Immunochemical studies on the putative plasmalemmal receptor for 1,25(OH)₂D₃. I. Chick intestine

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Immunochemical studies on the putative plasmalemmal receptor for 1,25(OH)₂D₃. I. Chick intestine. Am J Physiol Endocrinol Metab 278: E1104–E1114, 2000.—Antisera were raised against the NH₂-terminus of the putative basal lateral membrane (BLM) receptor for 1,25-dihydroxyvitamin D₃ ([1,25(OH)₂D₃; BLM-VDR]. In Western analyses of BLM proteins, antibody (Ab) 099 was monospecific for a 64.5-kDa band. A protein of 64.5 kDa was also labeled by the affinity ligand [¹⁴C]1,25(OH)₂D₃-bromacetate; label was diminished in the presence of excess unlabeled secostradiol. The monoclonal antibody against the nuclear VDR (9A7) failed to detect an appropriate band in BLM fractions. Precipitation of isolated intestinal cells with Ab 099, but not 9A7, affected the following two 1,25(OH)₂D₃-mediated signal transduction events: augmented intracellular calcium and protein kinase C activity. Subcellular distribution of Ab 099 reactivity by Western analyses and fluorescence microscopy revealed the highest concentrations in BLM followed by the endoplasmic reticulum. Exposure of isolated intestinal cells to 1,25(OH)₂D₃ for 10 s or vascular perfusion of duodena for 5 min resulted in a time-dependent increase in nuclear localization of the BLM-VDR antigen, as judged by electron microscopy, whereas 24,25-dihydroxyvitamin D₃ failed to increase antigen labeling in nuclei. Densitometric quantitation of Western blots of subcellular fractions prepared from isolated intestinal cells treated with vehicle or 1,25(OH)₂D₃ confirmed a hormone-induced increase of putative BLM-VDR in the nucleus. It is concluded that a novel cell surface binding protein for 1,25(OH)₂D₃ has been identified.

steroids; rapid effects; signal transduction; membrane receptor; calcium

MATERIALS AND METHODS

Preparation of subcellular fractions. Duodenal mucosae from vitamin D-replete white leghorn cockerels were fractionated as described previously (18). The procedure involves a combination of differential- and Percoll-gradient centrifugation; enrichment, recovery, and distribution of marker enzymes are described in detail elsewhere (18). Differential centrifugation fractions are P₁ (nuclei and brush-border membranes), P₂ (lysosomes, mitochondria, Golgi, and BLM), and P₃ (cytosol and microsomes). Percoll gradients of P₂ have been characterized for marker enzyme distribution as described elsewhere (18). Percoll was removed by dilution with homogenization medium and centrifugation (20). Protein was estimated by using the Bradford reagent (Bio-Rad, Hercules, CA) against bovine γ-globulin as standard (Sigma Chemical, St. Louis, MO).

PAGE and Western analyses. Appropriate fractions were resolved on 8% denaturing gels and were stained with either Coomassie brilliant blue to detect protein bands or used for Western analyses as follows: gels (containing colored molecular weight standards; Bio-Rad) were blotted on polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA) using the Hoeffer tank transfer system (10 V, 13 h at 4°C), and Western analyses were performed according to the Millipore protocol. For initial characterization, 60 μg of BLM proteins were used. No reaction product was visible with normal rabbit serum (NRS); antisera produced in three rabbits against the NH₂-terminal peptide sequence of the putative receptor revealed that Ab 099 and Ab 593 were monospecific for a protein of molecular weight 64,500, whereas Ab 980 recognized additional high-molecular-weight bands (22). Rabbit polyclonal antibody (PA1–711) to the COOH-terminal end of the human VDR (amino acids 395–413) and rat monoclonal antibody (9A7) to amino acids 89–105 of the

