3}H]D_3 (K_d = 1.2×10^{-10} M) that are comparable to those described for the cytoplasmic binding proteins isolated from intestinal mucosa of avian and mammalian species. In addition, as demonstrated by the binding of the 1,25-$(OH)_2$-[^{3}H]D_3-binding protein complex to DNA–cellulose, the ligand-'receptor' shows an affinity for DNA that is characteristic of steroid hormones [13]. These homogeneous, morphologically stable, and well-defined cells provide a valuable and convenient means for investigating the biochemical consequences of the binding of 1,25-$(OH)_2$-D_3 to its primary target cell.

Acknowledgements

We thank Ms Karen Batchelder for her secretarial assistance. This work was supported by USPHS AM06604, AM27334 and AM07028.

References

[1] Franceschi, R. T., Simpson, R. U. and DeLuca, H. F. (1981) Arch. Biochem. Biophys. 210, 1–13.
[2] Quaroni, A. and Isselbacher, K. J. (1981) J. Natl. Can. Inst. 67, 1353–1362.
[3] Quaroni, A. and May, R. J. (1980) Methods Cell Biol. 21B, 403–427.
[4] Eisman, J. A., Hamstra, A. J., Kream, B. E. and DeLuca, H. F. (1976) Arch. Biochem. Biophys. 176, 235–243.
[5] Santi, D. V., Sebley, C. H., Perriard, E. R., Tomkins, G. and Baxter, J. D. (1973) Biochemistry 12, 2412–2416.
[6] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[7] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.
[8] Haussler, M. R., Myrtle, J. F. and Norman, A. W. (1968) J. Biol. Chem. 243, 4055–4064.
[9] Brumbaugh, P. F. and Haussler, M. R. (1974) J. Biol. Chem. 249, 1251–1257.
[10] Lawson, D. E. M. and Wilson, P. W. (1974) Biochem. J. 144, 573–583.
[11] Brumbaugh, P. F. and Haussler, M. R. (1975) J. Biol. Chem. 250, 1588–1594.
[12] Inui, K., Quaroni, A., Tillotson, L. G. and Isselbacher, K. J. (1980) Am. J. Physiol. 239, C190–196.
[13] Yamammoto, K. R. and Alberts, B. M. (1976) Annu. Rev. Biochem. 45, 721–745.