Antagonistic effects of 24R,25-dihydroxyvitamin D₃ and 25-hydroxyvitamin D₃ on L-type Ca²⁺ channels and Na⁺/Ca²⁺ exchange in enterocytes from Atlantic cod (Gadus morhua)

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Abstract

There is mounting evidence that vitamin D and its metabolites play important roles in regulating plasma calcium concentrations in teleost fish as in other vertebrates. The aims of the present study were to elucidate the possible cellular target mechanisms for the rapid actions of 24R,25(OH)₂D₃, 25(OH)D₃ and 1,25(OH)₂D₃ in Atlantic cod enterocytes at physiological doses, and to establish the concentration and thus the physiological range of circulating 24R,25(OH)₂D₃, 25(OH)D₃ and 1,25(OH)₂D₃ in the Atlantic cod. The plasma concentrations of 25(OH)D₃, 1,25(OH)₂D₃ and 24R,25(OH)₂D₃ were 15·3±2·7 nM, 125·1±12·3 pM and 10·1±23·5 nM respectively. Exposure of enterocytes to 10 mM calcium (Ca²⁺) evoked an increase in intracellular Ca²⁺ concentrations ([Ca²⁺]ᵢ). This increase was suppressed by 24R,25(OH)₂D₃ dose-dependently, with an EC₅₀ of 4·9 nM and a maximal inhibition of 60%. 24R,25(OH)₂D₃ (20 nM) abolished an increase in [Ca²⁺]ᵢ (∼252%) in the control enterocytes exposed to 10 µM S(−)-BAYK-8644, suggesting that the hormone acts by inhibiting Ca²⁺ entry through L-type voltage-gated Ca²⁺-channels. Administration of 20 nM 24R,25(OH)₂D₃ to enterocytes in the absence of extracellular Ca²⁺ increased [Ca²⁺]ᵢ by ∼20%, indicating a release of Ca²⁺ from intracellular stores. Administration of 25(OH)D₃ (20 nM) resulted in a biphasic change in the enterocyte [Ca²⁺]: within 1–5 s, it decreased to 87±12 nM below its mean basal [Ca²⁺], (334±13 nM), followed by a rapid recovery of [Ca²⁺], to a new level, 10% lower than the initial [Ca²⁺]. The rapid decrease, the recovery rate and the final [Ca²⁺], were all affected dose-dependently by 25(OH)D₃, with EC₅₀ values of 8·5, 17·0 and 18·9 nM respectively. Furthermore, the effects of 25(OH)D₃ were sensitive to sodium (Na⁺), bepridil (10 µM) and nifedipine (5 µM), suggesting that 25(OH)D₃ regulates the activity of both basolateral membrane-associated Na⁺/Ca²⁺ exchangers and brush border membrane-associated L-type Ca²⁺ channels. Administration of 25(OH)D₃ (10 nM) to enterocytes in the absence of extracellular Ca²⁺ increased [Ca²⁺]ᵢ by ∼18%, indicating a release of Ca²⁺ from intracellular stores. 1,25(OH)₂D₃ also affected enterocyte [Ca²⁺], in a biphasic manner: the rapid decrease, the recovery rate, and the mean final [Ca²⁺], were all affected dose-dependently, with EC₅₀ values of 8·3, 24·5 and 7·7 nM respectively. The high EC₅₀ values for 1,25(OH)₂D₃ compared with circulating concentrations of 1,25(OH)₂D₃ (130 pM) suggest that this effect is pharmacological, rather than of physiological relevance in enterocyte Ca²⁺ homeostasis of the Atlantic cod. It is concluded that 24R,25(OH)₂D₃ has a physiological role in decreasing intestinal Ca²⁺ uptake via inactivation of L-type Ca²⁺ channels, whereas the physiological role of 25(OH)D₃ is to increase enterocyte Ca²⁺ transport via activation of Na⁺/Ca²⁺ exchangers, concurrent with activation of L-type Ca²⁺ channels.

