A specific binding moiety for 1,25-dihydroxyvitamin D₃ in basal lateral membranes of carp enterocytes

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A specific binding moiety for 1,25-dihydroxyvitamin D₃ in basal lateral membranes of carp enterocytes. Am J Physiol Endocrinol Metab 279: E614–E621, 2000.—Carp (Cyprinus carpio), a freshwater fish that lives in a low-calcium environment, and Atlantic cod (Gadus morhua), a seawater fish that lives in a high-calcium environment, were studied for the presence of a novel membrane binding protein (“receptor”) for the vitamin D metabolite, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]. Basal lateral membranes from enterocytes of either species were prepared and analyzed for specific saturable binding. Membranes from carp revealed a dissociation constant of 1.23 nM with a maximal binding capacity of 212 fmol/mg protein. In comparison, membranes from Atlantic cod enterocytes revealed very low and nonsignificant levels of specific binding. The [³H]1,25(OH)₂D₃ binding activity in carp was further characterized for protein dependence, detergent extractability, and competition for binding with the vitamin D metabolite, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]. Basal lateral membranes from enterocytes of either species were prepared and analyzed for specific saturable binding. Membranes from carp revealed a dissociation constant of 1.23 nM with a maximal binding capacity of 212 fmol/mg protein. In comparison, membranes from Atlantic cod enterocytes revealed very low and nonsignificant levels of specific binding. The [³H]1,25(OH)₂D₃ binding activity in carp was further characterized for protein dependence, detergent extractability, and competition for binding with the metabolites 25(OH)D₃ and 24R,25(OH)₂D₃. Finally, introduction of 1,25(OH)₂D₃ to isolated carp enterocytes enhanced protein kinase C activity within 5 min, whereas intracellular Ca²⁺ concentrations were unaffected by a range of 1,25(OH)₂D₃ concentrations, as judged by fura 2 fluorescence. Thus the binding moiety may be a putative plasma membrane receptor for vitamin D, because it is functionally coupled to at least one signal transduction pathway.

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MATERIALS AND METHODS

Animals. All procedures were approved by the Swedish Ethics Committee for Animal Research. Carp of both sexes (200–300 g body wt) were purchased from a local hatchery, (Aneboda Aqua Service) and were acclimated in recirculated, filtered, and aerated freshwater at 15°C for ≥5 days before the experiments. The carp were fed daily until satiation a plant diet consisting of duckweed (Lemma minor). Atlantic cod of both sexes (300–500 g body wt) were caught off the west coast of Sweden and kept in recirculated, filtered, and aerated seawater at 10°C for 5 days before they were killed. The Atlantic cod were not fed during the acclimation period.

Preparation of basal lateral membranes. The procedure was a modification of Flik and Verbost (9). Fish were anesthetized in 0.02% phenoxyethanol (Sigma Chemical, St. Louis, MO) before surgical removal of the first two-thirds of the intestine. After the intestinal segment was slit longitudinally and rinsed in 154 mM NaCl, the tissue was placed mucosa side up onto a glass petri dish inverted over ice. Unless otherwise specified, all steps were performed at 0–4°C. Each mucosa (2 per preparation) was scraped from the underlying circular muscle layer with a microscope slide into 5 ml of homogenization medium (in mM: 250 sucrose, 12.4 NaCl, 0.1 dithiothreitol, 5 HEPES, 0.15 imidazole, pH 7.8, 0.3 phenylmethylsulfonyl fluoride). The tissue was disrupted by 20 strokes using a Potter-Elvehjem homogenizer and motor-driven Teflon pestle set at low speed. Nuclei and cellular debris were removed by centrifugation at 1,400 g for 10 min, and the resulting supernatant fraction was centrifuged at 20,000 g for 20 min. The 20,000-g supernatant fraction contained crude basal lateral membranes. The 20,000-g pellet was washed 3 times, and the supernatant fractions were pooled for centrifugation at 125,000 g for 25 min. The resulting pellet contained a central core of brownish mitochondria and a surrounding white fluffy layer of basal lateral membranes that were carefully removed by aspiration. Further purification was achieved by resuspending the fluffy layer in 4 ml of homogenization medium, layering the suspension over 4 ml of 37% sucrose, and centrifugation at 200,000 g for 90 min. The membranes at the interface were carefully collected, pelleted at 180,000 g for 35 min, and washed twice with buffer (150 mM KCl, 0.8 mM MgCl2, 20 mM HEPES-Tris, pH 7.4). The final pellet was resuspended in the appropriate buffer by 20 passages through a 23-gauge needle.