membrane recognition sites for estrogen were first reported 20 years ago (28). Since then, cell surface “receptors” have been documented for every class of steroid hormone (16, 26), although experimental evidence for a direct action on membrane lipids (26) has not been found. For 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], a basal lateral membrane receptor (BLM-VDR) has been implicated in mediating rapid enhancement of intestinal calcium or phosphate transport (11, 14, 17, 19, 20, 24), activation of phospholipase-associated signaling pathways (1, 3, 8, 13, 31, 34), and opening of calcium channels (4–7, 32, 37). Unanswered questions regarding the BLM-VDR are whether it is related to the nuclear/cytoplasmic receptor (9, 12, 27), which has been proposed to “dock” at the inside of the plasma membrane (12), and whether ligand binding induces internalization or recompartmentalization. To begin addressing these questions, a synthetic peptide corresponding to the first 20 amino acids of the putative membrane receptor (20) was synthesized and used for production of antisera in rabbits. The current report confirms the receptor-like nature of the antigen, its localization on the plasmalemma and Golgi/endoplasmic reticulum (ER) elements, the ability of antisera to alter 1,25(OH)₂D₃-mediated signal transduction events, and detection of apparent ligand-induced redistribution to the nucleus.

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VDR were purchased from Affinity Bioreagents (Golden, CO) and were used at a 1:100 dilution or at 2 µg/ml, respectively.

Ligand binding assays. For affinity labeling, 100 µg of BLM protein and 100 µl of 10 mM Tris, 1.5 mM EDTA, and 1 mM dithiothreitol, pH 7.4 (TED buffer; see Ref. 20), were incubated in microfuge tubes with 20 µl of an ethanolic solution containing 0.13 nmol of [14C]1,25(OH)2D3-bromoacetate (29) in the absence or presence of a 200-fold molar excess of 1,25(OH)2D3 (overnight, 0°C). One milliliter of TED buffer was then added, and the mixture was centrifuged (15,000 rpm, 3 min). After the supernatant was decanted, the pellet was washed one time and then was resuspended in SDS-PAGE sample buffer for resolution on an 8% gel. The Coomassie-stained, dried gel was exposed to X-ray film (Kodak X-OMATAR) for 1 mo.

Loading of isolated intestinal cells with fura 2. Intestinal cells isolated by citrate chelation (18) were harvested by low-speed centrifugation and were resuspended in divalent cation-free PBS containing 100 µM EGTA, 5.55 mM glucose, 0.32 mM sodium pyruvate, 3 mg/ml BSA, 0.02% Pluronic F-127, and 2 µM fura 2-AM (Molecular Probes, Eugene, OR), as described elsewhere (36). Cells were incubated on ice for a 20-min loading period, diluted fourfold with PBS, collected by centrifugation, and resuspended in divalent cation-free PBS.

Intracellular calcium was determined by ratio changes of fluorescence intensity at an emission wavelength of 510 nm when excited at 340 and 380 nm. A camera control switched fluorescence intensity for transmission objective for transmission microscopy, segments of distal duodenal loop for viewing. Basal lateral membrane (BLM) proteins were resolved for 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] in chick intestine. Basal lateral membrane (BLM) proteins were resolved on 8% SDS-PAGE. After electroblotting on Immobilon-P, the polyvinylidene difluoride (PVDF) membranes were incubated in blocking buffer, washed three times, and incubated with primary antisera (antibody) (Ab) 099) at 1:1,000 dilution for 2 h at 23°C. After three additional washes, alkaline phosphatase-conjugated secondary antibody (1:30,000) was added for another 1-h incubation. Chromogenic visualization was accomplished with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Reaction product was visualized at a single band of molecular weight. MW was labeled by the affinity ligand [14C]1,25(OH)2D3-bromoacetate (2nd from right); the label was displaced when incubations were conducted in the presence of excess unlabeled 1,25(OH)2D3 (lane on right). For affinity labeling, 100 µg of BLM protein and 100 µl of Tris-EDTA-dithiothreitol (TED) buffer were incubated in microfuge tubes with 20 µl of an ethanolic solution containing 0.13 nmol [14C]1,25(OH)2D3-bromoacetate in the absence or presence of a 200-fold molar excess of 1,25(OH)2D3 (overnight, 0°C). TED buffer (1 ml) was then added, and the mixture was centrifuged (15,000 rpm/min). After the supernatant was decanted, the pellet was washed one time and then resuspended in SDS-PAGE sample buffer for resolution on an 8% gel. The Coomassie-stained, dried gel was exposed to X-ray film for 1 mo.