Journal of Molecular Endocrinology (2002) 28, 53–68
Introduction

The intestine is an important organ for Ca\(^{2+}\) uptake from ingested food and water. In mammals and birds, the intestinal Ca\(^{2+}\) uptake is under the control of the vitamin D\(_3\) endocrine system by two mechanisms: a slow genome-mediated response, and a rapid non-genome-mediated response (Nemere 1996, Sundell et al. 1996). Studies on rapid non-genomic effects in fish have demonstrated differences between freshwater- and seawater-adapted species. In freshwater-adapted European eel (Anguilla anguilla; Chartier et al. 1979) and Tilapia (Oreochromis mossambicus; Flik et al. 1982), 1,25(OH)\(_2\)D\(_3\) evoked a rapid increase in intestinal mucosa-to-serosa Ca\(^{2+}\) transport, whereas no rapid effects on in vitro intestinal Ca\(^{2+}\) transport were observed in the marine Atlantic cod (Gadus morhua), after treatment with 1,25(OH)\(_2\)D\(_3\) (Sundell & Björnsson 1990, Larsson et al. 1995). Instead, in Atlantic cod, in vitro intestinal Ca\(^{2+}\) uptake was increased and decreased by 25(OH)D\(_3\) and 24R,25(OH)\(_2\)D\(_3\) respectively, in physiological doses (Sundell & Björnsson 1990, Larsson et al. 1995).

The well-characterized rapid effects of 1,25(OH)\(_2\)D\(_3\) on intestinal Ca\(^{2+}\) transport in mammals and birds (transcaltachia) are mediated by a plasma membrane vitamin D receptor (pmVDR; Nemere et al. 1994). Depending on the animal system studied, the receptor–hormone interaction can result in altered phosphoinositide metabolism (Lieberherr 1989) with subsequent activation of protein kinase C (Nemere 1999), opening of L-type Ca\(^{2+}\) channels via a cAMP-dependent pathway (deBoland & Norman 1990, deBoland et al. 1990, Massheimer et al. 1994), enhancement in the content and reactivity of available sulphydryl groups (Mykkanen & Wasserman 1990, Tolosa de Talamoni et al. 1991), or an increased transcellular vesicular Ca\(^{2+}\) transport (Nemere & Norman 1990). Information regarding the mechanisms underlying the rapid responses mediated by 25(OH)D\(_3\) and 24R,25(OH)\(_2\)D\(_3\) on intestinal Ca\(^{2+}\) transport is, so far, scarce.

In teleosts, the mechanisms behind the rapid actions of vitamin D\(_3\) metabolites have not been investigated. However, recent studies on Ca\(^{2+}\)-transporting mechanisms in Atlantic cod enterocytes have demonstrated the presence of L-type Ca\(^{2+}\) channels, mainly located in the brush border membrane (Larsson et al. 1998). Furthermore, in apical membranes of Tilapia enterocytes, a P\(_2\) purinoceptor-mediated Ca\(^{2+}\) uptake through Ca\(^{2+}\) channels or carriers is indicated (Klaren et al. 1997). There is also ample evidence for the presence of both Ca\(^{2+}\)-ATPases and Na\(^{+}\)/Ca\(^{2+}\) exchangers for Ca\(^{2+}\) extrusion from enterocytes to the circulation, in both freshwater and marine teleosts (Flik et al. 1990, Schoenmakers & Flik 1992, Schoenmakers et al. 1993). In contrast to mammalian intestinal cells, in which Ca\(^{2+}\)-ATPases are the main extrusion mechanism, Na\(^{+}\)/Ca\(^{2+}\) exchangers are the main extrusion mechanism across teleost enterocyte basolateral membranes (Flik et al. 1990, Schoenmakers & Flik 1992, Schoenmakers et al. 1993). These cellular mechanisms, together with intracellular Ca\(^{2+}\) stores, are all potential targets for the rapid, non-genomic effects mediated by the vitamin D\(_3\) endocrine system.

The aims of the present study were to elucidate the possible cellular target mechanisms for the rapid actions of 24R,25(OH)\(_2\)D\(_3\), 25(OH)D\(_3\) and 1,25(OH)\(_2\)D\(_3\) in Atlantic cod enterocytes at physiological doses, and to establish the concentration and thus the physiological range of circulating 24R,25(OH)\(_2\)D\(_3\), 25(OH)D\(_3\) and 1,25(OH)\(_2\)D\(_3\) in the Atlantic cod.

Materials and methods

Chemicals

24R,25(OH)\(_2\)D\(_3\) was provided by Kureha Chemical Co., Ltd (Tokyo, Japan). Pluronic F-127, bepridil, 1,25(OH)\(_2\)D\(_3\) and 25(OH)D\(_3\) were purchased from Calbiochem (La Jolla, CA, USA), nifedipine from Sigma (St Louis, MO, USA), S(-)-BAY K-8644 from Research Biochemicals International (Natlick, MA, USA) and Fura-2/AM from Molecular Probes (Leiden, Netherlands). All other chemicals were bought from Sigma (St Louis, MO, USA) and were of analytical grade.

