Biochemical characterization and purification of a binding protein for 24,25-dihydroxyvitamin D₃ from chick intestine

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Abstract

An earlier study revealed that 24R,25-dihydroxyvitamin D₃ (24R,25(OH)₂D₃) inhibits the rapid actions of 1,25(OH)₂D₃ on stimulation of calcium transport in perfused duodena, as well as activation of protein kinases C and A. In the present work, a specific binding protein (24,25-BP) has been identified and partially characterized. Percoll-gradient resolution of differential centrifugation fractions from mucosal homogenates revealed the highest levels of specific [³H]24R,25(OH)₂D₃ binding to be in lysosomes (approximately eight to tenfold greater than in basal lateral membrane fractions). Incubation of isolated enterocytes with 6·5 nM [³H]24R,25(OH)₂D₃ for 10 s also demonstrated targeting of the steroid to lysosomal fractions. Using freshly isolated lysosomal fractions, time course studies indicated maximal specific binding after a 2-h incubation on ice. Western analyses revealed that the serum transport protein, DBP (vitamin D binding protein), was absent from both lysosomal and basal lateral membrane fractions. Protein dependence studies demonstrated linear binding between 0·05 and 0·155 mg of membrane fractions. Protein dependence studies demonstrated that 24,25(OH)₂D₃ had an EC₅₀ of 7·4 ± 1·8 nM, Bₘₐₓ = 142 ± 16 fmol/mg protein for lysosomes, and Kᵣ = 8·5 nM, Bₘₐₓ = 149 ± 25 fmol/mg protein for basal lateral membranes. Hill analyses of lysosomal binding yielded a Hill coefficient of 0·57 ± 0·11, indicative of negative cooperativity. Studies with lysosomal proteins revealed a 81% ± 7% competition of 24S,25(OH)₂D₃ with [³H]24R,25(OH)₂D₃ for binding (P<0·05, relative to competition with 24R,25(OH)₂D₃), while 25(OH)D₃ and 1,25(OH)₂D₃ yielded 53% ± 13% and 39% ± 11% competition respectively (each, P<0·05, relative to competition with 24R,25(OH)₂D₃). The apparent affinity of 24S,25(OH)₂D₃ for 24,25-BP led to testing of the metabolites effectiveness in the perfused duodenal loop system. Vascular perfusion with 130 pM 1,25(OH)₂D₃ stimulated ⁴⁵Ca transport to 2·5-fold above control levels after 40 min, while simultaneous perfusion with 6·5 nM 24S,25(OH)₂D₃ and 130 pM 1,25(OH)₂D₃ abolished the stimulatory activity completely. Purification of the 24,25-BP by chromatography revealed a single protein band upon SDS-PAGE and silver staining of 66 kDa. The combined results suggest that 24R,25(OH)₂D₃ may mediate its hormonal activities through a specific binding protein.


Introduction

Vitamin D is metabolized in the liver to 25-hydroxyvitamin D₃, and in the kidney to 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃). 1,25(OH)₂D₃ is well known for its hormonal activity in diverse cell types, and its ability to enhance intestinal calcium and phosphate absorption. 24,25(OH)₂D₃ has not been as widely studied, perhaps because of the perception that it is an inactivation product. However, a growing body of literature has indicated that 24,25(OH)₂D₃ has important effects in development (Henry & Norman 1978), bone (Sömjen et al. 1983, Lidor et al. 1987, Schwartz et al. 1988, Swain et al. 1993, Khoury et al. 1995, Yukihiro et al. 1994, Takeuchi & Guggino 1996, Ono et al. 1996, Seo & Norman 1996, 1997, Kato et al. 1998), and parathyroids (Carpenter et al. 1996). More recently (Nemere 1996, 1999), it has been demonstrated that in chick intestine, 24R,25(OH)₂D₃ inhibits the rapid stimulation of calcium transport mediated by 1,25(OH)₂D₃. The ability to block intestinal calcium transport further appears to be an important survival mechanism in marine fish (Sundell & Björnsson 1990, Larsson et al. 1995, Larsson 1999). The ability of 24R,25(OH)₂D₃ to inhibit 1,25(OH)₂D₃-enhanced protein kinases A and C activities in chick intestine (Nemere 1999) also suggests that a specific binding protein may exist for 24,25(OH)₂D₃ that has...
receptor-like properties. The current report describes a candidate protein.