**Fig. 1.** Characterization of antisera to the NH2-terminus of the putative basal lateral membrane receptor (BLM-VDR) for 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) in chick intestine. Basal lateral membrane (BLM) proteins were resolved on 8% SDS-PAGE. After electroblotting on Immobilon-P, the polyvinylidene difluoride (PVDF) membranes were incubated in blocking buffer, washed three times, and incubated with primary antisera [antibody (Ab) 099] at 1:1,000 dilution for 2 h at 23°C. After three additional washes, alkaline phosphatase-conjugated secondary antibody (1:30,000) was added for another 1-h incubation. Chromogenic visualization was accomplished with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Reaction product was visualized at a single band of molecular weight (MW). Basal lateral membrane (BLM) proteins were resolved on 8% SDS-PAGE. After electroblotting on Immobilon-P, the polyvinylidene difluoride (PVDF) membranes were incubated in blocking buffer, washed three times, and incubated with primary antisera [antibody (Ab) 099] at 1:1,000 dilution for 2 h at 23°C. After three additional washes, alkaline phosphatase-conjugated secondary antibody (1:30,000) was added for another 1-h incubation. Chromogenic visualization was accomplished with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Reaction product was visualized at a single band of molecular weight (MW).
chelation (18), pelleted at 500 g for 5 min (4°C), and resuspended in GBSS (pH 7.3) lacking glucose and bicarbonate and modified to contain 0.9 mM CaCl₂. Cells were then treated with either vehicle (0.1% vol/vol ethanol, final concentration) or 650 pM 1,25(OH)₂D₃ (final concentration) and were swirled for 10 s before removal of an aliquot to fixative (4% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.1) for 4 h. Cell pellets were dehydrated, infiltrated, and embedded in LR White for preparation of thin sections. After being exposed to blocking buffer (0.5% fish gelatin, 0.5% normal goat serum in 20 mM Tris, pH 7.4; 15 min), nonspecific staining controls from the hormone-treated sample were incubated with buffer (50 mM Tris, 150 mM NaCl, pH 7.4), whereas sections from both the control group and hormone-treated group were incubated with Ab 099 (1/500) in buffer overnight (4°C). After six washes in buffer, sections were labeled with 5 nm gold-conjugated goat anti-rabbit antibody (1:50 dilution in buffer) for 2 h (23°C). A final six washes were performed with reagent-grade water before enhancement with silver reagent and counterstaining with uranyl acetate and lead citrate. Representative nuclei were photographed under single-blind conditions, in which specimens were identified as treatment A, B, or C. Labeling densities were determined by overlaying a transparent film over the photograph with a 1 µm section delimited. Silver-enhanced gold particles were then counted.

Alternatively, duodena were vascularly perfused (18) for 5 min with either 6.5 nM 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃] or 650 pM 1,25(OH)₂D₃ before removal of a segment from the adjoining pancreas, slitting of the tissue, and immersion in fixative. Thick (16 µm) sections were then prepared for preembed labeling (21).

Electron spectrographic imaging technique. Immunolabeled sections were silver enhanced by immersion in silver enhancing solution (catalog no. sekl15; BB International) for 1 h. Ultrathin sections were cut on a Leica Ultracut E (Leica, Deerfield, IL), counterstained with uranyl acetate and lead citrate (30) for 2 and 4 min, respectively, analyzed with conventional transmission electron microscopic imaging, and recorded on Kodak film SO-163 (Eastman Kodak, Rochester, NY) with the Zeiss 902CEM transmission electron microscope (LEO, Thornwood, NY). Electron spectrographic images were also generated with the Zeiss 902CEM as digital images. Images were collected at the silver edge of 430 eV loss and the adjacent background of 355 eV loss. The background images were subtracted from the corresponding silver edge images, with the resultant being the elemental map for silver in the section. The elemental map was statistically analyzed and false colored according to McManus et al. (16). Pixels three SD above the mean background are yellow; pixels four SD above the mean background are green; pixels five and six SD above the mean background are light blue; pixels seven to nine SD above the mean background are dark blue; and pixels ten SD or greater above the mean background are black.

RESULTS

In the first series of experiments, the antibody was characterized with regard to specificity and protein dependence of Western analyses.