Plasma concentrations of vitamin D metabolites

Plasma was collected from Atlantic cod of both sexes (n=121) throughout the year of 1994 and was kept at −80°C until analysed for 25(OH)D\(_3\), 1,25(OH)\(_2\)D\(_3\) and 24R,25(OH)\(_2\)D\(_3\), according to a slight modification of the procedure described...
by Aksnes (1980a,b). Briefly, [3H]25(OH)D₃ and [3H]1,25(OH)₂D₃ (1500 d.p.m. each) were added to 1·5 ml fish plasma for determination of steroid recovery. [3H]24R,25(OH)₂D₃ was not commercially available at the time of the analyses. The plasma was mixed with 2 ml acetonitrile, vortexed and centrifuged at 10 000 g for 10 min to remove proteins. The supernatant was collected and 3·5 ml 0·1 M K₂HPO₄ pH 10·5 was added to the sample. The sample was applied to a C-18 hydroxy vacuum column (pressure of 127 mmHg; Varian, Walnut Creek, CA, USA), washed with 5 ml distilled H₂O, followed by a second wash with 5 ml methanol:H₂O (70:30, v/v), after which the steroids were eluted with hexan:isopropanol (95:5, v/v) and the collected samples were evaporated with nitrogen gas. The sample was dissolved in 250 µl hexan:isopropanol:ethanol (95:2·5:2·5, v/v) and the vitamin D metabolites were separated on a supelcosil silica column (15 cm × 4·6 mm, 3 µm; Supelco Inc., St Louis, MO, USA) by HPLC. Retention times for the vitamin D metabolites on the supelcosil silica column, determined by running standards for 25(OH)D₃, 24R,25(OH)₂D₃ and 1,25(OH)₂D₃ were 3 min, 5–6 min and 14–15 min respectively. The fractions containing the specific metabolites were evaporated with nitrogen gas. Ethanol was added to dissolve the metabolites, and 50 µl of the samples containing 25(OH)D₃ and 1,25(OH)₂D₃ were used for recovery assessment.

The recovery for 25(OH)D₃ and 1,25(OH)₂D₃ was 51·1±2·7% and 43·8±1·4% respectively. 1,25(OH)₂D₃ was quantified by radioreceptor assay (RRA), using the 1,25(OH)₂D₃ receptor from calf thymus. 25(OH)D₃ and 24R,25(OH)₂D₃ were quantified by RRA, using human vitamin D binding protein from blood plasma.

Enterocyte preparation and measurement of free intracellular Ca²⁺ concentrations

Atlantic cod of both sexes (body weight 300–500 g) were caught off the west coast of Sweden and kept in recirculated, filtered aerated seawater at 10 °C for 5 days before being killed. No experiments were performed on sexually mature fish during the spawning season, which occurs between January and April for Atlantic cod off the Swedish west coast. The fish were killed by a blow to the head and the proximal two-thirds of the intestine was dissected out, rinsed with 0·9% NaCl and cut open length-wise. In order to minimize possible contamination by excitable cells, the mucosal cell layer was stripped off the remainder of the intestinal wall along the submucosa. The mucosa was cut into 1-cm long segments and the enterocytes were isolated by Ca²⁺ chelation according to Larsson et al. 1998. To yield the enterocyte suspension used in each experiment, mucosa from four fish were pooled together.

Cell viability was determined by trypan blue exclusion in combination with phase-contrast microscopy and cell suspensions showing viability greater than 95% were used in the experiments.

The Fura-2/AM loading was performed as described by Thomas & Delaville (1991). Briefly, freshly dissected enterocytes were incubated for 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 KH₂PO₄, 1·2 mM MgSO₄, 2 mM CaCl₂ 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 with HBSS by centrifugation at 700 g for 10 min, and finally resuspended in HBSS.

