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Putative Basal Lateral Membrane Receptors for 24,25-Dihydroxyvitamin D_3 in Carp and Atlantic Cod Enterocytes: Characterization of Binding and Effects on Intracellular Calcium Regulation

Dennis Larsson,[1,2]* Ilka Nemere,[2] and Kristina Sundell[1]

[1]Department of Zoology, Fish Endocrinology Laboratory, Göteborg University, Göteborg, Sweden
[2]Department of Nutrition and Food Sciences and the Biotechnology Center, Utah State University, Logan, Utah

Abstract The vitamin D metabolite, 24*R*,25-dihydroxyvitamin D_3 (24*R*,25$(OH)_2D_3$), was tested for its ability to specifically bind to basal lateral membranes isolated from intestinal epithelium of Atlantic cod (a seawater fish), carp (a freshwater fish), and chicken. Specific saturable binding was demonstrated in membranes from all three species. Membranes from Atlantic cod, carp, and chicken revealed K_d's of 7.3±0.9, 12.5±0.9 and 7.8±0.1 nM, and a B_{max} for each species estimated to 57.9±2.9, 195.1±8.4 and 175±0.8 fmol/mg protein, respectively. Scatchard analyses indicated a convex curvature and Hill analyses revealed apparent Hill coefficients of 1.84±0.28, 1.80±0.29, and 1.78±0.27 for Atlantic cod, carp and chicken, suggesting a positive cooperative binding in all three species. Basal lateral membranes from Atlantic cod and carp were used to further characterize the binding moiety. In competition studies, basal lateral membranes from Atlantic cod or carp did not discriminate between 24*R*,25$(OH)_2D_3$ and the 24*S*,25$(OH)_2D_3$ isomer, whereas, 1,25$(OH)_2D_3$ and 25$(OH)D_3$, were less effective in competing with [^{3}H]24*R*,25$(OH)_2D_3$ for binding to basal lateral membranes in Atlantic cod and carp. In both the Atlantic cod and carp enterocyte basal lateral membranes, the binding activity could be extracted equally well with high salt as with detergent, indicating a peripheral membrane protein rather than an integral membrane binding protein. Finally, isolated Atlantic cod and carp enterocytes were chosen for analyses of signal transduction events mediated by the putative receptor. In both species, 24*R*,25$(OH)_2D_3$ but not 24*S*,25$(OH)_2D_3$, suppressed Ca^{2+}-uptake by enterocytes in a dose-dependent manner. Enterocytes from Atlantic cod and carp, acclimated to Ca^{2+}-free media, responded by an intracellular Ca^{2+}-release within seconds after addition of 24*R*,25$(OH)_2D_3$ or 24*S*,25$(OH)_2D_3$. The effects on intracellular Ca^{2+}-release were dose-dependent for both metabolites. 24*S*,25$(OH)_2D_3$ was effective at lower concentrations and triggered a higher response compared to 24*R*,25$(OH)_2D_3$. These results suggest that the binding molecule(s) for 24*R*,25$(OH)_2D_3$ and 24*S*,25$(OH)_2D_3$ is/are capable of acting as a receptor, mediating rapid, non-genomic responses in intestinal cells. J. Cell. Biochem. 83: 171–186, 2001.

Key words: fish; intestine; non-genomic effects; pmVDR; 24*R*,25-dihydroxyvitamin D_3; 24*S*,25-dihydroxyvitamin D_3

The vitamin D_3 endocrine system is an important regulator of calcium [Ca^{2+}] homeostasis. 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) has been considered as the principal mediator of biological activities, modulating ion transport processes in target tissues such as intestine, bone and kidney [Bouillon et al., 1995; Jones et al., 1998]. However, there is increasing evidence for a physiological function of the vitamin D_3 metabolite, 24*R*,25-dihydroxyvitamin D_3 (24*R*,25$(OH)_2D_3$). Specific binding for 24*R*,25$(OH)_2D_3$ has been demonstrated in

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Dennis Larsson's present address is Department of Nutrition & Food Sciences, Utah State University, Logan, UT 84322-8700.

*Correspondence to: Dennis Larsson, PhD, Department of Nutrition & Food Sciences, Utah State University, Logan, UT 84322-8700. E-mail: dennis.larsson@zool.gu.se or larsson@cc.usu.edu

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cytosol and nuclear fractions from chicken and rat bone [Sömjen et al., 1982a,b], rabbit cartilage [Corvol et al., 1980] and the chicken parathyroid gland [Merke and Norman, 1981]. Long-term exposure to 24*R*,25$(OH)_2D_3$ has been shown to inhibit parathyroid hormone secretion in the dog [Canterbury et al., 1978] and improve bone formation in chicken, rabbit, and rat [Ornoy et al., 1978; Malluche et al., 1980; Matsumoto et al., 1992; Nakamura et al., 1992]. In experiments with mice lacking the gene for 24-hydroxylase, and thus lacking the ability to produce 24,25$(OH)_2D_3$, unmineralized osteoid was found on the endothelial surface of cortical bone [St-Arnaud et al., 1996]. Thus, 24,25$(OH)_2D_3$ might have a classic steroid action on bone formation and parathyroid hormone secretion.

In addition to mediating physiological responses via the classical steroid receptor, a specific binding moiety for 24*R*,25$(OH)_2D_3$ has been demonstrated in basal lateral membranes and lysosomes from chicken enterocytes [Nemere et al., 1994; Nemere, 1999; Atkinson, Johns and Larsson, unpublished observations], rat chondrocytes [Pedrozo et al., 1999] and in fracture-healing callus from chicken tibial bone [Seo et al., 1996; Kato et al., 1998]. 24*R*,25$(OH)_2D_3$ binding to this putative receptor has been suggested to mediate rapid, nongenomic effects, in chondrocytes [Boyan et al., 1997], osteoblasts [Farach-Carson and Ridall, 1998] and on intestinal Ca^{2+}-uptake [Sundell and Björnsson, 1990; Larsson et al., 1995; Nemere, 1999]. In intestinal cells (enterocytes) and osteoblasts, 24*R*,25$(OH)_2D_3$ has been demonstrated to mediate rapid inhibition of L-type Ca^{2+} channels [Caffrey and Farach-Carson, 1989; Li et al., 1996; Takeuchi and Guggino, 1996; Larsson et al., 1997; Li et al., 1997; Larsson, 1999; Nemere, 1999] and Ca^{2+}-release from intracellular stores [Lieberherr, 1987; Larsson et al., 1997; Larsson, 1999].

Teleost fish comprises the greatest number of living species among the vertebrates. During evolution, new species have evolved and today, teleosts inhabit both marine and freshwater environments. Most species are strictly marine or freshwater living (stenohaline), but a few species, such as the eel (*Anguilla*) and salmon (*Salmonidae*) have the ability to migrate between the two environments. The great diversity among teleost fish and the different species ability to regulate their free circulating plasma Ca^{2+} levels within a narrow range, independent of a constant stress of either losing (freshwater) or gaining (seawater) Ca^{2+} from the environment, makes this vertebrate group a unique model for research on endocrine regulation of calcium homeostasis and the evolution of these endocrine systems. Long-term exposure (24 h) to 1,25$(OH)_2D_3$ increase intestinal Ca^{2+}-uptake together with total and free plasma Ca^{2+} concentrations in both freshwater and marine fish [Sundell et al., 1996]. No long-term effects have been reported for 24,25$(OH)_2D_3$ [Sundell et al., 1996]. In the short-term calcium regulation (affecting homeostasis within seconds to minutes), 1,25$(OH)_2D_3$ and 24*R*,25$(OH)_2D_3$ have different actions on intestinal Ca^{2+}-uptake in marine fish [Sundell and Björnsson, 1990; Larsson et al., 1995; Nemere et al., 2000] compared to freshwater living fish [Chartier et al., 1979; Flik et al., 1982; Nemere et al., 2000]. In the marine Atlantic cod (*Gadus morhua*), 24*R*,25$(OH)_2D_3$ decreases intestinal Ca^{2+}-influx, whereas 1,25$(OH)_2D_3$ is without effect [Sundell and Björnsson, 1990; Larsson et al., 1995]. In freshwater fish, on the other hand, 1,25$(OH)_2D_3$ increases intestinal Ca^{2+}-influx [Chartier et al., 1979; Flik et al., 1982], whereas no short-term effects of 24*R*,25$(OH)_2D_3$ have been reported for freshwater acclimated or freshwater living fish.

Thus, the aims of this study were to: (1) examine the possible existence of a specific binding moiety for 24*R*,25$(OH)_2D_3$ in basal lateral membranes of enterocytes from a bird, (White Leghorn cockerel), a freshwater teleost, carp (*Cyprinus carpio*), and a marine teleost, Atlantic cod (*Gadus morhua*), (2) investigate the cellular action of 24*R*,25$(OH)_2D_3$ on enterocyte Ca^{2+} homeostasis in carp and Atlantic cod, and (3) compare possible differences in the cellular action of 24*R*,25$(OH)_2D_3$ on enterocyte Ca^{2+} homeostasis and specific binding moiety, in animals living in Ca^{2+}-rich or Ca^{2+}-deficient environments.