Materials and Methods

Animals

White leghorn cockerels (Merrill Poultry, Poul, ID, USA) were raised for 4–7 weeks on a vitamin D-replete diet (Nemere 1996a). All animal procedures were approved by the Institutional Animal Care and Use Committee.

Subcellular fractionation

On the day of use, chicks were anesthetized, the duodenal loop surgically removed to ice-cold saline and chilled for 15 min. After removal of the pancreas, the segment was slit longitudinally, placed on an inverted Petri dish on ice, and the mucosa collected by scraping with a microscope slide. Scrapings from two duodena were homogenized in 40 ml of 250 mM sucrose, 5 mM histidine–imidazole, 1 mM EGTA, pH 7.0, as previously described (Nemere 1996b). Differential centrifugation yielded pellets P1 (1000 g, 20 min; containing nuclei and brush borders), P2 (20 000 g, 20 min, containing lysosomes, mitochondria, Golgi and basal lateral membranes) and P3 (105 000 g, 1 h, containing microsomes). Fractions P2 and P3 were further resolved on Percoll (Amersham Pharmacia, Piscataway, NJ, USA) gradients (Nemere 1996b), and 18, 52-drop fractions collected. Earlier reports (Nemere et al. 1986, Nemere 1996b) described characterization of the fractions by marker enzyme analyses, fold enrichment, and percent recovery. From the bottom of the gradient, fractions 1–3 were identified as lysosomes, fractions 4–13 as mitochondria, fractions 14–15 Golgi, and fractions 16–18 as basal lateral membranes (Nemere et al. 1986, Nemere 1996b). In the current work protein was determined with the Bradford reagent (BioRad, Hercules, CA, USA) against bovine gamma globulin as standard (Sigma Chemical Co., St Louis, MO, USA).

Binding of [3H]24R,25(OH)2D3 to isolated fractions

For each sample, five tubes were prepared to contain [3H]24R,25(OH)2D3 (76 Ci/mmol) in the absence (total binding, three tubes) or presence of a 200-fold molar excess of unlabeled competitor (nonspecific binding, two tubes) in 20 μl of ethanol. Each tube then received TED buffer (10 mM Tris, 1.5 mM EDTA, 2 mM dithiothreitol, pH 7.4) and sample. Incubations were conducted on ice and bound and free metabolites separated by hydroxylapatite (HAP) assay (Nemere et al. 1994). The radioactivity extracted from the final HAP pellet was dried in glass liquid scintillation vials. Six milliliters of scintillation cocktail were then added and the sample counted in a Beckman LS6500 counter. For saturation analyses specific 24R,25(OH)2D3 binding to membranes was tested by nonlinear regression. The concentration of the labeled hormone was plotted against the amount of specifically bound, labeled hormone (fmol/mg protein), and the data were fitted to a three-parameter sigmoid function:

\[ f(x) = a/(1 + e^{-(x - x_0)/b}) \]

The coefficient of variation (R2) and the adjusted coefficient of variation (adjR2) were used as a measure of how well the regression model described the data in the saturation and Hill analyses (Altman 1991). A one-way ANOVA with F statistics was used to gauge the contribution of the independent variable to predict the dependent variable (Altman 1991). A P<0.05 was considered as statistically significant.

A one-way ANOVA followed by a Student–Newman–Keuls post hoc test was used to test for significant differences between groups in the metabolite competition studies. The testing was two-tailed and the significance level was set to P<0.05. Data are presented as means ± s.e.m.

[3H]24R,25(OH)2D3 uptake by isolated intestinal cells

After removal of the pancreas from each of two duodenal loops, segments were everted, rinsed in 0.9% saline and then placed in citrate chelation solution (Nemere & Campbell 2000). Cells from the first two 15-min incubations were pooled into two 50 ml polypropylene tubes and collected by centrifugation (500 g, 5 min, 4 °C). The supernatants were decanted, and while still in the inverted position, the insides of the tubes were swabbed with a Kimwipe. The pellets were gently resuspended with a Teflon rod in 10 ml of Gey’s Balanced Salt Solution (GBSS) containing 0.1% BSA. The cell suspension was then rapidly combined with an equivalent volume of GBSS–BSA containing [3H]24R,25(OH)2D3 to give a final concentration of 6.5 nM steroid. The cells were swirled for 10 s and then collected by low speed centrifugation for homogenization and subcellular fractionation as described above.