Measurements of intracellular Ca²⁺ concentrations ([Ca²⁺]ᵢ) in Fura-2/AM loaded Atlantic cod intestinal cells (5 × 10⁵ cells/ml), were performed in a Photon Technology International Ratio Master model C-44 ratio fluorescence spectrometer (Photon Technology International Inc., Newton, PA, USA) at a 340/380 nm excitation ratio, with an emission wavelength of 510 nm (Larsson et al. 1999). Three millilitres of the cell suspension were placed in a quartz cuvette and stirred slowly at a constant temperature of 10 °C. According to the different experimental procedures (described below), stock solutions of the three hormones (10 µl), Ca²⁺ (100 µl), S(-)-BAYK 8644 (5 µl; L-type Ca²⁺ channel agonist), bepridil (10 µl; Na⁺/Ca²⁺ exchange antagonist) and nifedipine (5 µl; L-type Ca²⁺ channel antagonist) were added directly to the cuvette to give the final concentration stated under the appropriate experimental procedure. Ethanol served as vehicle for 25(OH)D₃, 1,25(OH)₂D₃ and 24R,25(OH)₂D₃, methanol was vehicle for bepridil, and DMSO was used as vehicle for S(-)-BAYK 8644 and nifedipine. In the control groups, the same type and volume of vehicle were used as for the corresponding stock solution of the treatment substances.
Recordings of [Ca²⁺]ᵢ were performed every 1 s, for a time period of 300 or 600 s. Fluorimetric calibrations were made by addition of digitonin (100 µg/ml) to lyse the cells and obtain the maximum fluorescence intensity of Ca²⁺-saturated Fura-2, followed by addition of 15 µl 400 mM EGTA/3 M Tris to measure the intensity of Ca²⁺-free Fura-2. [Ca²⁺]ᵢ was calculated according to Grynkiewicz et al. (1985) and a dissociation constant (Kᵣ) for Fura-2 of 362 nM at 10 °C was used (Larsson et al. 1999).

The measurements of enterocyte [Ca²⁺]ᵢ were conducted for 2–4 h after the loading of Fura-2. Control experiments revealed that the cell viability remained above 95% for at least 4 h after Fura-2 loading. The registration of basal [Ca²⁺]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 an increase in the basal [Ca²⁺]ᵢ results in increased cell death.

Effects of 24R,25(OH)₂D₃ on enterocyte Ca²⁺ homeostasis

Effects on Ca²⁺ uptake

Enterocytes were incubated (n=8 experiments per treatment group) in Ca²⁺-free HBSS, in the presence of vehicle or 24R,25(OH)₂D₃ (0·002, 0·02, 2·0, 5·0, 10, 20, 50, 101 and 202 nM) for 300 s before the start of the experiment. After the incubation, the basal [Ca²⁺]ᵢ was recorded for 300 s, then Ca²⁺ was added to the cuvette (to give a final concentration of 10 mM) and the [Ca²⁺]ᵢ recorded for another 300 s period.

Effects on L-type Ca²⁺ channels

Enterocytes were incubated (n=8 experiments per treatment group) in HBSS for 300 s, in the presence of vehicle or 20 nM 24R,25(OH)₂D₃. The basal [Ca²⁺]ᵢ was recorded for 150 s, then S(-)-BAYK 8644 (final concentration 5 µM) was added to the cuvette, and the [Ca²⁺]ᵢ recorded for an additional 150 s.

Effects on intracellular Ca²⁺ stores

Enterocytes were incubated (n=8 experiments per treatment group) in 2 mM EGTA for 300 s, in order to obtain a Ca²⁺-free incubation medium and to let the enterocytes restore a new basal [Ca²⁺]ᵢ. The basal [Ca²⁺]ᵢ was recorded for 150 s, then vehicle or 24R,25(OH)₂D₃ (to give a final concentration of 20 nM) was added to the cuvette, and the [Ca²⁺]ᵢ recorded for another 150 s.

Effects of 25(OH)D₃ on enterocyte Ca²⁺ homeostasis

Effects on intracellular Ca²⁺ concentrations

Enterocytes were incubated (n=8 experiments per treatment group) for 300 s in HBSS. The basal [Ca²⁺]ᵢ was recorded for 300 s. Then, vehicle or 25(OH)D₃ (to reach final concentrations of 0·002, 0·02, 0·2, 1, 5, 10, 20 or 50 nM) was added to the cuvette and the [Ca²⁺]ᵢ recorded for another 300 s period.

Effects on Na⁺/Ca²⁺ exchangers

The first set of experiments was designed to investigate whether the effects of 25(OH)D₃ on [Ca²⁺]ᵢ were Na⁺-dependent. Enterocytes were incubated (n=8 experiments per treatment group) for 300 s in HBSS containing 30, 60, 90 or 120 mM Na⁺. To compensate for differences in osmotic pressure in the four treatment groups, the enterocytes treated with 30, 60 and 90 mM Na⁺ also received 90, 60 and 30 mM sucrose respectively. After this, basal [Ca²⁺]ᵢ was recorded for 300 s, then the four enterocyte experimental groups were exposed to 25(OH)D₃ (final concentration 10 nM) and [Ca²⁺]ᵢ was recorded for another 300 s.

