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Binding Characteristics of a Membrane Receptor That Recognizes 1α,25-Dihydroxyvitamin D_3 and Its Epimer, 1β,25-Dihydroxyvitamin D_3

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Abstract The steroid hormone 1α,25-dihydroxyvitamin D_3 has been shown to exert rapid effects (15 s to 5 min) in osteoblasts. These effects occur in osteoblast-like cells lacking the nuclear vitamin D receptor, ROS 24/1, suggesting that a separate signalling system mediates the rapid actions. These non-genomic actions include rapid activation of phospholipase C and opening of calcium channels, pointing to a membrane localization of this signalling system. Previous studies have shown that the 1β epimer of 1α,25-dihydroxyvitmina D_3 can block these rapid actions, indicating that the 1β epimer may bind to the receptor responsible for the rapid actions in a competitive manner. We have assessed the displacement of ^{3}H-1α,25-dihydroxyvitamin D_3 by vitamin D compounds, as well as the apparent dissociation constant of 1α,25-dihydroxyvitamin D_3 and its 1β epimer for the membrane receptor in membrane preparations from ROS 24/1 cells. Increasing concentrations of 1α,25-dihydroxyvitamin D_3, 7.25 nM to 725 nM, displaced ^{3}H-1α,25-dihydroxyvitamin D_3 from the membranes with 725 nM of the hormone displacing 40–49% of the radioactivity. Similarly, 1β,25-dihydroxyvitamin D_3, 7.25 nM and 72.5 nM, displaced 1α,25-dihydroxyvitamin D_3 binding while 25-hydroxyvitamin D_3, 72.5 nM and 725 nM, did not. The apparent dissociation constant (K_D) for 1α,25-dihydroxyvitamin D_3 was determined from displacement of ^{3}H-1α,25-dihydroxyvitamin D_3 yielding a value of 8.1×10^{-7} M by Scatchard analysis. The K_D for the 1β epimer determined from displacement of ^{3}H-1β,25-dihydroxyvitamin D_3 was 4.8×10^{-7} M. The data suggest the presence of a receptor on the membranes of ROS 24/1 cells that recognizes 1α,25-dihydroxyvitamin D_3 and its 1β epimer, but not 25-hydroxyvitamin D_3. Its ability to recognize the 1β epimer which appears to be a specific antagonist of the rapid effects of the hormone suggests that these studies may be the initial steps in the isolation and characterization of the signalling system mediating the rapid actions of vitamin D.

Key words: osteoblast, ROS 24/1, receptor, calcium channels, vitamin D

1α,25-Dihydroxyvitamin D_3 exerts rapid effects in a variety of cell types. These effects have been most clearly delineated in osteoblasts where the hormone has been shown to increase intracellular [Lieberherr, 1987; Oshima et al., 1987; Civitelli et al., 1990; Baran et al., 1991] and nuclear [Sorensen et al., 1993a] calcium, calcium uptake [Kim et al., 1987; Caffrey and Farach-Carson, 1989; Farach-Carson et al., 1991; Norman et al., 1993], phospholipase C activity [Civitelli et al., 1990; Grosse et al., 1993; Sorensen et al., 1993b], and intracellular pH [Jenis et al., 1993]. These actions appear to be mediated by a signalling system distinct from interaction with the nuclear vitamin D receptor since 1α,25-dihydroxyvitamin D_3 rapidly increases intracellular calcium in a clonal rat osteosarcoma cell line lacking the vitamin D receptor [Baran et al., 1991]. 1β,25-Dihydroxyvitamin D_3, the epimer of 1α,25-dihydroxyvitamin D_3 inhibits the rapid effects of 1α,25-dihydroxyvitamin D_3 on intracellular calcium [Baran et al., 1991], calcium uptake [Norman et al., 1993], and intracellular pH [Jenis et al., 1993]. The 1β epimer binds to the nuclear receptor with very low affinity [Holick et al., 1980] and does not alter the binding of the vitamin D receptor complex to the vitamin D responsive elements [Baran et al., 1992]. The

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ability of 1β,25-dihydroxyvitamin D_3 to inhibit the rapid actions of 1α,25-dihydroxyvitamin D_3 without affecting the interaction of the hormone with its nuclear receptor is further evidence for a separate signalling system mediating the rapid actions.