Western analyses

SDS-PAGE was performed and proteins blotted onto PVDF membranes as described elsewhere (Jia & Nemere 1999, Nemere et al. 2000). Human vitamin D binding protein (DBP) was purchased from Sigma and goat anti-DBP from Incstar Corp. (Stillwater, MN, USA) and used at a dilution of 1/500. Alkaline phosphatase conjugated rabbit anti-goat antibody (1/30 000 dilution) was from Sigma.

Perfusion of duodenal loops

Procedures were as described (Nemere 1996a) for vascular perfusion of three duodenal loops with control medium during the treated phase, three with 130 pM 1,25(OH)2D3,
and three with 130 pM 1,25(OH)₂D₃ plus 6.5 nM 24S,25(OH)₂D₃ during the treated phase.

**Protein purification**

Lysoosomal fractions were collected and frozen. They were subsequently homogenized in ten volumes of cold TED buffer (25 strokes on ice) and Percoll removed by centrifugation. The supernatant fractions were passed through 0.45 μm syringe filters and then concentrated in Centriplus spin columns (Amicon, Beverly, MA, USA) with a 50 kDa pore size. Both the filtrate and retentate were tested for specific [³H]24,25(OH)₂D₃ binding. Protease inhibitors (Sigma) were added and the retentates stored at –20°C. Concentrated aliquots of protein were injected onto a Mono Q HR5 anion exchange column (Amersham Pharmacia) with a 1 ml loop. Chromatography was accomplished using the Pharmacia FPLC system with a flow rate of 1 ml/min. The column was washed for 7 min with buffer A (20 mM Tris, 1 mM EDTA, pH 8.0) and then eluted with a linear gradient formed with buffer B (20 mM Tris, 1 mM EDTA, 1 M NaCl, pH 8.0). The column was then washed an additional 5 min with high salt buffer before re-equilibration in starting buffer. Aliquots of each fraction were assayed for specific binding. Those highest in specific binding were pooled, concentrated in spin columns and subjected to gel exclusion chromatography on a Superose 12 column (Amersham Pharmacia). After filtration, sample was injected with a 200 μl loop and the column eluted (0.5 ml/min) with 150 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0. Aliquots (20 μl) were taken of each sample for determination of specific [³H]24,25(OH)₂D₃ binding and SDS-PAGE followed by silver staining (Nemere *et al.* 1994).

**Results**

**Binding of [³H]24R,25(OH)₂D₃ to isolated fractions**

Mucosal scrapings were homogenized, subjected to differential centrifugation and the particulate fractions analyzed for specific [³H]24R,25(OH)₂D₃ binding. Expressed as average fmol/mg protein ± range for whole homogenates, P₁, P₂ and P₃, values were 96 ± 17, 152 ± 33, 237 ± 33 and 239 ± 63 respectively. Pellet fractions P₂ or P₃ were subsequently further resolved on Percoll gradients and analyzed for specific [³H]24R,25(OH)₂D₃ binding. Figure 1 illustrates the results of replicate experiments in which a marked enrichment of specific binding was observed in lysosomal fractions 1–2 of P₂ Percoll gradients, while no fractions in P₃ exhibited a similar level of enrichment.

[³H]24R,25(OH)₂D₃ uptake by isolated intestinal cells

The results of binding studies with subcellular fractions suggested incubation of intact isolated intestinal cells with 6.5 nM [³H]24R,25(OH)₂D₃, to determine if the cellular sorting machinery directed the steroid to the same compartment. As shown in Fig. 2, a 10-s exposure to 6.5 nM radioactive seco-steroid (final concentration), followed by subcellular fractionation protocols, resulted in the highest levels of total [³H]24R,25(OH)₂D₃ localized in lysosomal fractions 1 and 2. Nonspecific binding could not be assessed in these experiments, since the level of unlabeled steroid would be supra-physiological.

One interpretation of the subcellular localization is that the binding activity resulted from the serum DBP adhering to the plasma membrane of the intestinal cells,
which upon binding of ligand, became internalized and sequestered in lysosomes. To assess such a possibility, authentic human DBP, lysosomal proteins and basal lateral proteins were separated on SDS-polyacrylamide gels for either Coomassie Blue staining, or for Western analyses. The composite shown in Fig. 3 demonstrates adequate protein staining of $1.5 \mu g$ of DBP (giving a single band), $100 \mu g$ of lysosomal protein, and $80 \mu g$ of basal lateral membrane protein. Western analyses of equivalent levels of DBP resulted in labeling of several bands (Fig. 3), one corresponding to the DBP protein band, while the smaller bands may have been due to nonspecific binding of secondary antibody to glycated contaminants. Lower levels ($15–60 \mu g$) of lysosomal or basal lateral proteins failed to give reaction product (data not shown), nor did $80–100 \mu g$ of protein (Fig. 3) at the molecular mass corresponding to DBP. Since the specificity of the commercially purchased antibody is not known, reaction product at lower molecular masses could be recognition of degradation products or cross-reactivity with related proteins/glycoproteins.