MATERIALS AND METHODS

Animals

Care and experimental procedures for fish were approved by the Swedish ethical committee for animal research, while care and experimental procedures for chickens were approved by the Utah State University Institutional Animal Care and Use Committee.

Carp of both sexes (body weight 200–300 g) were purchased from a local hatchery, Aneboda Aqua Service AB and were acclimated in recirculated, filtered, and aerated freshwater at 15°C for at least 5 days prior to the experiments. The carp were fed daily, until satiation, a plant diet consisting of duckweed (*Lemna minor*). Atlantic cod of both sexes (body weight 300–500 g) were caught off the west coast of Sweden and kept in recirculated, filtered, and aerated seawater at 10°C for 5–10 days before sacrifice. The Atlantic cod were not fed during the acclimation time. Chickens (White Leghorn cockerels) were obtained on the day of hatch (Merrill Poultry, Poul, ID) and raised on a vitamin D-replete diet for 4–6 weeks prior to use.

Preparation of Basal Lateral Membranes and Saturation Analyses of [^{3}H]24*R*,25(OH)$_2$D$_3$ Binding to Membranes

Preparation of chicken basal lateral membranes was as reported by Nemere et al. [1986] and Nemere [1996] using a combination of differential and Percoll gradient centrifugation. Basal lateral membranes from fish was prepared by differential centrifugation and sucrose gradient centrifugation as previously described by Nemere et al. [2000].

The protein concentrations of the different membrane preparations were measured according to Lowry et al. [1951].

Membranes isolated from Atlantic cod, carp, or chicken were adjusted to 50 μg protein/200 μl TED buffer and incubated in triplicate for total binding ([^{3}H]24*R*,25(OH)$_2$D$_3$; 76 Ci/mmol), and in duplicate for nonspecific binding ([^{3}H]24*R*,25(OH)$_2$D$_3$ 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. Bound hormone was separated from free by use of hydroxylapatite (HAP) as described by Nemere et al. [1994]. In short, the HAP-bound receptor (and ligand) were pelleted at 1500*g* for 4 min, the supernatant decanted, and the pellet washed three times with 0.5% Triton X-100 in TED. The pellet was treated with ethanol to extract ligand and centrifuged at 1500*g* for 4 min. The resulting supernatant was transferred to scintillation vials, the ethanol evaporated, and the amount of [^{3}H]24*R*,25(OH)$_2$D$_3$ in each sample was assessed using a Wallac 1409 β-scintillation counter.

Characterization of [^{3}H]24*R*,25(OH)$_2$D$_3$ Binding to Membranes

Time course of 24*R*,25(OH)$_2$D$_3$ binding. Time dependence of [^{3}H]24*R*,25(OH)$_2$D$_3$ binding to basal lateral membranes from Atlantic cod and carp was monitored. In both species, membranes (50 μg protein/200 μl TED) were incubated with 16 nM [^{3}H]24*R*,25(OH)$_2$D$_3$ in the absence or presence of unlabeled seco-steroid for 0.5, 1, 2, 4 or 24 h, and were analyzed for total and nonspecific binding.

Protein dependence of binding. [^{3}H]24*R*,25(OH)$_2$D$_3$ binding to basal lateral membranes of Atlantic cod and carp enterocytes was investigated for protein dependency. Increasing concentrations of protein (25–150 μg) were incubated (0°C, overnight) with 16 nM [^{3}H]24*R*,25(OH)$_2$D$_3$ in the absence or presence of unlabeled seco-steroid, and analyzed for total and nonspecific binding.

Membrane association. Membrane association of the [^{3}H]24*R*,25(OH)$_2$D$_3$ binding moiety was investigated in basal lateral membranes of Atlantic cod and carp enterocytes. Aliquots of membranes were homogenized in the presence of 300 mM KCl (final concentration) or 10 mM CHAPSO (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 incubated with 16 nM [^{3}H]24*R*,25(OH)$_2$D$_3$ and analyzed for total and nonspecific binding.

Isolation of Intestinal Epithelial Cells

Enterocytes from Atlantic cod and carp were isolated according to Larsson et al. [1998] and cell viability was determined by trypan blue exclusion in combination with phase contrast microscopy and was found to be larger than 95%.

Loading of Fura-2/AM

The fura-2/AM loading was performed as described by Thomas and Delaville [1991]. Briefly, freshly isolated intestinal epithelial cells were incubated 45 min in Hanks Balanced Salt Solution (HBSS; 120 mM NaCl, 20 mM Hepes-Tris, 10 mM glucose, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, at pH 7.3), with fura-2/AM (5 μM), pluronic F-127 (0.025%) and albumin (0.5%) at 37°C. The cells were washed three times by centrifugation at 700*g* for 10 min, and finally resuspended in HBSS.

Measurement of Free Intracellular Ca^{2+} Concentrations

Measurements of $[Ca^{2+}]_i$ in fura-2/AM loaded Atlantic cod and 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 Inc., 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 10°C for enterocytes from Atlantic cod and 15°C for enterocytes from carp. According to the different experimental protocols (described below), stock solutions of 24*R*, $25(OH)_2D_3$ and 24*S*,$25(OH)_2D_3$ (10 μl), calcium (100 μl) and EGTA (10 μl) were added directly to the cuvette to give the final concentration stated under a given experimental protocol. Ethanol, the vehicle for both vitamin D_3 metabolites, was added to control cell suspensions at a final concentration equivalent to that in treated cell suspensions. Recordings of $[Ca^{2+}]_i$ were performed every second, and followed throughout a time period of 300–450 s. Fluorometric calibrations were performed by addition of digitonin (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. Intracellular calcium concentrations $[Ca^{2+}{}_i]$ were calculated using the equation:

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

where K_d is the dissociation constant for fura-2 and corresponds to 360 nM at 10°C and 338 nM at 15°C [Larsson et al., 1999], R is the fluorescence of fura-2/AM, R_{min} is the intensity of Ca^{2+}-free fura-2, R_{max} is the maximum fluorescence intensity of Ca^{2+}-saturated fura-2, and S_f/S_b is the ratio of fluorescence intensities after excitation at 380 nm, for the probe at R_{min} and R_{max}, respectively [Grynkiewicz et al., 1985].

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 above 95% for at least 4 h after fura-2 loading. Furthermore, monitoring of basal $[Ca^{2+}]_i$ throughout the experiments served as an internal control, as viability tests (trypan blue exclusion and phase contrast microscopy) in combination with fluorospectrophotometry showed that increased cell death was associated with an increase in the basal $[Ca^{2+}]_i$.

Experimental Protocol

Effects on enterocyte Ca^{2+}-uptake. Intestinal cells were acclimated in a Ca^{2+}-free HBSS, in the presence of vehicle (n = 10), 24*R*,$25(OH)_2D_3$ (0.5, 1, 5.0, and 20 nM; n = 6–10 for each concentration tested) or 24*S*,$25(OH)_2D_3$ (0.5, 1, 5.0, and 20 nM; n = 6–10 for each concentration tested) for 300 s before the start of the experiment. The basal $[Ca^{2+}]_i$ was recorded for 150 s, then Ca^{2+} (final concentration 10 mM) was added to the cuvette and the fluorescence of $[Ca^{2+}]_i$ recorded for another 150 s period.

Effects on intracellular Ca^{2+} stores. Intestinal cells were acclimated in Ca^{2+}-free buffer in the presence of 2 mM EGTA for 150 s, in order to get a Ca^{2+}-free incubation media and to let the enterocytes establish a new basal $[Ca^{2+}]_i$. The basal $[Ca^{2+}]_i$ was recorded for 150 s, then vehicle (n = 8), 24*R*,$25(OH)_2D_3$ (0.5, 1, 5.0, and 20 nM; n = 6–10 for each concentration tested) or 24*S*,$25(OH)_2D_3$ (0.5, 1, 5.0, and 20 nM; n = 6–10 for each concentration tested) was added to the cuvette, and the fluorescence of $[Ca^{2+}]_i$ recorded for another 150 s.

Statistics

Specific 24*R*,$25(OH)_2D_3$ 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 three-parameter sigmoid equation:

$$Y = \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}}$$

The coefficient of variation (R^2) and the adjusted coefficient of variation ($adjR^2$), were used as a measure of how well the regression model describes the data in the saturation and Hill analysis [Altman, 1991]. A one-factorial analysis of variance (ANOVA) with F-statistics was used to gauge the contribution of the independent variable to predict the dependent variable [Altman, 1991]. $P < 0.05$ was considered as statistically significant.