The rapid effects of 1α,25-dihydroxyvitamin D_3 in osteoblasts appear to have a functional significance. Inhibition of the rapid actions with 1β,25-dihydroxyvitamin D_3 prevents the hormone-induced increments in osteocalcin mRNA transcription at 1 h and steady-state levels at 3 h [Baran et al., 1992]. However, rapid changes in intracellular calcium per se do not appear to be the sole signal for modulation of expression of these genes since substitution of extracellular sodium with choline prevents 1α,25-dihydroxyvitamin D_3-induced increases in intracellular pH and osteocalcin and osteopontin mRNA levels, without affecting increments in intracellular calcium [Jenis et al., 1993].

To begin to define the events coupling the rapid actions of vitamin D to genomic expression, we examined the binding characteristics of 1α,25-dihydroxyvitamin D_3 to membranes prepared from ROS 24/1 cells, a clonal osteosarcoma cell line that lacks the nuclear vitamin D receptor. Since 1α,25-dihydroxyvitamin D_3 has been shown to have very rapid effects on membrane phospholipids [Civitelli et al., 1990; Grosse et al., 1993], and membrane calcium channels [Kim et al., 1987; Caffrey et al., 1989; Farach-Carson et al., 1991; Norman et al., 1993], it follows that the hormone may interact with a membrane signalling system. The results of this study indicate that the binding of ^{3}H-1α,25-dihydroxyvitamin D_3 to membranes of ROS 24/1 cells is prevented by increasing concentrations of 1α,25-dihydroxyvitamin D_3 and also 1β,25-dihydroxyvitamin D_3, but not by 25-hydroxyvitamin D_3. The binding of ^{3}H-1α,25-dihydroxyvitamin D_3 to the membranes has a calculated K_D of 8.1×10^{-7} M, similar to that of ^{3}H-1β,25-dihydroxyvitamin D_3, 4.8×10^{-7} M. The analysis of the properties of a membrane receptor that recognizes both 1α,25-dihydroxyvitamin D_3 and its 1β epimer may be a first step in the characterization of the signalling system that mediates the rapid actions of the hormone.

METHODS

Cell Cultures

Osteoblast-like rat osteosarcoma cells, ROS 24/1, were grown in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) and F12 (50:50) plus 5% fetal calf serum.

Cells were grown for 6–7 days and harvested for experiments by trypsinization with 0.25% trypsin and 0.002% EDTA and by sedimentation at 200*g* for 8 min. Cell numbers were assessed by counting an aliquot of cells in a hemocytometer, and viability was determined by trypan dye exclusion.

Isolation of Membranes

Membranes were isolated as previously described [Wehling et al., 1992]. ROS 24/1 were suspended in a buffer containing Tricine 20 mM, $CaCl_2$ 2 mM, and $MgCl_2$ 1 mM, pH 7.9, 10^8 cells/ml buffer, and homogenized with 15 strokes in a tight fitting Dounce homogenizer. The mixture was centrifuged for 8 min at 650*g* to remove debris and nuclei. The supernatant was centrifuged for 60 min at 100,000*g* to yield a crude membrane pellet. The pellet was resuspended in a HEPES buffer, pH 7.4, to a final protein concentration of 3–4 mg/ml determined by the Pierce BCA protein assay (Pierce, Rockford, IL). This preparation contained microsomes and mitochondria as well as the membranes, but no intact cells or nuclei on light microscopy.