The second set of experiments was designed to investigate if the effects of 25(OH)D₃ on [Ca²⁺]ᵢ were sensitive to bepridil. Enterocytes were incubated (n=8 experiments per treatment group) for 300 s in HBSS containing vehicle or 10 µM bepridil. The basal [Ca²⁺]ᵢ was recorded for 300 s, then enterocytes preincubated with vehicle were exposed to ethanol (final concentration 0·3%) or 25(OH)D₃ (final concentration 10 nM), whereas enterocytes preincubated with bepridil were exposed to 25(OH)D₃ (final concentration 10 nM) and [Ca²⁺]ᵢ recorded for another 300 s.

Effects on L-type Ca²⁺ channels

Enterocytes were incubated (n=8 experiments per treatment group) for 300 s in HBSS in presence of
vehicle or 5 µM nifedipine. Basal $[\mathrm{Ca}^{2+}]_i$ was recorded for 300 s, then 10 nM 25(OH)D$_3$ was added to the cuvette and the $[\mathrm{Ca}^{2+}]_i$ was recorded for another 300 s.

**Effects on intracellular Ca$^{2+}$ stores**

Enterocytes were incubated ($n$=8 experiments per treatment group) in Ca$^{2+}$-free HBSS in the presence of 2 mM EGTA for 300 s, in order to obtain a Ca$^{2+}$-free incubation media and to let the enterocytes restore a new basal $[\mathrm{Ca}^{2+}]_i$. The basal $[\mathrm{Ca}^{2+}]_i$ was recorded for 300 s, then vehicle or 25(OH)D$_3$ (final concentration 10 nM) was added to the cuvette, and the $[\mathrm{Ca}^{2+}]_i$ recorded for another 300 s.

**Effects of 1,25(OH)$_2$D$_3$ on enterocyte Ca$^{2+}$ homeostasis**

**Effects on intracellular Ca$^{2+}$ concentrations**

Enterocytes were incubated ($n$=8 experiments per treatment group) for 300 s in HBSS. The basal $[\mathrm{Ca}^{2+}]_i$ was recorded for 300 s. Then, vehicle or 1,25(OH)$_2$D$_3$ (to reach final concentrations of 0·26, 130, 650, 6500, 13 000 and 32 500 pM) was added to the cuvette and the $[\mathrm{Ca}^{2+}]_i$ recorded for another 300 s period.

**Statistics**

Dose-dependent effects of 24R,25(OH)$_2$D$_3$, 25(OH)D$_3$, 1,25(OH)$_2$D$_3$ and Na$^+$-dependent effects of 25(OH)D$_3$ were tested by nonlinear regression. The logarithm of the administered dose was plotted against the observed response and the data were fitted to a three-parameter sigmoid equation:

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

where $Y$ is the observed effect, $x$ is the administered dose of metabolite, $a$ is the maximal effect observed, $b$ is the minimal effect observed, and $x_0$ is the metabolite concentration at half the maximal effect (EC$_{50}$). The coefficient of variation ($R^2$) and the adjusted coefficient of variation (adj$R^2$) were used as measures of how well the regression model described the data (Altman 1991). One-way analysis of variance (ANOVA) was used as measures to gauge the contribution of the independent variable to predict the dependent variable (Altman 1991). Differences at the $P<0·05$ level were considered statistically significant.

A one-factorial ANOVA was used to test for significant differences among control and treatment groups in each experiment, and in cases with significant differences among factors containing more than two levels, a Student–Newman–Keuls post hoc test was performed. The testing was two-tailed, and the significance level was set at $P<0·05$. Data are presented as mean ± s.e.m.

**Results**

**Plasma concentrations of vitamin D metabolites**

Circulating levels of 25(OH)D$_3$, 1,25(OH)$_2$D$_3$ and 24R,25(OH)$_2$D$_3$ were assessed to be 15·3 ± 2·7 nM ($n$=94), 125·1 ± 12·3 pM ($n$=121) and 10·1 ± 23·3 nM ($n$=73) respectively. The concentrations measured ranged from non-detectable to 78·0 nM ($n$=121) for 25(OH)D$_3$, from 21·2 to 692·3 pM for 1,25(OH)$_2$D$_3$ ($n$=121) and from non-detectable to 69·5 nM for 24R,25(OH)$_2$D$_3$ ($n$=121). The detection limits were approximately 4 pM for 1,25(OH)$_2$D$_3$ and 400 pM for 25(OH)D$_3$ and 24R,25(OH)$_2$D$_3$.