A one-factorial ANOVA followed by a Student–Newman–Keuls posthoc test was used to

test for significant differences between 24*R*, 25(OH)$_2$D$_3$, 24*S*,25(OH)$_2$D$_3$, 25(OH)D$_3$, and 1,25(OH)$_2$D$_3$ ability to compete with [^{3}H]24*R*, 25(OH)$_2$D$_3$, for binding to a putative membrane associated receptor protein in basal lateral membranes from Atlantic cod and carp. The testing used was two-tailed, and the significance level was set at $P < 0.05$. Data are presented as mean±SEM.

A two-factorial ANOVA was used to test for significant differences in Ca^{2+}-uptake and intracellular Ca^{2+}-release between groups exposed to equal concentrations of 24*R*,25 (OH)$_2$D$_3$ or 24*S*,25(OH)$_2$D$_3$ and between different concentrations of 24*R*,25(OH)$_2$D$_3$ or 24*S*, 25(OH)$_2$D$_3$, respectively. In cases with factors containing more than two levels a Student–Newman–Keuls posthoc test was performed. The testing used was two-tailed, and the significance level was set at $P < 0.05$. Data are presented as mean±SEM.

RESULTS

Saturation Analyses of 24*R*,25(OH)$_2$D$_3$ Binding to Membranes

Figure 1 illustrates the specific binding of [^{3}H]24*R*,25(OH)$_2$D$_3$ as a function of increasing concentrations of ligand in basal lateral membranes prepared from Atlantic cod (Fig. 1A), carp (Fig. 1B) and chicken intestine (Fig. 1C). The K_d of the specific binding in Atlantic

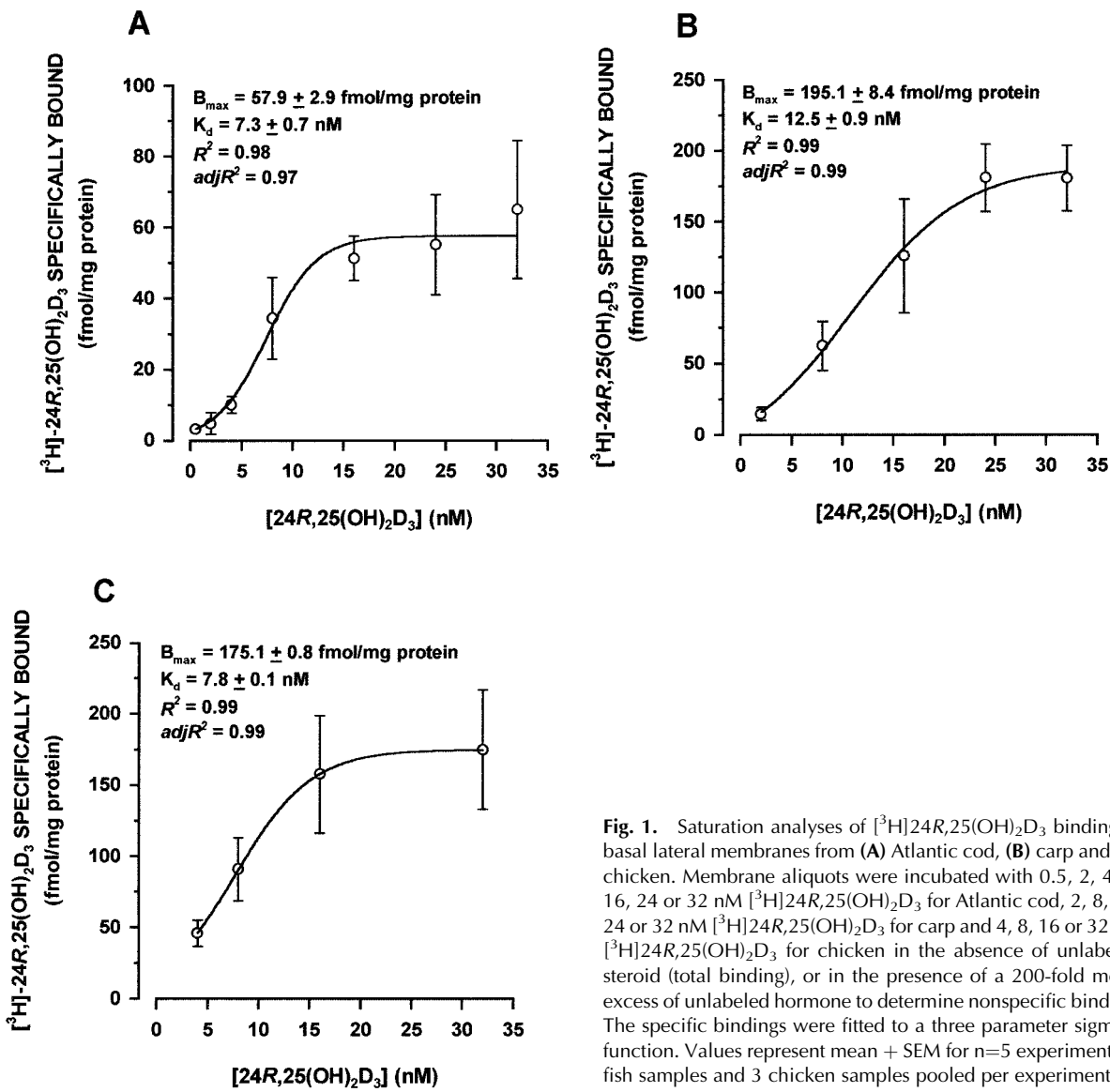

Fig. 1. Saturation analyses of [^{3}H]24*R*,25(OH)$_2$D$_3$ binding in basal lateral membranes from **(A)** Atlantic cod, **(B)** carp and **(C)** chicken. Membrane aliquots were incubated with 0.5, 2, 4, 8, 16, 24 or 32 nM [^{3}H]24*R*,25(OH)$_2$D$_3$ for Atlantic cod, 2, 8, 16, 24 or 32 nM [^{3}H]24*R*,25(OH)$_2$D$_3$ for carp and 4, 8, 16 or 32 nM [^{3}H]24*R*,25(OH)$_2$D$_3$ for chicken in the absence of unlabeled steroid (total binding), or in the presence of a 200-fold molar excess of unlabeled hormone to determine nonspecific binding. The specific bindings were fitted to a three parameter sigmoid function. Values represent mean + SEM for n=5 experiments, 2 fish samples and 3 chicken samples pooled per experiment.

cod, carp and chicken was determined to be 7.3±0.7 nM, 12.5±0.9 nM and 7.8±0.1 nM, respectively. The B_{max} were estimated to be 57.9±2.9 fmol/mg protein, 195.1±8.4 fmol/mg protein, 175.1±0.8 fmol/mg protein, respectively. The saturable, specific binding to basal lateral membranes followed a sigmoid function in Atlantic cod ($P < 0.05$; $F = 90.1$; $DF = 6$; $R^2 = 0.98$; $adjR^2 = 0.97$), carp ($P < 0.05$; $F = 129$; $DF = 4$; $R^2 = 0.99$; $adjR^2 = 0.99$) and chicken ($P < 0.05$; $F = 8393$; $DF = 3$; $R^2 = 0.99$; $adjR^2 = 0.99$). No specific binding was found in the brush border membranes in any of the species examined (data not shown).

The data presented in Figure 1 were further analyzed by Scatchard analysis (data not shown) and Hill analysis (Fig. 2). The Scatchard plots did not follow a linear relationship in Atlantic cod ($P > 0.05$; $F = 0.02$; $DF = 5$), carp ($P > 0.05$; $F = 0.62$; $DF = 4$) or chicken ($P > 0.05$; $F = 3.78$; $DF = 3$). The form of the Scatchard plots rather suggested a positive cooperativity for hormone-receptor binding in all three species, as indicated by the convex curvature. When the data were subjected to Hill analysis, the curves followed linear regression in Atlantic cod (Fig. 2A; $P < 0.05$; $F = 45.9$; $DF = 5$; $R^2 = 0.92$; $adjR^2 = 0.90$), carp (Fig. 2B; $P < 0.05$; $F = 99.8$; $DF = 4$; $R^2 = 0.97$; $adjR^2 = 0.96$) and chicken (Fig. 2C; $P < 0.05$; $F = 42.8$; $DF = 3$; $R^2 = 0.95$; $adjR^2 = 0.93$), with apparent Hill coefficients (n_{app}) of 1.84±0.28, 1.80±0.29 and 1.78±0.27, and with K_d's estimated to 6.91, 10.0, and 6.75 nM in Atlantic cod, carp and chicken, respectively.

Characterization of [^{3}H]24*R*,25(OH)$_2$D$_3$ Binding to Membranes

Time course of 24*R*,25(OH)$_2$D$_3$ binding. Using basal lateral membranes from Atlantic cod and carp enterocytes, the time course of specific binding of 16 nM [^{3}H]24*R*,25(OH)$_2$D$_3$ was monitored. The maximal binding was obtained after 4 h at 0°C and sustained for 24 h, for both species investigated.