Displacement Studies

Displacement studies were conducted in a total volume of 135 μl (125 μl membrane in HEPES, 5 μl ^{3}H-1α,25-dihydroxyvitamin D_3, 5 μl Ethanol, or unlabeled vitamin D compounds). Binding of 1α,25-dihydroxy [^{3}H-26,27] vitamin D_3, 165 Ci/mmol, (New England Nuclear, Boston, MA) to the membranes of ROS 24/1 cells was determined in the absence and presence of 1α,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 (courtesy Dr. M. Uskovic, Hoffman-LaRoche, Nutley, NJ), and 1β,25-dihydroxyvitamin D_3 at 4°C. After 90 min, 50 μl aliquots of the membrane suspension were filtered at 4°C through DEAE cellulose filter paper discs (DE81, 2.4 cm, Whatman Co., Maidstone, England) and washed five times with 1 ml of phosphate-buffered saline. The membranes were placed in 7 ml Optiflour (Packard Instrument B.V., The Netherlands) overnight before quantitating trapped radioactivity.

Binding Studies

1α,25-Dihydroxy [1β-^{3}H] vitamin D_3, 15 Ci/mmol, and 1β,25-dihydroxy [1α-^{3}H] vitamin D_3,

15 Ci/mmol synthesized as previously described [Holick et al., 1980], were incubated for 90 min on ice in 125 μl of membrane (3–4 mg/ml) in the presence of increasing concentrations of 1α,25-dihydroxyvitamin D_3 and 1β,25-dihydroxyvitamin D_3. After 90 min, 50 μl aliquots were filtered as described above and trapped radioactivity counted. Nonspecific radioactivity was assessed in the presence of 100 fold excess of cold ligand.

Statistics

For the displacement studies, probability of difference was determined by Duncan's test for multiple comparisons. For the Scatchard analyses, the best fit line was determined by linear regression.

RESULTS

The binding of ^{3}H-1α,25-dihydroxyvitamin D_3 to the membranes of ROS 24/1 cells reached a plateau by 20 min and remained at that level for 90 min (Fig. 1). The binding of the ^{3}H-1α,25-dihydroxyvitamin D_3 was diminished by increasing concentrations of 1α,25-dihydroxyvitamin D_3 (Fig. 2). At a concentration of 725 nM, 1α,25-dihydroxyvitamin D_3 decreased the binding of the radioactive hormone by 49%. Binding of the ^{3}H-1α,25-dihydroxyvitamin D_3 was also diminished by increasing concentrations of 1β,25-dihydroxyvitamin D_3 (Fig. 3). 1β,25-dihydroxyvitamin D_3 72.5 nM, decreased the binding of ^{3}H-1α,25-dihydroxyvitamin D_3 by 38%. The inhibition of ^{3}H-1α,25-dihydroxyvitamin D_3 binding to the plasma membrane by 1α,25-dihydroxyvitamin D_3 and 1β,25-dihydroxyvitamin D_3 was relatively specific since 25-hydroxyvitamin D_3 at 72.5 nM and 725 nM failed to diminish binding (Fig. 4). In the same experiment, 1α,25-dihydroxyvitamin D_3, 725 nM decreased the binding of ^{3}H-1α,25-dihydroxyvitamin D_3 by 40%.

The binding constant for 1α,25-dihydroxyvitamin D_3 to the membranes was estimated by displacement curves for the tritiated hormone by increasing concentrations of unlabelled 1α,25-dihydroxyvitamin D_3 (Fig. 5) or 1β,25-dihydroxyvitamin D_3 (Fig. 6). The binding of ^{3}H-1α,25-dihydroxyvitamin D_3 to the membranes had a calculated K_D of 8.1×10^{-7} M in the presence of 1α,25-dihydroxyvitamin D_3 (Fig. 5) and a calculated K_D of 1.6×10^{-7} M in the presence of 1β,25-dihydroxyvitamin D_3 (Fig. 6). Likewise, the binding of ^{3}H-1β,25-dihydroxyvitamin D_3 to the membranes had a K_D of 3.2×10^{-7} M in the presence of 1α,25-dihydroxyvitamin D_3 (Fig. 7) and a K_D of 4.8×10^{-7} M in the presence of 1β,25-dihydroxyvitamin D_3 (Fig. 8).