**Characterization of the lysosomal binding activity for $[3H]24R,25(OH)_{2}D_{3}$**

To assess certain properties of the lysosomal protein, time course studies and protein dependence of binding were undertaken. Incubation of $50 \mu g$ of lysosomal protein with $16 \text{nM} [3H]24R,25(OH)_{2}D_{3}$, in the absence or presence of excess unlabeled steroid ($0–4 ^{\circ}C$), produced an increase in specific binding between 0.5 and 2 h (data not shown). Increasing the duration of incubation did not result in further specific binding (data not shown). Using the 2-h incubation time, three levels of protein, 50, 100 and $155 \mu g$ in $0.2 \text{mL}$ final volume, were tested for specific binding. A linear increase ($P<0.05$; $f(\chi)=0.2303 \pm 137.5X$; $R^2=0.96$; adj$R^2=0.94$; $F=49.73$; d.f.=3) in specific $[3H]24R,25(OH)_{2}D_{3}$ binding was observed between 50 and $155 \mu g$ of protein.

Saturation analyses were conducted with both basal lateral membranes (Fig. 4A) and lysosomal fractions (Fig. 4B–D). Using $50 \mu g$ of protein per tube, samples were incubated with increasing concentrations of $[3H]24R,25(OH)_{2}D_{3}$ in the absence or presence of a 200-fold molar excess of unlabeled seco-steroid. Under these conditions, binding parameters for basal lateral
Figure 4  Saturation analyses of specific \([^{3}H]24\text{R,25(OH)}_2\text{D}_3\) binding to basal lateral membrane and lysosomal fractions. Isolated membranes or lysosomes were removed from Percoll as described in the legend to Fig. 3. Each concentration of steroid was tested (50 \(\mu\)g of protein per tube; 2 h, 0 °C) for total binding in triplicate with the indicated amount of labeled steroid, and in duplicate for nonspecific binding in the presence of a 200-fold molar excess of unlabeled steroid. Bound and free steroid were separated by HAP. Values represent means ± S.E.M.s for three independent experiments. (A) Saturation binding to basal lateral membranes; (B) saturation binding to lysosomal fractions; (C) Scatchard analyses of data from (B); (D) Hill analyses of data from (B). \(S_b\): specifically bound hormone; \(S_f\): free hormone.
Perfusion studies

The ability of 24S,25(OH)₂D₃ to compete efficiently with [³H]24R,25(OH)₂D₃ for binding to a lysosomal protein was further tested in terms of physiological significance in the perfused duodenal loop system. Figure 6 depicts the results of duodena that were either vascularly perfused with control medium, 130 pM 1,25(OH)₂D₃ or 130 pM 1,25(OH)₂D₃ plus 6·5 nM 24S,25(OH)₂D₃. While 1,25(OH)₂D₃ stimulated calcium transport to levels that were 2-5-fold above controls, inclusion of 24S,25(OH)₂D₃ in the vascular perfusate abolished the stimulatory effect of 1,25(OH)₂D₃. These results implied a receptor-like function for the 24S,25(OH)₂D₃ binding protein.

Protein purification

Optimal conditions for the extraction of the binding protein from lysosomal fractions were assessed by comparing homogenization with media containing detergent (10 mM CHAPSO, final concentration) and hypotonic media (TED buffer), followed by centrifugation at 160 000 × g, 1 h. Since equivalent specific binding activity was extracted by either method, detergent was subsequently omitted. Elution of the crude lysosomal extract from an anion exchange column yielded the chromatogram shown in Fig. 7A. The fractions highest in specific [³H]24R,25(OH)₂D₃ binding (21–33) were pooled and concentrated. Analyses of content by SDS-PAGE and silver staining revealed numerous bands, necessitating a
further purification step on gel exclusion chromatography (Fig. 7B). Binding activity was concentrated in fractions 28–30, corresponding to a molecular mass of approximately 60 000. A representative silver-stained gel is shown in Fig. 7C. The protein consistently present in active fractions corresponded to the 66 kDa band.