Saturation analyses of \([3H]1,25(OH)_2D_3\) binding to membranes. Membranes isolated from carp or Atlantic cod were adjusted to 50 μg protein/200 μl TEB buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, pH 7.4) and incubated in triplicate for total binding (isotopically labeled hormone; 178 Ci/mmol, Amersham Pharmacia Biotech, Uppsala, Sweden), as well as for nonspecific binding (radioactively labeled hormone in the presence of a 200-fold molar excess of unlabeled hormone) for each concentration tested. Samples were incubated on ice overnight to achieve equilibrium binding conditions. The next morning, bound hormone was separated from free hormone by perchloric acid precipitation, as described elsewhere (12, 25). Although this technique is suitable for hydrophobic membrane-associated proteins, it does not measure binding to the soluble classical vitamin D receptor (12, 25). The precipitated protein was pelleted at 14,000 g for 10 min, the supernatant was decanted, and the insides of the tubes were carefully swabbed to remove residual liquid. The pellet was dissolved in 6 M guanidine-HCl and transferred to scintillation vials for counting.

Protein dependency of \([3H]1,25(OH)_2D_3\) binding to membranes. Increasing concentrations of membrane protein from carp enterocytes were incubated (0°C, overnight) with 1 nM \([3H]1,25(OH)_2D_3\) in the absence or presence of unlabeled secosteroid and were then analyzed for total and nonspecific binding, as described in the previous section.

Membrane association of the binding protein. Aliquots of membranes from carp enterocytes were homogenized in the presence of 300 mM KCl (final concentration) or 10 mM [3-(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate (CHAPS, final concentration) with 25 strokes on ice. After centrifugation (14,000 g, 10 min), aliquots of the supernatants and pellets (resuspended to a volume equivalent to that of the supernatant) were taken for incubation with 1 nM \([3H]1,25(OH)_2D_3\) and were analyzed for total and nonspecific binding, as described in the previous section.

Isolation of intestinal epithelial cells. Enterocytes from carp were isolated according to Larsson et al. (16) by a chelation protocol. Cell viability was determined by trypan blue exclusion in combination with phase contrast microscopy and was found to be >95%.

Loading of fura 2-AM. Fura 2-AM loading was performed as described earlier (17). Briefly, freshly isolated intestinal epithelial cells were incubated for 45 min in Hank’s balanced salt solution (HBSS) with fura 2-AM (5 μM), pluronnic F-127 (0.025%), and albumin (0.5%) at 37°C. The cells were washed three times with HBSS by centrifugation at 700 g for 10 min and were finally resuspended in HBSS.

Measurement of free intracellular Ca$^{2+}$ concentrations. Measurements of intracellular Ca$^{2+}$ concentrations (Ca$^{2+}$i), in fura 2-AM-loaded carp intestinal cells (5 × 10^5 cells/ml) were performed in a Photon Technology International Ratio Master model C-44 ratio fluorescence spectrometer (Photon Technology International, Monmouth Junction, NJ) at a 340/380 nm excitation ratio, with a 510-nm emission wavelength. The cells were placed in a quartz cuvette and slowly stirred at a constant temperature of 15°C. According to the different experimental protocols (described below), stock solutions of 1,25(OH)2D3 and 24R,25(OH)2D3 (10 μl, calcium (100 μl), and EGTA (10 μl) were added directly to the cuvette to give the final concentration stated under the experimental protocol (see below). Controls received the vehicle ethanol [for 1,25(OH)2D3 and 24R,25(OH)2D3 at the same final concentration. Fluorometric calibrations were performed by addition of 0.1% Triton (100 μg/ml) to lyse the cells and obtain the maximum fluorescence intensity of Ca$^{2+}$-saturated fura 2-AM, followed by addition of 15 μl of 400 mM EGTA-3 M Tris to measure the intensity of Ca$^{2+}$-free fura 2-AM. [Ca$^{2+}$i] were calculated with the equation

$$[Ca^{2+}]_i = K_d \left( \frac{R - R_{min}}{R_{max} - R} \right) S_T$$

where $K_d$ is the dissociation constant for fura 2-AM and corresponds to 338 nM at 15°C (19); $R$ is the fluorescence of fura 2-AM; $R_{min}$ is the intensity of Ca$^{2+}$-free fura 2-AM after treatment with EGTA; $R_{max}$ is the maximum fluorescence intensity of Ca$^{2+}$-saturated fura 2-AM after treatment with...
digitonin, and $S_o/S_b$ is the ratio of fluorescence intensities after excitation at 380 nm for the probe at $R_{\min}$ and $R_{\max}$ (17).