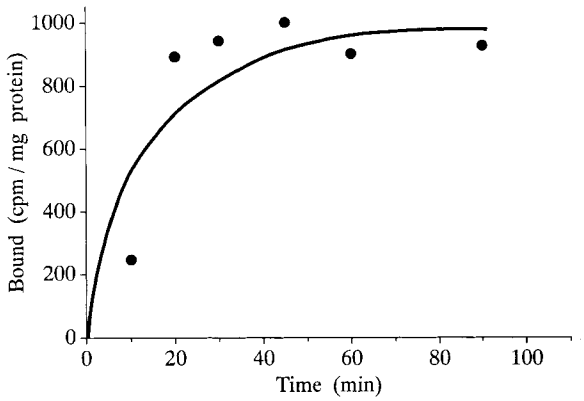

Fig. 1. Binding of ^{3}H-1α,25-dihydroxyvitamin D_3 to the membrane of ROS 24/1 cells. Membranes (3–4 mg protein/ml) were incubated on ice for the designated times with ^{3}H-1α,25-dihydroxyvitamin D_3 165 Ci/mmol. Values represent the mean of four observations at each time point.

DISCUSSION

The results of this study demonstrate that membranes of ROS 24/1 cells specifically bind both 1α,25-dihydroxyvitamin D_3 and 1β,25-dihydroxyvitamin D_3 and that the epimers interact with a common site. This binding activity cannot be due to the classical nuclear vitamin D receptor since these cells lack the mRNA for the nuclear vitamin D receptor [Baran et al., 1991] and 1β,25-dihydroxyvitamin D_3 does not bind to the nuclear vitamin D receptor [Holick et al., 1980; Baran et al., 1992]. This membrane binding activity appears to recognize the hydroxyl group at the C_1 position, since 25-hydroxyvitamin D_3 is unable to displace ^{3}H-1α,25-dihydroxyvitamin D_3 from the membrane (Fig. 4). The binding affinities of both epimers were similar (Figs. 5, 8) indicating that the binding activity did not distinguish the axial from equatorial configurations of the hydroxyl group at the C_1 position.

The interaction of the nuclear vitamin D receptor with 1α,25-dihydroxyvitamin D_3 is characterized by an equilibrium dissociation constant of 10^{-10} M at 4°C [Mellon et al., 1979], although studies with pure protein have yet to be carried out [Pike, 1991]. The equilibrium dissociation constant for membrane binding was several orders of magnitude less, approximately 8×10^{-7} M (Fig. 5).

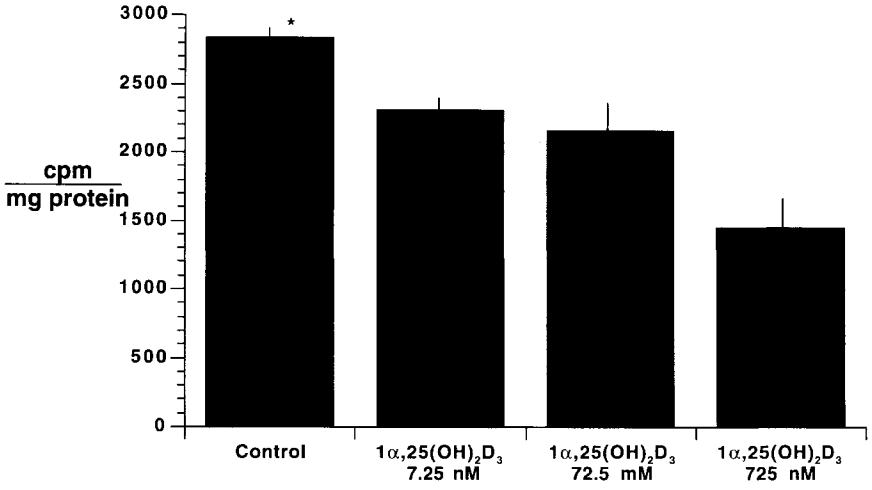

Fig. 2. Displacement of ^{3}H-1α,25-dihydroxyvitamin D_3 from membranes of ROS 24/1 cells by 1α,25-dihydroxyvitamin D_3. Membranes (3–4 mg/ml protein) were incubated on ice with ^{3}H-1α,25-dihydroxyvitamin D_3, 165 Ci/mmol in the presence or absence of increasing concentration of 1α,25-dihydroxyvitamin D_3. At 90 min, 50 μl aliquots were filtered and trapped radioactivity determined. Values represent the mean ± SD of 3–4 observations in each group. Probability of difference was determined by Duncan's test for multiple comparisons.