Western analyses and affinity labeling. Figure 1 demonstrates the protein dependence of Western analyses with Ab 099 (lanes in middle) and shows that only one band (mol wt 64,500) was recognized at the concentrations tested. The affinity reagent [¹⁴C]1,25(OH)₂D₃-bromoacetate (29) labeled a band of equivalent molecular weight (second from right), which was reduced by excess unlabeled 1,25(OH)₂D₃ (lane on right). The

Fig. 2. Fura 2 determination of intracellular calcium in intestinal cells exposed to 130 pM 1,25(OH)₂D₃ in the absence or presence of Ab 099. Intestinal cells isolated by citrate chelation were loaded with fura 2 for 20 min on ice as described in MATERIALS AND METHODS. Aliquots (200 µl) were then taken for imaging. Conditions were as follows. A: cells in the absence of extracellular calcium were treated with secoesteroid after a 60-s basal period. B: aliquots of the cell suspension used in A were mixed with 1 mM CaCl₂ and then were treated with hormone at 50 s. C: cells in the presence of CaCl₂ were mixed with Ab 099 (1:500 dilution, final concentration) on ice; after a 60-s basal period in the presence of antibody (23°C), cells were treated with hormone. Values represent fluorescence ratio of fura 2 emission at 510 nm relative to excitation at 340 and 380 nm. Data are representative of 6 experiments. t, Time.
substantial degree of competition shown in Fig. 1 was not always the case; in other experiments, the label was reduced by ~50%. Although a number of other bands were faintly labeled by affinity reagent in these overnight incubation protocols (Fig. 1), these bands were completely absent when shorter incubation times (90 min) were used.

Western blots performed with the monoclonal antibody to the VDR (9A7) or the polyclonal PA1-711 faintly labeled many bands in nuclear and supernatant fractions of intestinal epithelium, including one band that comigrated with authentic recombinant VDR; this band was absent in BLM preparations (data not shown).

In the next series of experiments, Ab 099 was tested for its ability to inhibit signal transduction events initiated by 1,25(OH)₂D₃, including intracellular calcium increases and protein kinase activation.

Figure 2 depicts the results of fura 2 fluorescence intensity (expressed as a ratio of emission and excitation wavelengths; see Ref. 36) in intestinal cells incubated in the absence of calcium (A) or the presence of 1 mM extracellular calcium (B and C). As reported elsewhere (7), the absence of extracellular calcium abolished the augmentation of intracellular calcium by 1,25(OH)₂D₃. In Fig. 2, A-C, the average response of several hundred cells is depicted. In the absence of extracellular calcium, intracellular fluorescence of fura 2 declined over time, with no noticeable increase elicited by addition of 130 pM secosteroid (Fig. 2A).

In contrast, when an aliquot of the same cell suspension was made 1 mM in CaCl₂, a stable baseline of fura 2 fluorescence was observed for 60 s before the addition of hormone (Fig. 2B). Exposure of cells to 130 pM 1,25(OH)₂D₃ led to very rapid oscillations in intracellular calcium. When cells were combined with Ab 099 (1:500 dilution, final concentration), a stable baseline was observed only for 30 s, followed by antibody-induced oscillations in intracellular calcium that were apparently unaffected by addition of secosteroid at 60 s (Fig. 2C). Incubation of cells with an irrelevant antibody (anti-calmodulin kinase; Zymed, South San Francisco, CA) at an equivalent dilution, or 9A7 at 2 µg/ml, failed to produce oscillatory spikes and did not hinder subsequent stimulation by 1,25(OH)₂D₃ (data not shown).

The data in Fig. 3 were normalized as follows. The average basal (0–60 s) fluorescence (emission-excitation) ratio was calculated for a field of cells. Fura 2 fluorescence (emission-excitation ratios) after the test substance was then divided by basal values for the corresponding field of cells. The normalized data for two independent experiments are presented in Fig. 3. Addition of 130 pM 1,25(OH)₂D₃ (Fig. 3, A and C) resulted in oscillatory spikes of intracellular calcium that were 6- to 10-fold above basal values. Cells in the

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**Fig. 3.** Fura 2 determination of intracellular calcium in intestinal cells exposed to 130 pM 1,25(OH)₂D₃ (at **t** = 0) in the absence or presence of Ab 099, as indicated. Fluorescence during the treated phase was normalized to the basal phase for each corresponding treatment. A and C: hormone alone. B and D: Ab 099 followed by hormone.
presence of Ab 099, however, did not respond to the addition of secosteroid hormone with increased oscillations (Fig. 3, B and D).