Protein dependence of 24*R*,25(OH)$_2$D$_3$ binding. Basal lateral membranes prepared from Atlantic cod and carp enterocytes were assessed for specific binding of 16 nM [^{3}H]24*R*,25(OH)$_2$D$_3$ in the presence of increasing concentrations of protein. Binding was linear between 25–150 μg of membrane protein from Atlantic cod ($f = -0.640 + 0.087 \cdot X$; $P < 0.05$; $F = 28.69$; $DF = 3$; $R^2 = 0.93$; $AdjR^2 = 0.90$) and

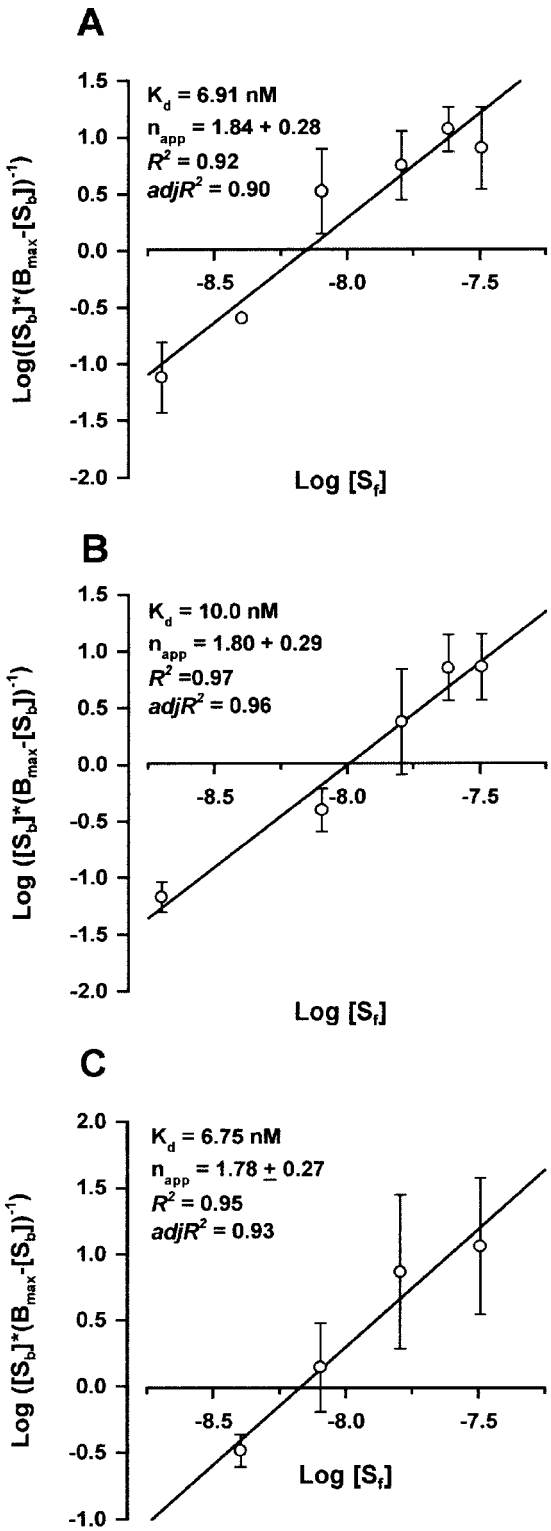

Fig. 2. Hill analyses of binding data from **(A)** Atlantic cod, **(B)** carp and **(C)** chicken. Data presented in Figure 1A were transformed for further analyses. [S_b] represent the specifically bound fraction of the administrated total free ([S_f]) [^{3}H]24*R*,25(OH)$_2$D$_3$. The K_d and the apparent Hill coefficient (n_{app}) were calculated using linear regression.

between 25–100 μg of protein in carp ($f = 1.756 + 0.033 \cdot X$; $P < 0.05$; $F = 55.67$; $DF = 2$; $R^2 = 0.98$; $adjR^2 = 0.96$).

Specificity of 24*R*,25(OH)$_2$D$_3$ binding. Competition studies were undertaken to determine the relative specificity of [^{3}H]24*R*,25(OH)$_2$D$_3$ binding to basal lateral membranes isolated from Atlantic cod or carp intestine. For both Atlantic cod (Fig. 3A) and carp (Fig. 3B), the 24*S*,25(OH)$_2$D$_3$ isomer was as effective as 24*R*,25(OH)$_2$D$_3$ in competing with [^{3}H]24*R*,25(OH)$_2$D$_3$ for binding to basal lateral membranes (92.3±5.9% and 96.5±6.0%, respectively). The ability of 1,25(OH)$_2$D$_3$ to compete with [^{3}H]24*R*,25(OH)$_2$D$_3$ for binding to basal lateral membranes was 51.7±22.6% of the value obtained for 24*R*,25(OH)$_2$D$_3$ in membranes from Atlantic cod (Fig. 3A; $P < 0.05$), whereas the corresponding value for carp was 23.4±16.6% (Fig. 3B; $P < 0.05$). The ability of 25(OH)D$_3$ to compete with [^{3}H]24*R*,25(OH)$_2$D$_3$ for binding to basal lateral membranes in Atlantic cod was 49.8±28.7% (Fig. 3A; $P < 0.05$), whereas in carp the value was 72.8±13.3%.

Membrane association of [^{3}H]24*R*,25(OH)$_2$D$_3$-binding. To determine the association of the 24*R*,25(OH)$_2$D$_3$ binding moiety with the membrane, basal lateral membranes were homogenized (25 strokes on ice) in either 300 mM KCl in TED or 10 mM CHAPSO in TED. After centrifugation, both the supernatant and the resuspended pellet were assayed for specific binding to 16 nM [^{3}H]24*R*,25(OH)$_2$D$_3$. No difference in specific binding was observed between extraction with high salt or detergent for membranes from Atlantic cod (Fig. 4A) or carp (Fig. 4B).

Effects of 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ on $[Ca^{2+}]_i$ in Enterocytes

Effects on extracellular Ca^{2+}-uptake. Representative recordings from spectrofluorometric analyses of Ca^{2+}-uptake in enterocytes preexposed to vehicle, 20 nM 24*R*,25(OH)$_2$D$_3$ or 20 nM 24*S*,25(OH)$_2$D$_3$ are presented in Figure 5A. Addition of Ca^{2+} (final concentration 10 mM) to the incubation medium resulted in an initial increase in $[Ca^{2+}]_i$ of vehicle treated enterocytes (Fig. 5A, ~70 % for Atlantic cod; Fig. 5B, ~45 % for carp), which leveled out at a new, stable $[Ca^{2+}]_i$, after 300 s (Fig. 5A,B). Thus, the rate of initial $[Ca^{2+}]_i$ change during the first 18 s (t = 150–168 s) after addition of

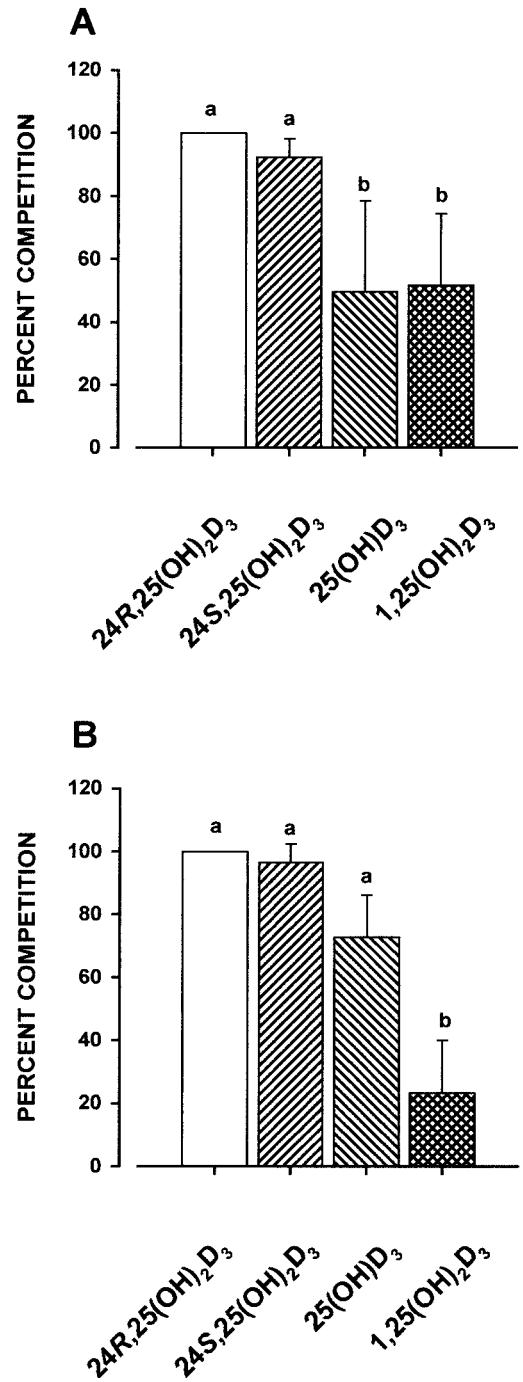

Fig. 3. Competition of vitamin D metabolites with [^{3}H]24*R*,25(OH)$_2$D$_3$ for binding to Atlantic cod **(A)** or carp **(B)** basal lateral membranes. Membrane aliquots were incubated with 16 nM [^{3}H]24*R*,25(OH)$_2$D$_3$ alone (total binding), or in the presence of a 200-fold molar excess of 24*R*,25(OH)$_2$D$_3$ (nonspecific binding), 24*S*,25(OH)$_2$D$_3$, 25(OH) D$_3$, or 1,25 (OH)$_2$D$_3$. Specific binding with [^{3}H]24*R*,25(OH)$_2$D$_3$ and homologous competing ligand was set to 100%. Different letters indicate significant differences between groups using a one-factorial ANOVA with 4 levels followed by Student–Newman–Keuls multiple comparison test ($P < 0.05$).