The measurements of enterocyte [Ca$^{2+}$]$_i$ were conducted for 2–4 h after the loading of fura 2. Control experiments revealed that the cell viability remained at >95% for ≥4 h after fura 2 loading. Furthermore, the registration of basal [Ca$^{2+}$]$_i$ throughout the experiments served as an internal control, because viability tests (trypan blue exclusion and phase contrast microscopy) in combination with fluorospectrophotometry show that increased cell death is associated with an increase in the basal [Ca$^{2+}$]$_i$.

Effects of 1,25(OH)$_2$D$_3$ on [Ca$^{2+}$]$_i$. Effects of 1,25(OH)$_2$D$_3$ on [Ca$^{2+}$]$_i$ were investigated in enterocytes from carp. Intestinal cells were acclimated for 5 min in HBSS. The basal [Ca$^{2+}$]$_i$ was recorded for 150 s; then vehicle (10 μl ethanol; $n = 8$) or 1,25(OH)$_2$D$_3$ (130, 260, 650, 6,500, and 13,000 pM; $n = 8$ for each concentration tested) was added to the cuvette, and the [Ca$^{2+}$]$_i$ was recorded for another 150 s period.

Effects of 1,25(OH)$_2$D$_3$ on intracellular stores. Intestinal cells were acclimated in HBSS in the presence of 2 mM EGTA for 150 s, to produce Ca$^{2+}$-free incubation media and to let the enterocytes equilibrate at a new [Ca$^{2+}$]$_i$. The basal [Ca$^{2+}$]$_i$ was recorded for 150 s; then vehicle (10 μl ethanol; $n = 8$), 1,25(OH)$_2$D$_3$ (130, 260, 650, 6,500, and 13,000 pM; $n = 8$ for each concentration tested) or 20 nM 24,25(OH)$_2$D$_3$ was added to the cuvette, and the [Ca$^{2+}$]$_i$ was recorded for another 150 s.

Determination of PKC activity. For time course studies, 200 μl of the cell suspension (containing ~10$^6$ cells in HBSS, 0.1% BSA) were pipetted into microfuge tubes. Zero-time samples received 200 μl of medium, and subsequent time point samples received an equivalent volume containing either 130 pM 1,25(OH)$_2$D$_3$ (final concentration) or the vehicle ethanol (0.05%, final concentration). At the appropriate times, the tubes were removed to ice and then were centrifuged at 500 g for 10 min. The cell pellets were extracted with 0.6 ml of 20 mM Tris, pH 7.5, 10 mM mercaptoethanol, 0.5 mM EGTA, 0.5 mM EDTA, 0.5% Triton X-100, and 25 μg each of aprotinin and leupeptin, as previously described (23). The high-speed supernatants (14,000 g, 3 min) were diluted 1:10 with buffer that did not contain detergent, and 20 μl of the diluted extract were taken for analysis of enzyme activity, according to instructions supplied with the kit (GIBCO Life Technologies, Waverly, MA). Aliquots were incubated with lipid activator in the absence or presence of a specific PKC inhibitor for 20 min before the addition of buffer containing [35P]ATP-20 μM ATP and acetylated myelin basic protein (3–14). After an additional 5-min incubation at 23°C, 25 μl were spotted onto phosphocellulose disks, washed, and placed in vials for liquid scintillation spectrophotometry.

Values were related to extract protein as described by Lowry et al. (21).

Statistics. Specific 1,25(OH)$_2$D$_3$ 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 two-parameter hyperbolic equation

$$Y = \frac{a \cdot x}{b + x}$$

The coefficient of variation ($R^2$) and the adjusted coefficient of variation (adj$R^2$) were used as a measure of how well the regression model described the data (1). A one-way ANOVA with $F$-statistics was used to gauge the contribution of the independent variable to predict the dependent variable (1). $P < 0.05$ was considered statistically significant.

ANOVA was used to test for significant differences between control and treatment groups in each experiment, and an ANOVA followed by a Student-Newman-Keuls post hoc test was performed when a factor with more than two levels was used for comparison. The testing used was two tailed, and the significance level was set at $P < 0.05$. Data are presented as means ± SE.