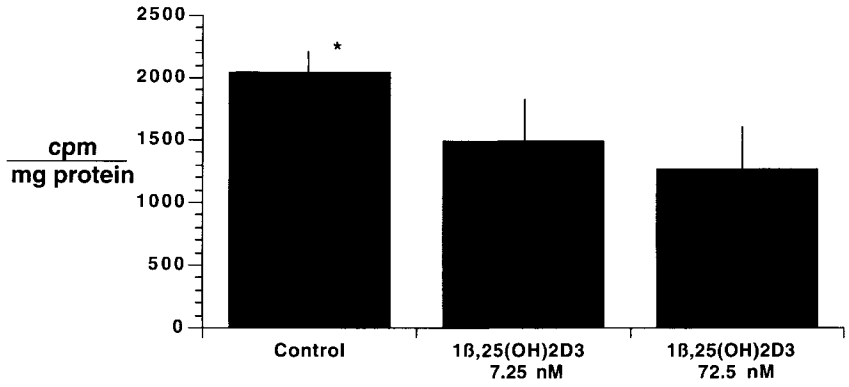

Fig. 3. Displacement of ^{3}H-1α,25-dihydroxyvitamin D_3 from membranes of ROS 24/1 cells by 1β,25-dihydroxyvitamin D_3. Membranes (3–4 mg protein/ml) were incubated on ice with ^{3}H-1α,25-dihydroxyvitamin D_3 165 Ci/mmol in the presence or absence of 1β,25-dihydroxyvitamin D_3. At 90 min, 50 μl aliquots were filtered and trapped radioactivity counted. Values represent the mean ± SD of 5–6 observations in each group. Probability of difference was determined by Duncan's test for multiple comparisons.

The affinity of hormone binding to membranes is less than the concentrations of the 1α,25-dihydroxyvitamin D_3 which have been shown to produce rapid effects in ROS 24/1 cells. The hormone at a concentration of 200 pM increases intracellular calcium by 70% in ROS 24/1 cells [Baran et al., 1991], while individual ROS 24/1 cells respond to 1α,25-dihydroxyvitamin D_3, 10 nM, with an increase in Fura 2 fluorescence [Civitelli et al., 1990].

The discrepancy between the calculated binding constant and biological activity argues against the possibility that the binding activity reflects interaction with the "signalling system" which initiates the rapid actions of the hormone. On the other hand, the rapid actions of 1α,25-dihydroxyvitamin D_3 are blocked by the 1β epimer [Baran et al., 1991; Norman et al., 1993; Jenis et al., 1993], suggesting that it may bind competitively to the signalling system. The

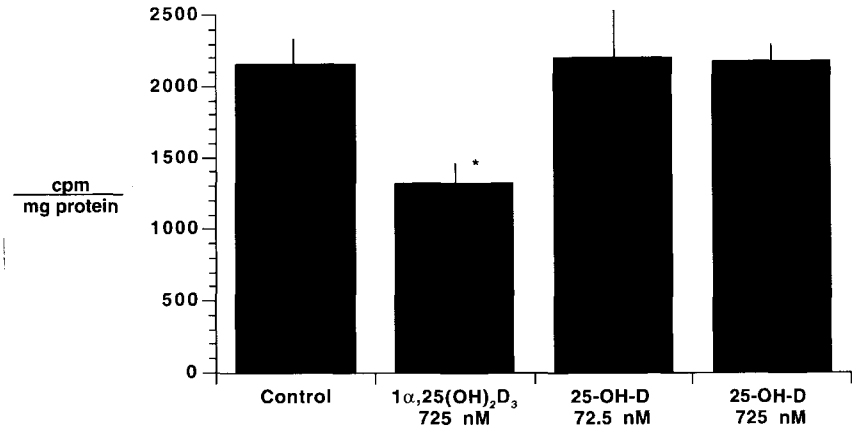

Fig. 4. Specificity of inhibition of binding of ^{3}H-1α,25-dihydroxyvitamin D_3 to membranes of ROS 24/1 cells. Membranes (3–4 mg protein/ml) were incubated on ice with ^{3}H-1α,25-dihydroxyvitamin D_3, 165 Ci/mmol, with either 1α,25-dihydroxyvitamin D_3 or 25 hydroxyvitamin D_3. At 90 min, 50 μl aliquots were filtered and trapped radioactivity counted. Values represent the mean ± SD of four observations in each group. Probability of difference was determined by Duncan's test for multiple comparisons.