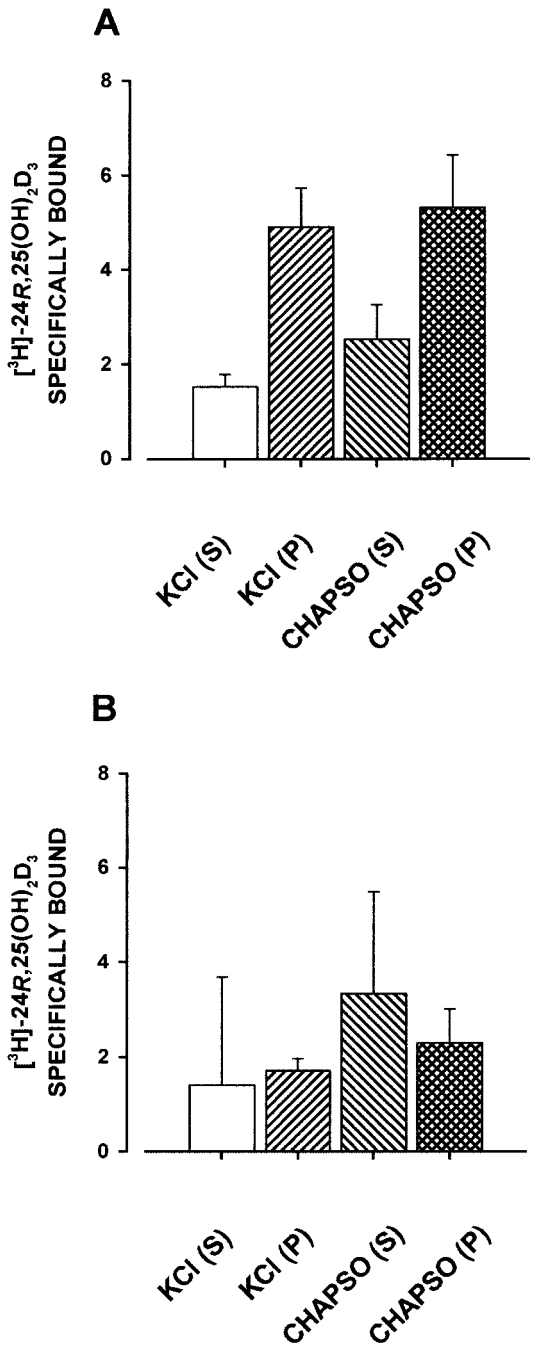

Fig. 4. Membrane association of the [^{3}H]24*R*,25(OH)$_2$D$_3$ binding moiety in basal lateral membranes from **(A)** Atlantic cod and **(B)** carp. Membrane aliquots were homogenized in the presence of 300 mM KCl or 10 mM CHAPSO to determine if the 24,25(OH)$_2$D$_3$ binding proteins were associated or incorporated in the membrane. After centrifugation, aliquots of the supernatants (S) and pellets (P) were incubated with 16 nM [^{3}H]24*R*,25(OH)$_2$D$_3$ in the absence or presence of unlabeled seco-steroid. Values represent mean±SEM for n = 3 experiments.

Ca^{2+} and the mean final $[Ca^{2+}]_i$ (t = 250–300 s) were calculated as percent change from the mean basal $[Ca^{2+}]_i$ (t = 0–149 s) and are presented in Table I. The initial $[Ca^{2+}]_i$ increase was dose-dependently (0.5–20 nM) suppressed by 24*R*,25(OH)$_2$D$_3$ in both Atlantic cod and carp (Table I), whereas 24*S*,25(OH)$_2$D$_3$ in the same concentration range did not affect the initial $[Ca^{2+}]_i$ increase (Table I). The final $[Ca^{2+}]_i$ was not affected by any of the two 24,25(OH)$_2$D$_3$ isoforms, in either of the two species (Table I; Fig. 5).

Effects on intracellular Ca^{2+} stores. Representative recordings of intracellular Ca^{2+}-release after treatment with vehicle (10 μl ethanol), 20 nM 24*R*,25(OH)$_2$D$_3$ or 20 nM 24*S*,25(OH)$_2$D$_3$ are presented in Figure 6A–F, respectively. The typical pattern of changes in $[Ca^{2+}]_i$ after administration of 24,25(OH)$_2$D$_3$ to enterocytes, in the absence of extracellular Ca^{2+}, was an initial increase in $[Ca^{2+}]_i$, which leveled out at a new stable $[Ca^{2+}]_i$ level (Fig. 6C–F). For comparison of the physiological effects of the two 24,25(OH)$_2$D$_3$ isomers, the maximal $[Ca^{2+}]_i$ concentration (peak value) occurring within 10 s after hormone administration (t = 150–160 s) and the mean final $[Ca^{2+}]_i$ (t = 275–300 s), were calculated as percent change from the mean basal $[Ca^{2+}]_i$ (t = 0–149 s) and are presented in Table II. Administration of 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ resulted in a rapid, dose-dependent increase in $[Ca^{2+}]_i$, as judged by the peak $[Ca^{2+}]_i$, in both Atlantic cod and carp (Table II). Enterocytes exposed to 24*S*,25(OH)$_2$D$_3$ showed a dose-dependent increase in the final $[Ca^{2+}]_i$ in both species examined, whereas 24*R*,25(OH)$_2$D$_3$ only increased the final $(Ca^{2+})_i$ in carp (Table II). Treatment with 5 nM and 20 nM 24*S*,25(OH)$_2$D$_3$ evoked a significantly higher initial $[Ca^{2+}]_i$ increase than administration of the same concentrations of 24*R*,25(OH)$_2$D$_3$ (Table II). The same pattern was observed when the effects of 24*S*,25(OH)$_2$D$_3$ and 24*R*,25(OH)$_2$D$_3$ was compared for their ability to increase the final $[Ca^{2+}]_i$, showing that enterocytes treated with 24*S*,25(OH)$_2$D$_3$ have higher final $[Ca^{2+}]$ compared to enterocytes treated with 24*R*,25(OH)$_2$D$_3$ (Table II).

DISCUSSION

The present data on Atlantic cod, carp and chicken demonstrate specific binding of 24*R*,25(OH)$_2$D$_3$ to basal lateral membranes in