RESULTS

Saturation analyses of [3H]1,25(OH)$_2$D$_3$ binding to membranes. Figure 1 illustrates the results of specific binding activity as a function of increasing concentrations of ligand in basal lateral membranes prepared from carp or Atlantic cod intestine. Specific binding in carp was observed to become half-saturable at slightly more than 1 nM ($K_d = 1.23 ± 0.77$ nM) with a maximal binding capacity ($B_{\text{max}}$) of 212 ± 52 fmol/mg protein. The saturable specific binding to carp basal lateral membranes of 1,25(OH)$_2$D$_3$ could be described as a hyperbolic function ($P < 0.05; F = 35.1, DF = 2; R^2 = 0.73; \text{adj}R^2 = 0.71$). No specific binding was observed in brush border membranes from this species (data not shown). Basal lateral membranes prepared from Atlantic cod exhibited much lower specific binding (Fig. 1), with values approaching the limits of sensitivity for the perchloric acid precipitation assay ($B_{\text{max}} = 28 ± 22$ fmol/mg protein and $K_d = 1.3 ± 2.6$ nM). The specific

![Fig. 1 Saturation analyses of [3H]1,25-dihydroxyvitamin D$_3$ ([3H]1,25(OH)$_2$D$_3$) binding in basal lateral membranes of fish. $B_{\text{max}}$, maximal binding capacity; $K_d$, dissociation constant; $R^2$, coefficient of variation; adj$R^2$, adjusted $R^2$. Membranes were isolated from carp (A) or Atlantic cod (B) by a combination of differential- and sucrose-gradient centrifugation, and membranes from 2 fish were pooled together in one sample and adjusted to 50 μg protein/200 μl TED buffer (10 mM Tris, 1.5 mM EDTA, 1mM dithiothreitol, pH 7.4). Triplicate aliquots were incubated (0°C, overnight) with 0.5, 1, 2, or 4 nM [3H]1,25(OH)$_2$D$_3$ in the absence of unlabeled steroid (total binding), or in the presence of a 200-fold molar excess of unlabeled hormone, also in triplicate, to determine nonspecific binding. Bound radioactivity was precipitated with perchloric acid in the presence of carrier bovine γ-globulin. The precipitate was collected by centrifugation, dissolved in 6 M guanidine-HCl, and transferred to vials for liquid scintillation counting. Values represent means ± SE; n = 3 experiments.](http://ajpendo.physiology.org/Downloadedfromhttp://ajpendo.physiology.org)
binding to Atlantic cod basal lateral membranes of 1,25(OH)_{2}D_{3} could not be described by a saturable hyperbolic function (P > 0.05; F = 1.35; DF = 12). Thus the level of the putative plasma membrane receptor for vitamin D (pmVDR) for 1,25(OH)_{2}D_{3} is distinctly different in intestinal membranes from freshwater and seawater fish. Subsequent binding studies were confined to basal lateral membranes from carp.

The data depicted in Fig. 1 for carp membranes were recalculated for Scatchard analysis (Fig. 2A) and Hill analysis (Fig. 2B). The Scatchard analysis showed no correlation when data were subjected to linear regression analysis (P > 0.05; R^2 = 0.48; adjR^2 = 0.22; F = 1.86; DF = 10; Fig. 2A). Subsequent Hill analysis yielded a curve which could be described by a linear regression (P < 0.05; R^2 = 0.73; adjR^2 = 0.70; F = 24.21; DF = 10) with an apparent Hill coefficient (n_{app}) of 1.22 ± 0.25 and a K_d of 2.04 nM (Fig. 2B).

**Protein dependency of [3H]1,25(OH)_{2}D_{3} binding to membranes.** Additional experiments were undertaken to characterize the binding protein for 1,25(OH)_{2}D_{3} in basal lateral membranes of carp intestine. Increasing amounts of membrane revealed that specific binding of the secosteroid hormone was linearly dependent on protein concentration (P < 0.05; F = -0.162 + 0.032x; Fig. 2A). (Fig. 2A) and Hill analysis (Fig. 2B). The Scatchard analysis showed no correlation when data were subjected to linear regression analysis (P > 0.05; R^2 = 0.48; adjR^2 = 0.22; F = 1.86; DF = 10; Fig. 2A). Subsequent Hill analysis yielded a curve which could be described by a linear regression (P < 0.05; R^2 = 0.73; adjR^2 = 0.70; F = 24.21; DF = 10) with an apparent Hill coefficient (n_{app}) of 1.22 ± 0.25 and a K_d of 2.04 nM (Fig. 2B).