Effect of 1,25(OH)2D3 and Ab 099 on PKC activity. The secosteroid hormone has also been shown to activate the PKC signal transduction pathway (8, 33, 34), perhaps in part as a consequence of calcium mobilization. In time course studies with isolated intestinal cells, 130 pM 1,25(OH)2D3 was found to increase PKC activity 5 min after hormone and protein kinase A (PKA) activity 7 min after hormone (19). PKC activity in intestinal cells was then tested in the absence or presence of a 5-min preincubation with Ab 099. As a control, PTH-mediated stimulation of PKA activity was monitored under equivalent incubation conditions. As revealed in Fig. 4A, cells incubated with control medium or Ab 099 in the absence of hormone exhibited a low level of activity. Addition of 130 pM 1,25(OH)2D3 resulted in a twofold increase in activity (P < 0.05, relative to controls) that was abolished by preincubation with Ab 099 (P < 0.005, relative to hormone alone; Fig. 4A). In contrast, 19A7 had no effect on 1,25(OH)2D3-stimulated PKC activity. The average values (pmol min–1 mg protein–1 ± range) for replicate experiments were 93 ± 11 for controls, 98 ± 9 for controls plus 9A7, 199 ± 28 for 1,25(OH)2D3-treated cells, and 211 ± 26 for hormone plus 9A7.

Preincubation with Ab 099 was also found to abolish steroid hormone-enhanced PKA activity in a pilot study: cells incubated with control medium, 130 pM 1,25(OH)2D3, and Ab 099 followed by secosteroid yielded values of 137, 321, and 146 pmol min–1 mg protein–1, respectively.

The intestine has been shown to have receptors for PTH (18) that are most likely unrelated to cell surface receptors for 1,25(OH)2D3. As demonstrated in Fig. 4B, PTH stimulates PKA activity in intestinal cells, whereas PKC activity remains unchanged (18). Stimulation of PKA activity by PTH was unaffected by preincubation with Ab 099 (Fig. 4B), demonstrating that Ab 099 is not a general inhibitor of signal transduction. The absence of a general inhibitory effect of Ab 099 was confirmed in experiments using an unrelated steroid hormone, estradiol 17β. Because estrogen receptors have been found in intestinal cell lines (35), the effect of Ab 099 on estradiol activation of PKC was investigated. Enzyme specific activity (pmol min–1 mg protein–1; given as average ± range) for isolated intestinal cells incubated with control media was 104 ± 31, whereas a 5-min treatment with 1 nM estradiol 17β yielded a modest increase to 164 ± 11, and preincubation with Ab 099 followed by 1 nM estradiol 17β indicated a lack of inhibitory action (185 ± 12).

A duplicate set of experiments was also conducted to investigate the tentative connection between calcium channel activation and PKC activation. Intestinal cell suspensions were preincubated with control media or with 1 µM nifedipine, a calcium channel blocker, before addition of control media or 130 pM 1,25(OH)2D3. Determination of PKC specific activities (pmol min–1 mg protein–1) yielded values (expressed as average ± range) that were 103 ± 3 for controls, 106 ± 3 for controls with nifedipine, 199 ± 23 for 1,25(OH)2D3, and 192 ± 17 for nifedipine followed by 1,25(OH)2D3.

The final series of experiments was designed to elucidate the subcellular localization of the putative BLM-VDR by Western analyses and microscopy and to determine whether exogenous hormone resulted in recompartmentalization of the antigen.