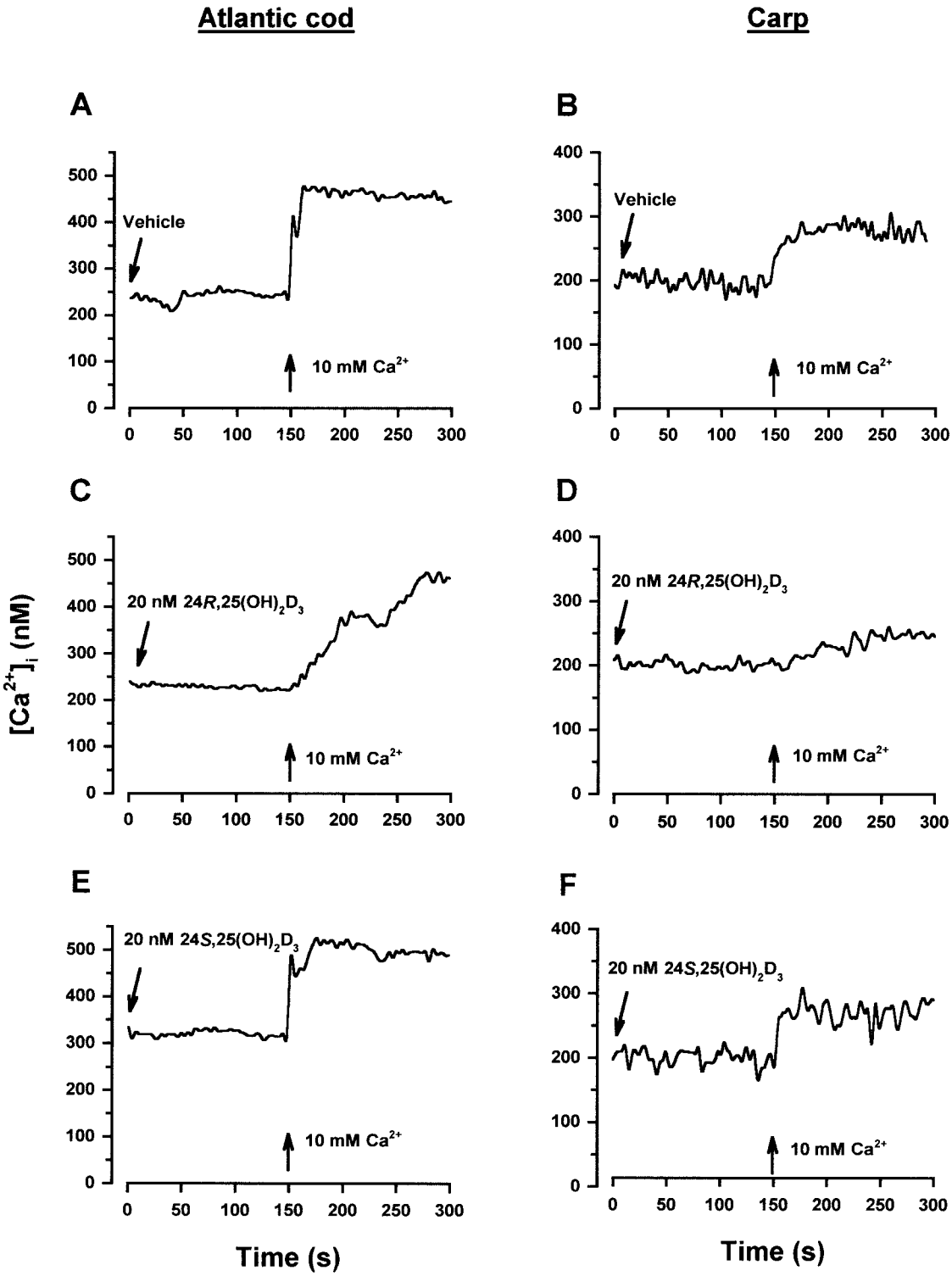

Fig. 5. Effects of 24*R*,25(OH)$_2$D$_3$ or 24*S*,25(OH)$_2$D$_3$ on Ca^{2+} uptake were investigated in enterocytes from Atlantic cod (**A, C, E**; left panel) and carp (**B, D, F**; right panel). Baseline fura-2 fluorescence (t = 0–150 s) was determined with vehicle (10 µl ethanol; A, B), 20 nM 24*R*,25(OH)$_2$D$_3$; (C, D) or 20 nM 24*S*,25(OH)$_2$D$_3$ (E, F) present from the start of the experiment. The arrow denotes addition of 10 mM Ca^{2+} to the incubation medium and the fluorescence measurements were continued in the presence of 10 mM Ca^{2+} for an additional 150 s.

TABLE I. Effects of 0–20 nM 24*R*,25(OH)$_2$D$_3$ or 24*S*,25(OH)$_2$D$_3$ on Ca^{2+} Uptake in Enterocytes From Atlantic Cod and Carp

Metabolite (nM)	24*R*,25(OH)$_2$D$_3$	24*S*,25(OH)$_2$D$_3$	24*R*,25(OH)$_2$D$_3$	24*S*,25(OH)$_2$D$_3$
	Initial Ca^{2+} uptake velocity (% change from basal $[Ca^{2+}]_i \cdot min^{-1}$)		Final $[Ca^{2+}]_i$ (t=250–300 s) (% change from basal $[Ca^{2+}]_i$)	
Atlantic cod				
0	43.00±4.0[a]	45.07±6.4	73.32±5.3	66.89±5.6
0.5	45.01±9.3[a]	48.47±10.0	79.62±14.3	73.90±14.4
1	43.59±8.6[a]	42.71±8.7	73.91±12.7	73.81±7.1
5	30.51±4.4[b*]	46.56±6.3	67.10±6.0	63.82±8.4
20	20.59±7.2[b*]	38.67±4.7	73.71±12.2	60.29±6.7
Carp				
0	69.00±6.3[a]	70.56±17.0	43.57±3.6	44.34±3.4
0.5	58.88±10.4[a]	59.79±9.5	45.70±3.5	50.45±2.7
1	52.96±98[a,b]	56.28±5.2	44.45±4.4	48.84±4.2
5	52.84±6.1[a,b]	59.23±10.3	47.22±5.3	43.40±4.3
20	44.62±8.2[b]	52.90±10.0	40.44±4.7	35.11±4.6

Enterocytes were either pre-exposed to 0, 0.5, 1, 5, and 20 nM of 24*R*,25(OH)$_2$D$_3$ or 24*S*,25(OH)$_2$D$_3$ for 300 s.The basal $[Ca^{2+}]_i$ was recorded for 150 s, then 10 mM Ca^{2+} was added to the media and the $[Ca^{2+}]_i$ was recorded for another 150 s. Data obtained were compared for: (1) Initial Ca^{2+} uptake velocity, as percent change from its mean basal $[Ca^{2+}]_i$ during the first 18 s after addition of 10 mM Ca^{2+}. (2) The final $[Ca_{2+}]_i$ from 250–300 s, as percent change from the mean basal $[Ca^{2+}]_i$. A two-factorial ANOVA followed by a Student–Newman–Keuls posthoc test was used to test for statistical differences between administrated doses for each of the Vitamin D metabolites and between metabolites for each dose. Different uppercase letters indicate significant differences between the groups treated with one of the metabolites and * indicate statistical differences between 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ at the same administered concentration. Data are expressed as mean±SEM (n=6–10) and the level of significance was set at $P < 0.05$.

enterocytes from all three species, suggesting the presence of membrane receptors for 24*R*,25(OH)$_2$D$_3$. Furthermore, the two isoforms; 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$, both reduced Ca^{2+}-uptake and triggered an intracellular Ca^{2+}-release within seconds after administration. 24*S*,25(OH)$_2$D$_3$ was more effective in triggering intracellular Ca^{2+}-release, whereas 24*R*,25(OH)$_2$D$_3$ was more effective in inhibiting extracellular Ca^{2+}-uptake into enterocytes. Comparing the binding characteristics for the putative 24*R*,25(OH)$_2$D$_3$ membrane receptor to the concentration of 24*R*, 25(OH)$_2$D$_3$ affecting enterocyte intracellular Ca^{2+} signaling to the circulating levels of 24,25(OH)$_2$D$_3$ in these species [1–69 nM; Bishop et al., 1980; Takeuchi et al., 1991; Larsson, 1999], revealed that the effective metabolite concentrations are well within the physiological range. Thus, this supports for a physiological function for these vitamin D$_3$ metabolites.

What is the physiological relevance of rapid, non-genomic effects of the vitamin D$_3$ system on intestinal regulation? The physiological relevance of rapid, non-genomic, effects on Ca^{2+} transport may be to provide a system that is capable of keeping the free Ca^{2+} concentration in blood and extracellular fluids of an organism at a constant level. Freshwater fish and terrestrial vertebrates, in most cases, face a situation where they have either no direct exchange of Ca^{2+} with the environment or are at risk to lose Ca^{2+} to the environment. Marine fish, on the other hand, live in a Ca^{2+}-rich environment and are confronted with the problem of excess Ca^{2+} entering the body fluids. Marine fish [Sundell and Björnsson, 1990; Larsson et al., 1995] compared to freshwater fish and terrestrial vertebrates [Chartier et al., 1979; Flik et al., 1982; Yoshimoto and Norman, 1986; Massheimer et al., 1994; Nemere et al., 1994], display different rapid responses in intestinal Ca^{2+} transport to 1,25(OH)$_2$D$_3$, 25(OH)D$_3$ and 24*R*, 25(OH)$_2$D$_3$. These observed differences in the non-genomic actions of vitamin D$_3$ metabolites in species living in Ca^{2+}-rich and Ca^{2+}-deficient environments, may be due to an adaptation of the vitamin D$_3$ endocrine system to the environmental Ca^{2+} availability encountered by the different species [Larsson et al., 1995; Sundell et al., 1996; Larsson, 1999]. The current data demonstrate that both the marine Atlantic cod and the freshwater carp possess an endocrine system in which 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ are able to rapidly decrease the intestinal Ca^{2+}-uptake. However, it must be considered that circulating 24,25(OH)$_2$D$_3$ concentrations could not be detected in carp [Takeuchi et al., 1991] indicating that the normal metabolite concentration in the circulation is not sufficient to evoke a rapid