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$R^2 = 0.98; \text{adj} R^2 = 0.98; F = 191.2, DF = 10)$, up to 100 µg.

**Membrane association of the binding protein.** Studies were undertaken to determine whether the binding activity was associated with basal lateral membranes as a peripheral protein extractable with high salt or whether the protein was integral to the lipid bilayer and required detergent for extraction. Membranes were homogenized (25 strokes on ice) in the presence of 300 mM KCl or 10 mM CHAPSO and then centrifuged. Both the supernatant fractions and resuspended pellet were tested for specific [3H]1,25(OH)₂D₃ binding. As illustrated in Fig. 3, high salt concentrations failed to solubilize the binding activity. In contrast, treatment with detergent at levels above the critical micellar concentration resulted in two successful solubilizations and one preparation in which binding activity was marginally increased in the supernatant after detergent treatment (Fig. 3).

**Specificity of [3H]1,25(OH)₂D₃ binding.** Basal lateral membranes prepared from carp intestine were incubated in the presence of 1 nM [3H]1,25(OH)₂D₃ and in the absence or presence of 200 nM unlabeled 1,25(OH)₂D₃, 25(OH)D₃, or 24R,25(OH)₂D₃. The amount of competition between labeled and unlabeled homologous ligand was set to 100%. As indicated in Fig. 4, neither of the naturally occurring heterologous ligands was an effective competitor with [3H]1,25(OH)₂D₃ ($P < 0.05$) for binding to basal lateral membranes. Approximately 40 and 20% competition was observed with 25(OH)D₃ or 24R,25(OH)₂D₃, respectively (Fig. 4).

**Effects of 1,25(OH)₂D₃ on [Ca²⁺].** The specific saturable binding observed in basal lateral membranes isolated from carp enterocytes suggested that the bind-

![Fig. 5. Representative tracings of intracellular Ca²⁺ concentration ([Ca²⁺]) from either extracellular or intracellular sources (A, B) or release of Ca²⁺ from intracellular stores (C-F) after administration of vehicle (A, C, E) 130 pM 1,25(OH)₂D₃ (B, D) or 20 nM 24R,25(OH)₂D₃ (F). Carp enterocytes were isolated by chelation as described in the text, loaded with fura 2, washed, and resuspended in Hanks’ balanced salt solution. Aliquots were removed to a cuvette for determination of baseline fluorescence before the addition ($t = 150$ s) of ethanol or the indicated vitamin D metabolite. Fluorescence measurements were continued for an additional 150 s.](http://ajpendo.physiology.org/)

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ing protein might have receptor-like properties, in particular, activation of intracellular signal transduction pathways. Studies were undertaken to determine whether 1,25(OH)₂D₃ altered intracellular Ca²⁺ levels in isolated intestinal cells of carp analogous to observations in other systems (14, 17, 23, 26). A range of 1,25(OH)₂D₃ concentrations was tested (130–1,300 pM hormone). Figure 5 depicts representative tracings of cells treated with either the vehicle ethanol (Fig. 5, A, C, and E), 130 pM 1,25(OH)₂D₃ (Fig. 5, B and D), or 20 nM 24R,25(OH)₂D₃ (Fig. 5F). As shown in Fig. 5, B and D, 130 pM 1,25(OH)₂D₃ had no influence on influx from external Ca²⁺ stores (Fig. 5B) or Ca²⁺ release from intracellular stores (Fig. 5D), as judged by fura 2 fluorescence. A similar lack of responsiveness was observed for the other hormone concentrations tested (data not shown). As a control of hormone-activated Ca²⁺ release, Fig. 5F illustrates a representative tracing in which 20 nM 24R,25(OH)₂D₃ induced a rapid increase in intracellular Ca²⁺ within the same preparation of enterocytes. This concentration of the metabolite 24R,25(OH)₂D₃ reproducibly induced a rapid increase in intracellular Ca²⁺ in each of eight preparations tested (data not shown).