Subcellular distribution of antigen. Studies on the subcellular distribution of the BLM-VDR antigen were undertaken by Western analyses. In four independent experiments, 15 µg of protein from fraction P1 (nuclei and brush borders), supernatant fraction S2 (microsomes and cytosol), lysosomes, mitochondria, Golgi, and BLM were loaded in individual lanes. Figure 5 shows a representative Coomassie-stained gel (A) and Western blot with Ab 099 (B), and the results of densitometric analyses of the Western blots are shown in Fig. 5C. Only low amounts of the BLM-VDR antigen were found (average ± SE) in P1 (26 ± 4%), S2 (31 ± 6%), lysosomes (21 ± 7%), and mitochondria (36 ± 6%).
whereas the Golgi contained larger amounts (64 ± 12%) compared with BLM (set to 100%). For comparison, the relative distribution of the BLM marker enzyme activity, Na^+-K^+-ATPase, in P1, S2, lysosomes, mitochondria, and Golgi, respectively, was 17, 17, 20, 44, and 52% compared with BLM (set to 100%). Golgi and BLM contained 7–10% of Percoll gradient acid phosphatase activity (a lysosomal marker enzyme) and 4–5% of gradient succinate dehydrogenase activity (a mitochondrial marker enzyme). Distribution of the putative membrane receptor antigen parallels earlier findings that BLM and Golgi/ER fractions from the postnuclear 20,000-g pellet contained reproducible specific binding of [3H]1,25(OH)2D3 (20).

Immunofluorescence microscopy. To confirm and extend the observations obtained from Western analyses, immunofluorescence microscopy of fixed frozen sections of chick duodena was undertaken. Use of standard epifluorescence or confocal microscopy of 4-µm sections revealed only basal lateral staining with Ab 099 or Ab 593 in epithelium separated from the villus core (data not shown). Confocal microscopy of whole villi permeabilized with either 0.1% Triton X-100 or lysophosphatidylcholine revealed only lateral staining with Ab 099 (data not shown). In comparison, confocal microscopy of 16-µm sections revealed basal membrane staining and labeling of structures that may be ER/Golgi or lateral membranes from lower cell layers (Fig. 6B). Nuclei and brush borders were devoid of label. Incubation of 16-µm sections with preabsorbed antisera resulted in a complete absence of immunofluorescence (Fig. 6A).

Electron microscopy. To investigate the possibility of ligand-induced redistribution of the BLM-VDR, isolated intestinal epithelial cells were treated with 0.1% ethanol (final concentration) or with 650 pM 1,25-(OH)2D3 for 10 s, after which 500 µl of cells were removed from each suspension and placed in fixative (4 h, 23°C). Samples were then prepared for electron microscopy.

With the use of postemebd labeling with Ab 099 as primary antisera, followed by gold-conjugated secondary antibody, concentrated areas of electron-dense particles were not observed in controls, most likely due to decreased antigenicity caused by the plastic embedding media. However, nuclei from hormone-treated cells were found to contain a relative increase in label.
Figure 7 displays pseudocolored labeling densities from representative nuclei selected under single-blind conditions. Averaging the number of gold particles per square micrometer for three nuclei (± SD) in the nonspecific staining group (prepared from hormone-treated cells) yielded 28 ± 5. When corrected for background, nuclei of freshly isolated cells had 53 ± 16 gold particles/µm² (not shown), vehicle controls had 40 ± 6, and nuclei of cells treated with 1,25(OH)₂D₃ had 122 ± 17 (P < 0.05, relative to controls). These data indicate that a low level of antigen exists in nuclei of intestinal cells from vitamin D-replete chicks that is not visible at the light microscopic level (Fig. 6). However, treatment of intact cells with secosteroid hormone for very brief periods results in the apparent ligand-induced translocation of the BLM-VDR to the nucleus.

These studies were subsequently extended to include tissue exposed to either 6.5 nM 24,25(OH)₂D₃ or 650 pM 1,25(OH)₂D₃ for 5 min by vascular perfusion of duodena before fixation. Using electron spectrographic imaging and false color displays as described by McManus et al. (16), silver enhancement of gold-labeled secondary antibody can be more quantitatively assessed. As shown in Fig. 8A, a nucleus from tissue vascularly perfused with 6.5 nM 24,25(OH)₂D₃ for 5 min revealed only sparse labeling, as indicated by the yellow color, whereas in Fig. 8B, a nucleus from tissue vascularly perfused with 650 pM 1,25(OH)₂D₃ for 5 min exhibits more abundant label and greater intensity, as indicated by the green and blue colors. The smaller area of labeling was not identified.