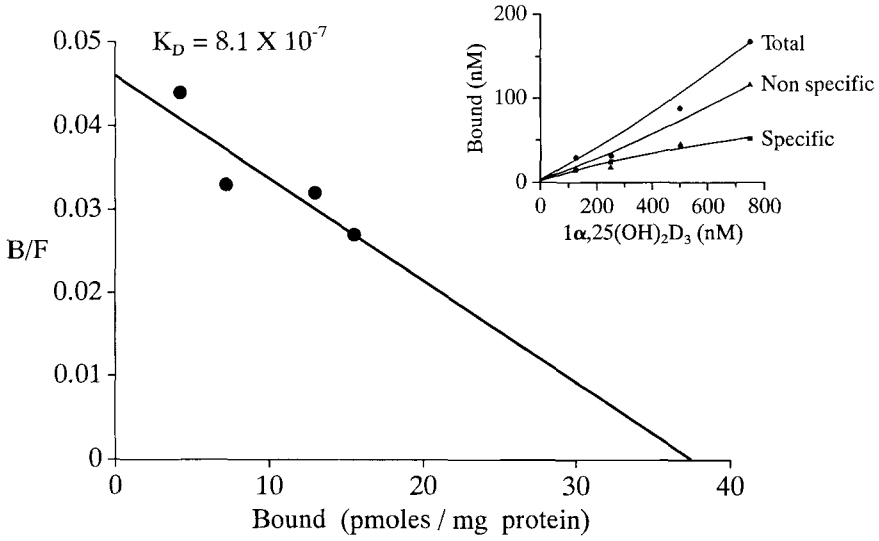

Fig. 5. Binding characteristics of ^{3}H-1α,25-dihydroxyvitamin D_3 15 Ci/mmol, to membranes of ROS 24/1 cells in the presence of 1α,25-dihydroxyvitamin D_3. Nonspecific binding was determined in the presence of 100-fold excess of 1α,25-dihydroxyvitamin D_3, 75 μM. Each value for the binding curve represents the mean of two observations.

finding that the 1β epimer will displace 1α,25-dihydroxyvitamin D_3 (Figs. 2, 6) is consistent with the possibility that the binding activity reflects the binding activity of the receptor that mediates the rapid effects.

The apparent K_D for binding to membranes may be influenced by many factors. Clearly, it is dependent upon knowledge of the precise concentrations of free hormone. Because vitamin D is fat soluble and there is fat in the membrane preparation, the free monomeric concentration of the vitamin D analogs may be much lower than that assumed for K_D calculation. This would lead to a much lower calculated affinity. In addition, disruption of the cell may affect proteins and lipids associated with the signalling system. These factors may influence the ligand binding properties of this putative receptor.

The presence of membrane receptors for other steroid hormones has been reported. Aldosterone, 70 pM, rapidly stimulates the membrane system for sodium transport in human mono-