Effect of 1,25(OH)₂D₃ on PKC activity. The effect of 130 pM 1,25(OH)₂D₃ on isolated intestinal cells from carp was tested. Figure 6 illustrates the results of replicate time course studies in which enhanced activity relative to control incubations was evident within 3 min of hormone treatment (P < 0.05), was optimal within 5 min (P < 0.05), and declined to control levels 10 min after 1,25(OH)₂D₃. In duplicate pilot studies, a 5-min exposure of isolated carp enterocytes to 20 nM 24R,25(OH)₂D₃ yielded a twofold stimulation in PKC activity relative to vehicle controls.

**DISCUSSION**

Freshwater fish and terrestrial vertebrates, in most cases, face a situation where they have either no direct exchange of Ca²⁺ with the environment or are subjected to a risk of losing Ca²⁺ to the environment. In contrast, seawater fish such as Atlantic cod are challenged by an overabundance of Ca²⁺. Thus hormones involved in increasing intestinal Ca²⁺ uptake, such as 1,25(OH)₂D₃, might be physiologically more important in freshwater than in seawater fish (14, 15, 31). Studies on rapid effects in fish reveal differences between freshwater- and seawater-adapted species. Freshwater-adapted European eel [Anguilla anguilla (5)] and tilapia [Oreochromis mossambicus (8)] both showed an acute increase in intestinal mucosa-to-serosa Ca²⁺ transport after treatment with 1,25(OH)₂D₃. In the marine teleost, Atlantic cod, no rapid effects on intestinal Ca²⁺ transport in vitro or [Ca²⁺]o of enterocytes could be observed by 1,25(OH)₂D₃ treatment (14, 15, 30). Instead, 25(OH)₂D₃ and 24,25(OH)₂D₃ exerted rapid effects on both Ca²⁺ transport in vitro (15, 30) and [Ca²⁺]i (14). The current work demonstrates that basal lateral membranes from carp enterocytes, but not from Atlantic cod intestinal epithelial cells, exhibit specific saturable binding for 1,25(OH)₂D₃. Both the affinity of the putative membrane receptor and the number of binding sites were found to be comparable to those observed in chick intestine (25). Furthermore, the affinity of the binding moiety was in the physiological range for circulating concentrations of 1,25(OH)₂D₃, as previously reported for carp (36), suggesting a physiological function. As in chick (25), the putative receptor was found to be an integral membrane protein that exhibited a higher affinity for 1,25(OH)₂D₃ than for 25(OH)₂D₃ or 24R,25(OH)₂D₃. Thus the data presented in this study, demonstrating differences in the expression of the putative pmVDR for 1,25(OH)₂D₃ in the freshwater carp compared with the seawater Atlantic cod, agree well with the previous studies on freshwater- and seawater-living fish. Specifically, the data indicate that the different outcomes in rapid effects of the vitamin D₃ metabolites may be due to an adaptation of the vitamin D₃ endocrine system to the specific Ca²⁺ availability encountered by each animal group. The combined results on rapid effects and expression of the putative pmVDR could also indicate a divergent evolution of the vitamin D₃ endocrine system in seawater fish compared with freshwater fish, birds, and mammals (14, 15, 31).

The specific binding moiety for 1,25(OH)₂D₃ in basal lateral membranes of enterocytes from carp showed indications for positive cooperative binding; Scatchard analysis indicated a convex curvature, and Hill analysis revealed an apparent Hill coefficient >1. These results are indicative of a putative receptor with at least two binding sites or the presence of several receptors. Positively cooperative binding has previously

![Fig. 6](http://ajpendo.physiology.org/DownloadedFrom/10.1152/ajpendo.00290.2017)
been reported for the classical 1,25(OH)₂D₃ nuclear receptor in chick intestine (18, 40) and in rat intestine and kidney (11). Furthermore, a membrane-associated form of the nuclear receptor for 1,25(OH)₂D₃ was reported in ROS 17/2.8 cells (13). These earlier results suggested that the 1,25(OH)₂D₃ binding protein in carp basal lateral membrane could be a membrane-associated form of the nuclear receptor for 1,25(OH)₂D₃. However, the patterns in which different vitamin D metabolites compete for the 1,25(OH)₂D₃ membrane receptor in carp differ from the patterns described for the classical nuclear receptor in fish (29) and terrestrial vertebrates (3). In the present work, supra-physiological concentrations of 24R,25(OH)₂D₃ and 25(OH)₂D₃ exhibited, respectively, 20 and 40% competition with [³H]1,25(OH)₂D₃ for binding to membrane preparations, whereas neither metabolite competed significantly with [³H]1,25(OH)₂D₃ for binding to the nuclear receptor (29). This indicates that the membrane receptor for 1,25(OH)₂D₃ in carp is of a different nature than the nuclear receptor for 1,25(OH)₂D₃. The binding activity, however, was not recognized by the antibody generated toward the NH₂ terminus of the putative chick membrane receptor by Western analyses (D. Larsson, unpublished observations).