Western analyses of subcellular fractions after hormone treatment. Increased nuclear labeling after hormone treatment of isolated cells might be attributable also to increased antibody binding to the liganded form of the BLM-VDR. In an attempt to resolve these possibilities, Western analyses were performed with Ab 099 on subcellular fractions prepared from isolated intestinal epithelial cells treated with vehicle or 650 pM 1,25(OH)₂D₃ for 10 s. Although visual inspection of the blots revealed small changes, densitometric analyses revealed that they were consistent changes. The results presented in Table 1 indicate a significant hormone-mediated increase in antigenicity to Ab 099 in P₁ (containing nuclei) and significant decreases in lysosomes and BLM prepared from cells exposed to 1,25(OH)₂D₃, relative to corresponding controls. Increasing the 1,25(OH)₂D₃ treatment period to 5 min further augmented BLM-VDR antigenicity in P₁ relative to controls (Table 1).

DISCUSSION

The secosteroid hormone 1,25(OH)₂D₃ has been known for many years to stimulate ⁴⁵Ca uptake in isolated rat intestinal cells (24) and transport in perfused chick duodena (19, 20, 25). The physiological importance of these findings has been controversial, because these phenomena are difficult to observe in vivo (2). However, this may be either completely or in part due to the suppressive effect of endogenous 24,25(OH)₂D₃ levels (19). Indeed, 24-hydroxylase knockout mice, in which the suppressive hormone is absent, have been reported to be hypercalcemic (10). Thus calcium homeostasis is more complicated than was
once thought, and studies on the rapid actions of 1,25(OH)₂D₃ are warranted.

The current work provides further characterization of a receptor-like protein for 1,25(OH)₂D₃ identified in BLM of chick intestinal epithelium (20). Affinity labeling of the 65-kDa integral membrane protein strengthens the candidacy of this binding moiety as a cell surface receptor. Treatment of isolated intestinal cells with either 1,25(OH)₂D₃ or Ab 099 dramatically increased calcium oscillations, as judged by fura 2 fluorescence. These results may be due to a conformational change produced by antibody binding to the putative receptor, with subsequent activation of a coupled calcium channel (6). Enterocytes pretreated with Ab 099 did not exhibit additional oscillations in intracellular calcium when cells were subsequently exposed to 130 pM 1,25(OH)₂D₃. At present, these results can only be interpreted as a lack of additivity between secosteroid and antibody-induced oscillations.

Clear-cut antibody-mediated abolition of 1,25(OH)₂D₃ signal transduction was evident in the PKC pathway. These results confirm the observations in chondrocytes (23). In contrast, interaction of PTH with its receptor to stimulate PKA activity, or estradiol 17β with its receptor to simulate PKC activity, was unaffected by the presence of antibody to the putative 1,25(OH)₂D₃ hormone membrane receptor. The dual observations that Ab 099 activated calcium channels yet blocked secosteroid-enhanced PKC activity are difficult to explain without further research into signal transduction. However, it may indicate that the two pathways can be separated and may lead to different physiological end points. Preliminary studies with the calcium channel blocker nifedipine support this hypothesis. The specificity of the effect of Ab 099 on receptor-coupled calcium channel activation was supported by the inability of the Ab 9A7 to produce similar effects.

Earlier work (20) indicated that, within the 20,000-g pellet fraction, [3H]1,25(OH)₂D₃ exhibited reproducible, specific binding only in BLM fractions and ER/Golgi elements. With the use of Western analyses of the same subcellular fractions, the present study likewise reveals that BLM and Golgi fractions were enriched in the candidate receptor protein. Thus the binding activity parallels the distribution of the BLM-VDR antigen. Although the BLM-VDR antigen may be related to the nuclear receptor in the ligand-binding domain, since both molecules manifest an equivalent affinity for 1,25(OH)₂D₃ (20), several experimental findings suggest that the receptor proteins are different molecular entities. It is evident from epifluorescence studies of immunologically labeled sections that the receptor is not merely “docking” at the cytoplasmic surface as suggested by Kim et al. (12). Specific staining along the BLM in fixed frozen sections of duodena was observed only when the lamina propria was separated from the epithelium. A cytoplasmic localization would allow the observation of labeling even in tissue sections where the BLM was in contact with the substratum. These results, together with the finding that the receptor activity requires detergent for solubilization (20), suggest that the protein is integral to the membrane rather than peripheral. Confocal microscopy with Ab 099 confirmed a basal lateral localization and an absence of
brush-border and nuclear labeling at the light level. With the use of 16-µm sections (~3 cell layers thick), immunofluorescent staining was also observed in what could either be intracellular membranes or lateral membranes of underlying cells. Subcellular distribution of antigen in Western analyses paralleled distribution of the BLM marker enzyme activity Na\(^+\)-K\(^-\)-ATPase. The presence of antigen in Golgi fractions could thus either be due to contamination with BLM, or it could represent a vesicular compartment for export or retrieval of the putative BLM-VDR.