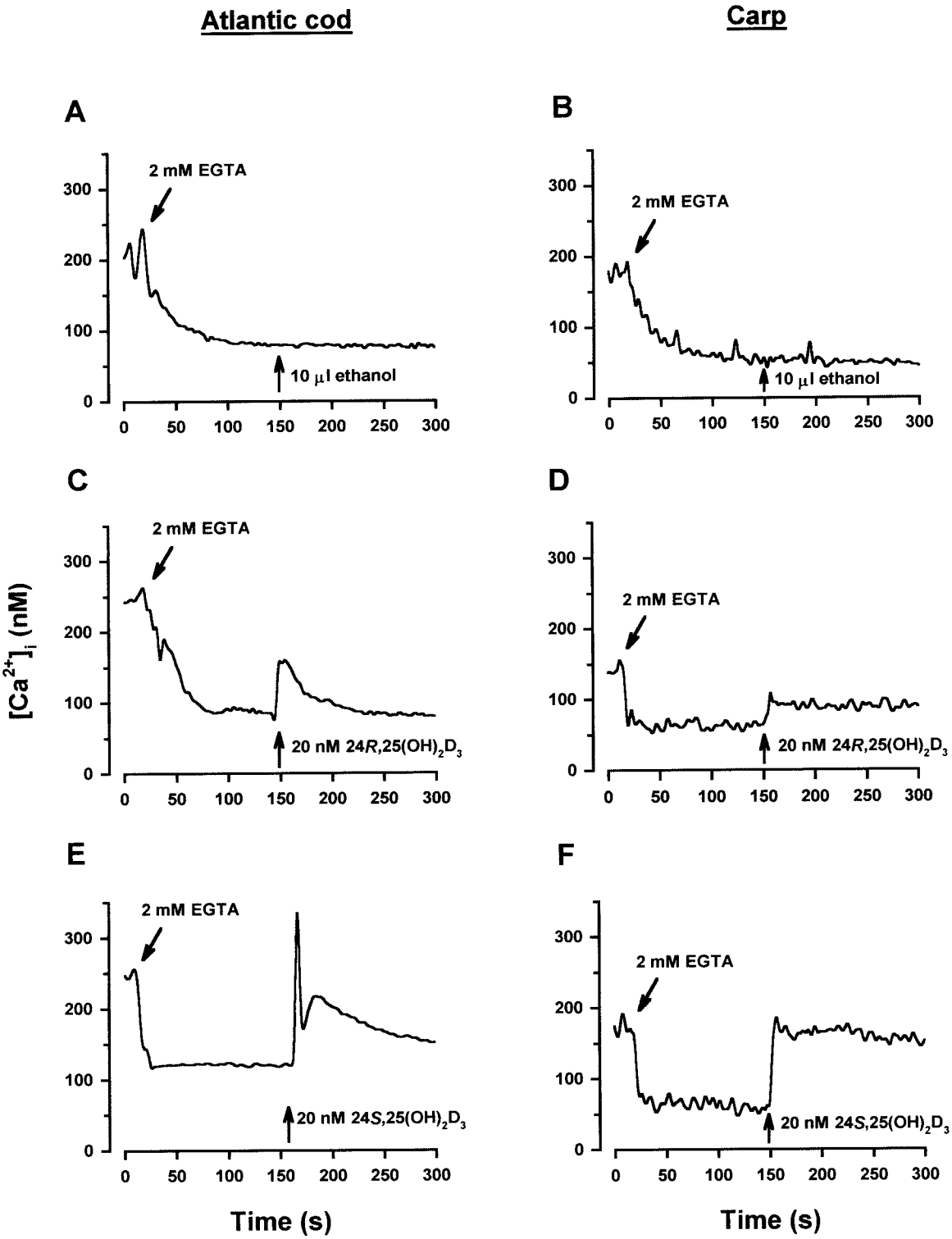

Fig. 6. Effects of 24*R*,25(OH)$_2$D$_3$ or 24*S*,25(OH)$_2$D$_3$ on intracellular Ca^{2+} release in enterocytes from Atlantic cod (**A, C, E**; left panel) and carp (**B, D, F**; right panel). Baseline fura-2 fluorescence was determined with 2 mM EGTA present from the start of the experiment (t = 0–150 s). The arrow denotes addition of vehicle (10 µl ethanol; A, B), 20 nM 24*R*,25(OH)$_2$D$_3$ (C, D) or 20 nM 24*S*,25(OH)$_2$D$_3$ (E, F) to the incubation medium, the fluorescence measurements were then continued for an additional 150 s.

TABLE II. Effects of 0–20 nM 24*R*,25(OH)$_2$D$_3$ or 24*S*,25(OH)$_2$D$_3$ on Intracellular Ca^{2+} Release in Enterocytes From Atlantic Cod and Carp

	24*R*,25(OH)$_2$D$_3$	24*S*,25(OH)$_2$D$_3$	24*R*,25(OH)$_2$D$_3$	24*S*,25(OH)$_2$D$_3$
Metabolite (nM)	Peak $[Ca^{2+}]_i$ (t=150–160 s) (% change from basal $[Ca^{2+}]_i \cdot min^{-1}$)		Final $[Ca^{2+}]_i$ (t=250–300 s) (% change from basal $[Ca^{2+}]_i \cdot min^{-1}$)	
Atlantic cod				
0	6.15±3.1[a]	−2.68±3.3[a]	−13.68±4.1	−8.49±6.0[a]
0.5	16.11±9.6[a]	88.41±47.5[a,b]	−12.89±4.6	1.49±11.8[a]
1	16.25±10.0[a]	78.18±48.3[a,b]	−10.19±4.2	−10.6±4.6[a]
5	9.15±4.9[b*]	71.86±22.1[b]	−9.65±2.7	8.05±7.4[a]
20	51.85±22.1[b*]	159.7±42.3[b]	7.16±11.1[*]	44.84±10.8[b]
Carp				
0	−3.69±5.3[a]	−0.51±4.6[a]	−20.73±7.8[a]	−23.14±7.2[a]
0.5	−2.99±5.8[a]	42.34±23.2[a,b]	22.01±5.3[a]	14.37±7.0[a]
1	4.25±4.4[a*]	48.52±16.7[a,b]	−11.81±3.7[a]	3.05±13.0[a]
5	25.66±6.9[b*]	83.86±22.6[b,c]	−2.39±7.5[a]	28.88±19.3[a]
20	65.86±13.4[c*]	132.87±23.0[c]	23.96±14.4[b]	83.60±23.0[b]

Enterocytes were acclimated with 2 mM EGTA for 150 s in order to get a Ca^{2+} free incubation media and to let enterocytes restore a new $[Ca^{2+}]_i$ The basal $[Ca^{2+}]_i$ was recorded for 150 s, then 0, 0.5, 1, 5 and 20 nM of 24*R*,25(OH)$_2$D$_3$ or equivalent concentrations of 24*S*,25(OH)$_2$D$_3$ was added to the incubation media and the $[Ca^{2+}]_i$ was recorded for another 150 s. Data obtained were compared for: (1) The maximal initial increase in $[Ca^{2+}]_i$ during the first 10 s (t = 150–160 s) after addition of 24*R*,25(OH)$_2$D$_3$ or 24*S*,25(OH)$_2$D$_3$, as percent change from the mean basal $[Ca^{2+}]_i$. (2) The final $[Ca^{2+}]_i$ from 250–300 s as percent change from the mean basal $[Ca^{2+}]_i$. A two-factorial ANOVA followed by a Student–Newman–Keuls posthoc test was used to test for statistical differences between administrated doses for each of the vitamin D metabolites and between metabolites for each dose. Different uppercase letters indicate significant difference between groups treated with one of the metabolites and * indicate statistical difference between 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ at the same administrated concentration. Data are expressed as mean±SEM (n = 6–8) and the significance was set at $P < 0.05$.

inhibition of Ca^{2+}-influx. Furthermore, Atlantic cod showed a higher affinity and lower B_{max} of the 24,25(OH)$_2$D$_3$ binding, compared to carp and chicken, indicating a rapid receptor turnover and suggesting a higher activity of 24*R*,25(OH)$_2$D$_3$ and maybe 24*S*,25(OH)$_2$D$_3$ in the seawater fish.