**Discussion**

Analyses of enterocyte subcellular organelles indicates that a specific binding protein for 24,25(OH)₂D₃ is enriched in lysosomal fractions, when binding is determined by the HAP method for separation of bound and free steroid. The lysosomal enrichment exceeds that of binding in basal lateral membrane fractions (Nemere et al. 1994, Fig. 1 of the current work), although affinity and sites/mg protein are similar (Fig. 4A and B). Isolated intestinal cells incubated with [³H]24R,25(OH)₂D₃ also sequestered the highest levels of seco-steroid in lysosomal fractions. Thus, both the unliganded and liganded forms of the 24R,25(OH)₂D₃ binding protein (24,25-BP) are localized in the same vesicles that are strong candidates as transporters of calcium and phosphate across the intestinal cell (Nemere et al. 1986, Nemere 1996b). Indeed, in mucosae from duodenal loops fractionated after vascular perfusion with 1,25(OH)₂D₃ and 24R,25(OH)₂D₃, lysosomal phosphate increases from the luminal media are blocked relative to equivalent preparations from tissue vascularly exposed to 1,25(OH)₂D₃ alone (Nemere 1996b). Thus, two possible explanations for the inhibitory actions of 24R,25(OH)₂D₃ on ion transport are (i) a direct effect of the liganded 24,25-BP on the lysosomal ATPase identified as the enzyme for loading the organelles with phosphate (Pisoni & Lindley 1992), and/or (ii) blocking of signal transduction pathways stimulated by 1,25(OH)₂D₃ (Nemere 1999).

A number of considerations indicate that the putative membrane receptor for 1,25(OH)₂D₃ and the 24,25-BP are different molecular entities. Neither protein exhibits substantial levels of competition for binding with heterologous ligand (Nemere et al. 1994, Fig. 5 in the current work). While the putative receptor for 1,25(OH)₂D₃ is
located largely in the basolateral/endoplasmic reticulum (Golgi) fractions (Nemere et al. 1994, 2000), the 24,25-BP is enriched in lysosomes when equivalent subcellular fractionation protocols are used. Electron microscopy of thin sections labeled with Ab099 directed against the putative membrane receptor for 1,25(OH)2D3 indicates that exposure of intestinal tissue to 1,25(OH)2D3, but not 24R,25(OH)2D3, results in apparent translocation of the antigen to the nucleus. In contrast, both liganded and unliganded forms of the 24,25-BP appear to be in lysosomes (Figs 1 and 2). A definitive relationship or lack of homology between the two proteins will be provided by N-terminal microsequencing of the 24,25-BP for comparison with the sequence obtained from the putative 1,25(OH)2D3 membrane receptor.

The enrichment of the 24,25-BP in lysosomal fractions suggested that the protein might be the serum transporter DBP, which through endocytosis, became sequestered in vesicles. However, Western analyses with antibody against authentic DBP failed to detect a reaction product in either lysosomal or basolateral membrane fractions. The possibility remains that the 24,25-BP may have structural homology to the intracellular DBP characterized in New World primate cells (Gacad et al. 1997, Wu et al. 2000), although competition studies indicate a greater affinity for 24R,25(OH)2D3 over 25(OH)2D3. Work is in progress to obtain the sequence of the 24,25-BP which will provide definitive data regarding potential similarities to the 25(OH)D3 binding protein; the latter protein has been shown to be related to heat shock proteins (Gacad et al. 1997, Wu et al. 2000).

Competition studies also indicate that the 24S,25(OH)2D3 isomer has a high affinity for the lysosomal 24,25-BP. Since both isomers exist naturally (Ishizuka et al. 1984), this result is not unexpected. Nor is the finding that 24S,25(OH)2D3 is effective in inhibiting 1,25(OH)2D3-stimulated calcium transport in the perfused duodenal system, since the physiological activity agrees with the competitive binding data.

The binding of [3H]24R,25(OH)2D3 to lysosomal and basolateral membranes showed no difference in affinity or number of receptors/mg protein. This suggests that the 24R,25(OH)2D3 binding proteins in lysosomal and basolateral membranes are of a common origin. However, one difference between the lysosomal and basal lateral binding proteins is in analyses of cooperativity. In basolateral membranes, binding exhibits positive cooperativity (Larsson et al. 1999, 2001), while in lysosomal fractions binding exhibits negative cooperativity. One explanation for these observations might be that the binding proteins in the two subcellular compartments have different naturally occurring ligands, resulting in differences in cooperative binding. Efforts are currently underway to purify sufficient quantities of these two 24,25-BP for sequencing, which in turn may yield additional information about the observed differences in cooperativity.

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