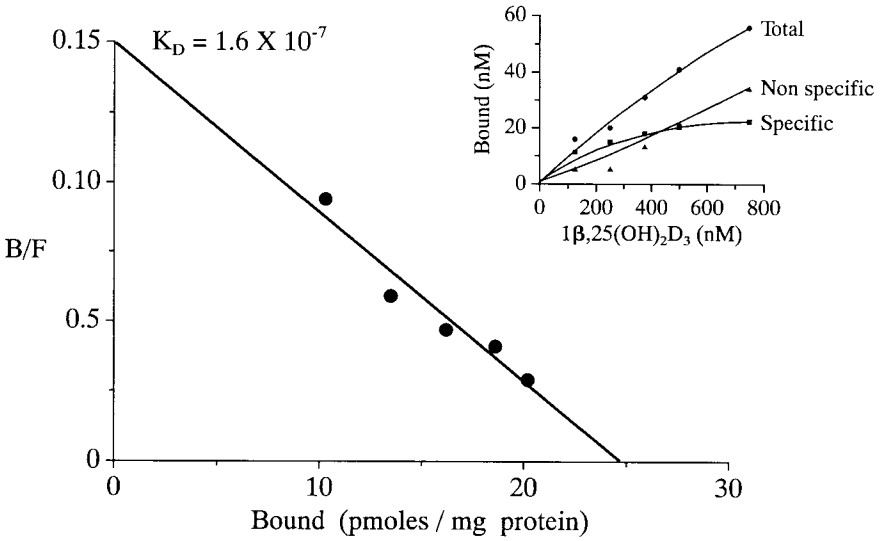

Fig. 6. Binding characteristics of ^{3}H-1α,25-dihydroxyvitamin D_3 15 Ci/mmol to membranes of ROS 24/1 cells in the presence of 1β,25-dihydroxyvitamin D_3. Nonspecific binding was determined in the presence of 100-fold excess of 1β,25-dihydroxyvitamin D_3, 75 μM. Each value for the binding curve represents the mean of two observations.

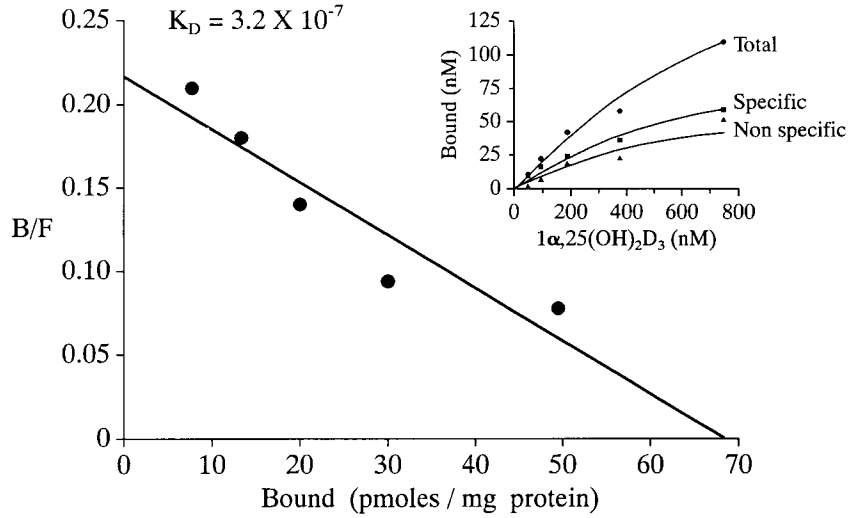

Fig. 7. Binding characteristics of ^{3}H-1β,25-dihydroxyvitamin D_3 15 Ci/mmol to membranes of ROS 24/1 cells in the presence of 1α,25-dihydroxyvitamin D_3. Nonspecific binding was determined in the presence of 100-fold excess of 1α,25-dihydroxyvitamin D_3, 75 μM. Each value for the binding curve represents the mean of two observations.

nuclear leukocytes [Wehling et al., 1991]. Iodinated aldosterone binds to the membranes of these cells with an apparent dissociation constant of 10^{-10} M [Wehling et al., 1992]. Therefore, despite being fat soluble, aldosterone binds to its membrane receptor with a K_D that is 100-fold greater than that observed for the binding of 1α,25-dihydroxyvitamin D_3 to the membranes of ROS cells. A portion of that difference may be explained by the ability to iodinate aldosterone with a resultant specific activity of 2,000 Ci/mmol. Our studies do not exclude 1α,25-dihydroxyvitamin D_3 binding to membranes with high affinity since relatively low specific activity of ^{3}H ligands (15 Ci/mmole) precludes demonstration of high affinity binding unless a relatively large numbers of receptors are present. Purification of the plasma membrane from the total homogenate can increase the density of binding sites per mg of protein, but it is usually accompanied by a substantial loss of total protein [Limbird, 1986]. For these reasons, the