Our data demonstrate that membrane receptors for 24,25(OH)$_2$D$_3$ in both Atlantic cod and carp could be described as associated with the membrane rather than as an integral protein, as previously reported for the 1,25(OH)$_2$D$_3$ plasma membrane receptor in both chicken [Nemere et al., 1994] and carp [Nemere et al., 2000]. Furthermore, when the specific binding of 24*R*,25(OH)$_2$D$_3$ was subjected to competition with other metabolites, 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ showed an equally high affinity for the receptor. The two other vitamin D metabolites tested, 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$, showed a lower affinity for the 24,25(OH)$_2$D$_3$ receptor protein. The pattern in which different vitamin D metabolites compete for the 24,25(OH)$_2$D$_3$ membrane receptors in Atlantic cod and carp differ from the patterns described for chicken tibial fracture-healing callus [Kato et al., 1998], plasma vitamin D binding protein in fish [Hay and Watson, 1977] and DBP in birds and mammals [Hay and Watson, 1977; Kato et al., 1998], where 25(OH)D$_3$ was superior or equivalent to 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ in competing for the binding site. This then indicates that the 24,25(OH)$_2$D$_3$ binding protein in Atlantic cod and carp enterocyte membranes is different from the plasma vitamin D binding proteins in fish, birds and mammals and the binding protein in chicken tibial fracture-healing callus. Another possible candidate for the 24*R*,25(OH)$_2$D$_3$ binding protein in Atlantic cod and carp enterocytes could be a membrane associated form of a cytosolic/nuclear vitamin D receptor. Kim et al. [1996] reported that the classic cytosolic/nuclear vitamin D receptor could be associated with cellular acceptance sites in ROS 17/2.8 cells. Putative cytosolic/nuclear receptors for 24*R*,25(OH)$_2$D$_3$ have been demonstrated in several tissues from different animals [Corvol et al., 1980; Merke and Norman, 1981; Sömjen et al., 1982a,b, 1986], and there are several lines of evidence that 24*R*,25(OH)$_2$D$_3$ induces transcription [Sömjen et al., 1986] and protein synthesis [Sömjen et al., 1984, 1986]. However, no effects on transcription, protein synthesis, or specific binding for 24*R*,25(OH)$_2$D$_3$ in the cytosol fraction, were found in rat intestinal mucosal cells [Sömjen et al., 1982a, 1983], suggesting that the binding moiety for 24*R*,25(OH)$_2$D$_3$ in enterocyte membranes is a different protein.

The 24*R*,25(OH)$_2$D$_3$ binding protein in Atlantic cod, carp and chicken basal lateral membranes all exhibit the characteristics of an allosteric protein. Positive cooperativity of steroid hormones binding to plasma membranes is not restricted to 24*R*,25(OH)$_2$D$_3$. The steroids 1,25(OH)$_2$D$_3$ [Larsson, 1999; Nemere et al., 2000], estradiol [Pietras and Szego, 1980; Horvat et al., 1995; Marsigliante et al., 1999] and progesterone [Tetel et al., 1997] have been demonstrated to bind to plasma membranes with positive cooperativity. Thus, the binding characteristics of steroid binding membrane proteins indicate a common feature, cooperative hormone binding, and suggest that these allosteric proteins have at least two binding sites or for the existence of a receptor dimer in the cell membranes.

No previous reports have addressed possible rapid, membrane associated, effects of 24*S*, 25(OH)$_2$D$_3$. However, biological effects of 24*S*, 25(OH)$_2$D$_3$ have been investigated in rat bone and kidney [Sömjen et al., 1983, 1984] and in chicken tibial fracture-healing callus [Kato et al., 1998]. These studies showed that 24*R*,25(OH)$_2$D$_3$, but not 24*S*,25(OH)$_2$D$_3$, stimulated creatin kinase activity, increased ornithine decarboxylase activity, and incorporation of [^{3}H]thymidine into DNA [Sömjen et al., 1983], as well as bone mechanical strength parameters together with bone ash content [Kato et al., 1998]. The present study demonstrates that 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$, both rapidly affect the Ca^{2+}-homeostasis in enterocytes: 24*R*,25(OH)$_2$D$_3$ primarily inhibits Ca^{2+}-uptake whereas 24*S*, 25(OH)$_2$D$_3$ mainly triggers intracellular Ca^{2+}-release. Thus, the rapid biological effects of 24*S*,25(OH)$_2$D$_3$ in fish enterocytes, suggest that the membrane associated receptor protein(s) for the 24,25(OH)$_2$D$_3$ isomers in Atlantic cod and carp, is/(are) of a different nature than the receptor protein for 24*R*,25(OH)$_2$D$_3$ reported in rat bone and kidney, and chicken fracture-healing callus, as the responsiveness to 24*S*, 25(OH)$_2$D$_3$ differs between the systems studied.

Treatment with 24*R*,25(OH)$_2$D$_3$ and 24*S*,25 (OH)$_2$D$_3$ affected intracellular Ca^{2+} signaling in Atlantic cod and carp, by inhibition of Ca^{2+}-uptake and by triggering of Ca^{2+}-release from intracellular stores. Previous reports on osteoblast like cells [Caffrey and Farach-Carson, 1989; Yukihiro et al., 1994; Takeuchi and Guggino, 1996; Li et al., 1997] and enterocytes [Larsson et al., 1997; Larsson, 1999] have indicated that 24*R*,25(OH)$_2$D$_3$ inhibits Ca^{2+}-uptake through L-type Ca^{2+} channels. The exact mechanism behind this rapid inhibition of the activity of L-type Ca^{2+} channels is not known. A possible explanation for Ca^{2+} channel inactivation was put forward by Plant et al. [1983], who demonstrated that increased intracellular Ca^{2+} concentrations and/or activation of protein kinase C evoked Ca^{2+} channel inactivation in neurons from *Helix aspersa*. Extrapolating the hypothesis from Plant et al. [1983], to the data presented in this study, suggests that 24*S*,25(OH)$_2$D$_3$ should be more effective than 24*R*,25(OH)$_2$D$_3$ in inhibiting the activity of Ca^{2+} channels since 24*S*, 25(OH)$_2$D$_3$ increased the $[Ca^{2+}]_i$ to significantly higher concentrations compared to 24*R*,25 (OH)$_2$D$_3$. However, in the present study, 24*R*, 25(OH)$_2$D$_3$ was a more powerful inhibitor of Ca^{2+}-uptake than 24*S*,25(OH)$_2$D$_3$ in both Atlantic cod and carp enterocytes. Thus, in Atlantic cod and carp enterocytes, the intracellular Ca^{2+}-release observed after 24*S*,25 (OH)$_2$D$_3$ or 24*R*,25(OH)$_2$D$_3$ treatment is probably independent of the metabolites ability to inactivate L-type Ca^{2+} channels.

Ishizuka et al., 1984, demonstrated that the naturally occurring pool of 24,25(OH)$_2$D$_3$ in serum from rats is a mixture of the 24*R*,25 (OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ isomers, in a ratio of 89.5:10.5. Extrapolating this ratio to circulating levels of 24,25(OH)$_2$D$_3$ in fish, where the concentration of 24,25(OH)$_2$D$_3$ in tuna [Takeuchi et al., 1991] and Atlantic cod [Larsson, 1999] were measured to be in the range of 1–69 nM, would mean that the level of 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ should be 0.89–61.7 nM and 0.11–7.3 nM, respectively. Thus, the concentrations used to test for effects of 24*R*,25 (OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$ in this study, are in the physiological range and argue for a biological function of both 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$, but it still remains to be elucidated whether the isomers mediate their effects through the same or separate receptors.

In conclusion, our results demonstrate putative receptors for 24,25(OH)$_2$D$_3$, associated with the basal lateral membrane in enterocytes from Atlantic cod, carp and chicken with biochemical characteristics different from previously described receptor proteins in rat chondrocytes [Pedrozo et al., 1999], chicken tibial fracture-healing callus [Seo et al., 1996; Kato et al., 1998]

and more extensively studied then the earlier investigation in chicken enterocytes [Nemere et al., 1994]. The K_d of the specific binding in Atlantic cod, carp and chicken were in the physiological range for circulating levels of 24*R*,25$(OH)_2D_3$ as reported for fish [Takeuchi et al., 1991, Larsson, 1999] and chicken [Bishop et al., 1980], and a positive cooperativity with at least two binding sites was indicated. Furthermore, this is to our knowledge the first demonstration of different physiological functions for 24*S*,25$(OH)_2D_3$ and 24*R*,25$(OH)_2D_3$; 24*S*,25$(OH)_2D_3$ was more effective in triggering intracellular Ca^{2+}-release, whereas 24*R*,25$(OH)_2D_3$ was more effective in inhibiting Ca^{2+}-uptake into the enterocytes. Thus, both binding studies on purified basal lateral membranes and spectrofluorometric analyses of Ca^{2+}-fluxes suggest the presence of receptors with at least two separate binding sites, or presence of two or more receptors for 24*R*,25$(OH)_2D_3$ and 24*S*,25$(OH)_2D_3$, with different physiological functions.

ACKNOWLEDGMENTS

[^{3}H]24*R*,25$(OH)_2D_3$, 24*R*,25$(OH)_2D_3$, and 24*S*,25$(OH)_2D_3$ were generously supplied by Kureha Chemical Co. Ltd., Tokyo, Japan.

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