In addition, Western analyses with the antinuclear VDR receptor indicate an absence of the classical receptor in membrane preparations.

In the presence of hormone, the BLM-VDR antigen is apparently capable of recompartmentalization. On Western analyses, the BLM-VDR is increased in crude nuclei after a brief (10-s) exposure of isolated cells to 1,25(OH)\(_2\)D\(_3\), with a concomitant decrease in antigen in the BLM fraction. This may represent endocytosis of the membrane antigen and delivery to the nucleus. A 5-min exposure of intestinal cells to secosteroid further increases Ab 099 antigenicity in the subsequently prepared low-speed pellet.

A far larger hormone-stimulated increase in nuclear localization of the BLM-VDR was observed with electron microscopy, using either isolated cells (10-s treatment) or vascularly perfused duodena (5-min treatment). The lower sensitivity of the biochemical procedure is undoubtedly due in part to redistribution of antigen during homogenization. A similar occurrence was observed for the loss of calbindin-D\(_{28k}\) from lysosomes (21). Electron microscopy of isolated intestinal cells did not, however, allow quantitation of plasmalemmal BLM-VDR in the presence or absence of hormone due to the randomness of the sectioning angle through unaligned cells and loss of antigenicity due to plastic embedding before labeling. The observation that ligand induces the apparent translocation of the BLM-VDR to the nucleus within 10 s of exposure to 1,25(OH)\(_2\)D\(_3\) has several interesting ramifications. It has been widely believed that steroid hormones, due to their lipophilic properties, simply diffuse through membranes. However, as pointed out elsewhere (17, 26), this theory fails to explain why the steroid would leave such a preferred environment. Ligand-induced translocation of the membrane receptor to the nucleus is perhaps a more efficient means of hormone delivery. The swiftness of such delivery (10 s) further indicates that a nonnuclear

![False color imaging of label in nuclei of tissues exposed to 6.5 nM 24,25-dihydroxyvitamin D\(_3\) (A) or 650 pM 1,25(OH)\(_2\)D\(_3\) (B) for 5 min. Sections were preimm labeled with Ab 099, followed by 5 nm gold-conjugated secondary antibody. Yellow color indicates density 3 SD above the mean background value, green represents 4 SD, light blue represents 5–6 SD, and dark blue represents 7–9 SD above the mean.](image)

Table 1. Quantitation of Western analyses performed with Ab 099 of subcellular fractions prepared from intestinal cells exposed to 650 pM 1,25(OH)\(_2\)D\(_3\) or vehicle

<table>
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<th>Fraction</th>
<th>+D/Con (10 s)</th>
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</thead>
<tbody>
<tr>
<td>P1</td>
<td>1.37 ± 0.1</td>
<td>&lt;0.03</td>
<td>1.77 ± 0.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>S2</td>
<td>1.18 ± 0.3</td>
<td>NS</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>0.54 ± 0.2</td>
<td>&lt;0.01</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.82 ± 0.1</td>
<td>NS</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Golgi</td>
<td>1.06 ± 0.3</td>
<td>NS</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BLM</td>
<td>0.73 ± 0.0</td>
<td>&lt;0.006</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE for 3 independent experiments; significance was determined between treated and control groups. P1, 1000-g pellet containing nuclei and brush borders; S2, 20,000-g supernatant containing microsomes and cytosol; BLM, basal lateral membrane; Ab, antibody; 1,25(OH)\(_2\)D\(_3\), 1,25-dihydroxyvitamin D\(_3\) (+D); Con, vehicle control; NS, not significant; ND, not determined.
signaling system must be operational within this time frame to be valid mediators of the so-called rapid effects. A number of second messengers, such as hormone- and receptor-mediated opening of calcium channels (4; Fig. 2 of the present work) and activation of phospholipase C-initiated systems (13, 34), appear to occur within a suitable time frame.

Studies are currently underway to clone and characterize the cDNA for the putative membrane receptor.

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