In many systems of diverse cell types and species, rapid membrane-mediated effects of 1,25(OH)₂D₃, 25(OH)₂D₃, and 24R,25(OH)₂D₃ have been related to changes in intracellular Ca²⁺ as determined by fura 2 fluorescence (4, 6, 14, 19, 23, 26, 35). In the present study, administration of physiological 1,25(OH)₂D₃ concentrations to carp enterocytes failed to produce a change in [Ca²⁺]. 24R,25(OH)₂D₃ induced a rapid increase in intracellular Ca²⁺ release within the same preparation of intestinal epithelial cells, which agrees with previous findings in Atlantic cod and carp (14) and our unpublished observations. Thus the lack of 1,25(OH)₂D₃-mediated changes in [Ca²⁺], was not due to compromised or decreased activity. However, a very small localized change in [Ca²⁺] may yet be identified by ion imaging. Recent evidence indicates that changes in [Ca²⁺] are not necessarily responsible for signal transduction (23, 26). Furthermore, it is important to point out that the use of fluorospectrometry with fura 2 as a Ca²⁺ probe fails to detect any change in intracellular vesicular Ca²⁺ transport. In the chick duodenum, there is an increased vesicular Ca²⁺ transport associated with 1,25(OH)₂D₃-enhanced Ca²⁺ transport in vitamin D-deficient chicks (22), whereas 1,25(OH)₂D₃ was without effect on [Ca²⁺] in enterocytes from vitamin D-deficient chicks (24). Thus, in the current work, the inability of 1,25(OH)₂D₃ to mediate alterations in [Ca²⁺] in carp enterocytes is not without precedent, and a lack of fura 2 fluorescence does not necessarily mean an inability to influence Ca²⁺ transport.

As an indicator of receptor-mediated hormone action, PKC activation has also been reported to occur in response to 1,25(OH)₂D₃ in a variety of systems (6, 20, 23, 26, 34, 39). In the present work, 130 pM 1,25(OH)₂D₃ was observed to mediate a greater than threefold increase in PKC activity within 5 min, relative to control incubations. The maximal time point for stimulation, and the subsequent decline in PKC activity to levels comparable to controls, is similar to the time course observed in isolated chick intestinal cells (23). Furthermore, the increased PKC activity in carp enterocytes was not associated with increased [Ca²⁺]. These results are contradictory to previous reports from isolated rat colonocytes and Caco2 cells, where treatment with 1,25(OH)₂D₃ increased both [Ca²⁺] and the activity of PKC-α (2, 39). It may be possible that 1,25(OH)₂D₃ mediated an increase in the activity of a PKC isoform that is different from PKC-α in carp enterocytes. This has been demonstrated in rat colonocytes, where 1,25(OH)₂D₃ activated Ca²⁺-dependent (PKC-α) as well as Ca²⁺-independent (PKC-δ, -ε and -ζ) isoforms of PKC. Little is known about the regulation and functions of the Ca²⁺-independent PKC isoforms. Frawley et al. (10) demonstrated that PKC-ζ was implicated in regulating gene expression induced by growth factors and hormones. Thus activation of PKC is not necessarily dependent on elevation of the [Ca²⁺]. Indeed, in enterocytes from vitamin D-deficient chicks, 1,25(OH)₂D₃ fails to stimulate increases in intracellular Ca²⁺ and protein kinase A; the hormone does stimulate PKC and 45Ca extrusion (24). Thus PKC activation may be associated with exocytosis of vesicular transport calcium (24).

In conclusion, a receptor-like moiety for 1,25(OH)₂D₃ is present in the basal lateral membranes of carp intestine but is absent in basal lateral membranes of Atlantic cod. The biochemical characteristics are similar to those reported earlier for chick intestinal basal lateral membranes (23). Thus the results presented in this paper, together with data on rapid effects of vitamin D₃ metabolites in different animal groups, suggest that the system mediating rapid responses to 1,25(OH)₂D₃ is evolutionarily conserved and therefore physiologically important for animals living in a low-Ca²⁺ environment.

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REFERENCES