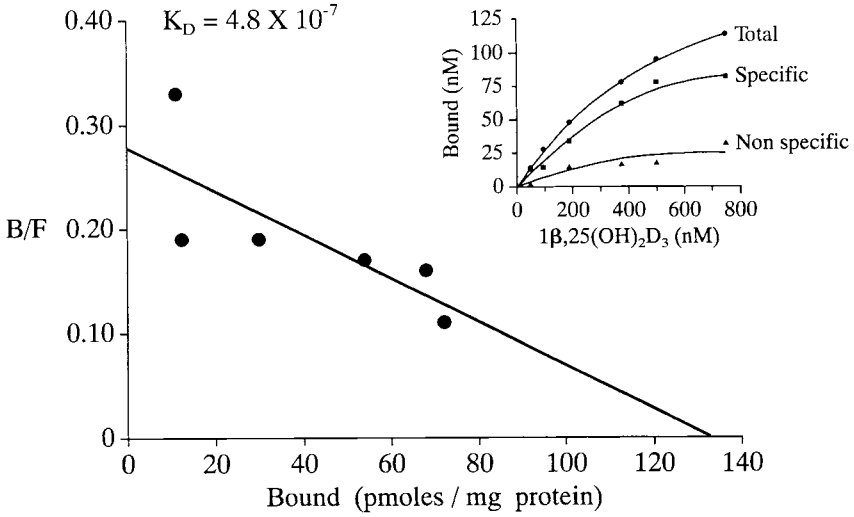

Fig. 8. Binding characteristics of ^{3}H-1β,25-dihydroxyvitamin D_3 15 Ci/mmol to membranes of ROS 24/1 cells in the presence of 1β,25-dihydroxyvitamin D_3. Nonspecific binding was determined in the presence of 100-fold excess of 1β,25-dihydroxyvitamin D_3, 75 μM. Each value for the binding curve represents the mean of two observations.

higher specific activity of iodinated ligands provides a more accurate assessment of binding constants.

The observation that the binding of 1β,25-dihydroxyvitamin D_3 to the membranes has a similar apparent dissociation constant to 1α,25-dihydroxyvitamin D_3 binding and that each epimer can displace the other suggests that this binding activity may have some relationship to the signalling system that mediates the rapid effects of the hormone. 1β,25-dihydroxyvitamin D_3 has been shown to inhibit the rapid effects of 1α,25-dihydroxyvitamin D_3 in osteoblasts [Baran et al., 1991, 1992; Jenis et al., 1993; Sorensen et al., 1993; Norman et al., 1993] without binding to the nuclear vitamin D receptor [Baran et al., 1992; Norman et al., 1993]. Thus, the 1β epimer seems to be relatively specific in its binding to the signalling system mediating the rapid effects. The receptor in the membranes appears to recognize both the 1α and 1β epimers with comparable affinity.

In summary, we report the binding characteristics of membranes prepared from ROS 24/1 cells that recognize both 1α,25-dihydroxyvitamin D_3 and its 1β epimer. Both 1α,25-dihydroxyvitamin D_3 and its epimer are able to displace the binding of ^{3}H-1α,25-dihydroxyvitamin D_3 to the membrane receptor, while 25-hydroxyvitamin D_3 cannot. The apparent dissociation constants for the binding of the 1α and 1β epimers to the membrane receptor, 4–8 × 10^{-7} M, are considerably lower than the binding of hormone to the nuclear vitamin D receptor and the concentrations needed to elicit rapid actions in biologic systems. These differences may reflect the difficulties in dealing with fat soluble, low specific activity ligands. Nevertheless, the observation that the 1β epimer, but not 25-hydroxyvitamin D, can displace 1α,25-dihydroxyvitamin D_3 binding to membranes coupled with the previous findings that the 1β epimer is a specific inhibitor of the rapid actions of the hormone suggests that this binding activity may be involved in the receptor signalling system that mediates the rapid effects of vitamin D.

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