**Effects of 24R,25(OH)$_2$D$_3$ on enterocyte Ca$^{2+}$ homeostasis**

**Effects on Ca$^{2+}$ uptake**

Representative recordings from fluorospectrophotometric analysis of $[\mathrm{Ca}^{2+}]_i$ in enterocytes preincubated with vehicle (10 µl ethanol) or 20 nM 24R,25(OH)$_2$D$_3$ are presented in Fig. 1A. Exposure of vehicle-preincubated enterocytes to 10 mM Ca$^{2+}$ evoked a typical increase in $[\mathrm{Ca}^{2+}]_i$ (264 ± 16 nM) from basal values (203 ± 13 nM), which levelled out at a new stable $[\mathrm{Ca}^{2+}]_i$ (Fig. 1A, upper panel). The initial rate of change in $[\mathrm{Ca}^{2+}]_i$ during the first 18 s ($t$=300 to 318 s) after addition of 10 mM Ca$^{2+}$ and the mean final $[\mathrm{Ca}^{2+}]_i$ ($t$=550 to 600 s) were calculated and expressed as % change from the mean basal $[\mathrm{Ca}^{2+}]_i$ ($t$=0 to 299 s). The initial $[\mathrm{Ca}^{2+}]_i$ increase (Fig. 1B) and the final $[\mathrm{Ca}^{2+}]_i$ (Fig. 1C) were dose-dependently suppressed by 24R,25(OH)$_2$D$_3$, from 0·2 pM to 202 nM ($P<0·05$). The dose-dependent suppression of initial $[\mathrm{Ca}^{2+}]_i$ changes had an EC$_{50}$ of 4·9 nM ($P<0·05$,
$R^2=0.97$, adj$R^2=0.97$) and the final $[\text{Ca}^{2+}]_i$ had an EC$_{50}$ of 30 nM ($P<0.05$; $R^2=0.97$, adj$R^2=0.94$). The maximal inhibition of the Ca$^{2+}$ uptake (approximately 60%) occurred at 20 nM 24$R,25$(OH)$_2$D$_3$.

Effects on L-type Ca$^{2+}$ channels

When control enterocytes were exposed to S(-)-BAYK-8644, a transient response of the $[\text{Ca}^{2+}]_i$ was observed (Fig. 2A, lower panel). A rapid increase in $[\text{Ca}^{2+}]_i$ (429 ± 24 nM) was followed by a decrease back to basal values (170 ± 10 nM). The rate of initial $[\text{Ca}^{2+}]_i$ change during the first 10 s ($t=150$ to 160 s) after addition of S(-)-BAYK-8644 and the mean final $[\text{Ca}^{2+}]_i$ ($t=275$ to 300 s) were calculated as % change from the mean basal $[\text{Ca}^{2+}]_i$ ($t=0$ to 149 s). Addition of 20 nM 24$R,25$(OH)$_2$D$_3$ resulted in an initial increase in $[\text{Ca}^{2+}]_i$ (91.5 ± 12.6% change from basal $[\text{Ca}^{2+}]_i$; $P<0.05$; Fig. 3B) and an increase in the final $[\text{Ca}^{2+}]_i$ (12.6 ± 2.0% of basal $[\text{Ca}^{2+}]_i$; $P<0.05$; Fig. 3C), whereas addition of vehicle had no effect on initial $[\text{Ca}^{2+}]_i$ changes (Fig. 3B) or the final $[\text{Ca}^{2+}]_i$ (Fig. 3C).

Effects of 25(OH)D$_3$ on enterocyte Ca$^{2+}$ homeostasis

The typical pattern of changes in enterocyte $[\text{Ca}^{2+}]_i$ evoked by treatment with 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ in Ca$^{2+}$-containing and Ca$^{2+}$-free medium could be divided into three parts (Figs 4A, 7A and 8A). Therefore, the events calculated and compared in all experiments with 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ described below are: the mean initial change in $[\text{Ca}^{2+}]_i$ during the first 5 s after hormone or vehicle treatment ($t=300$ to 305 s), the rate of
change in $[\text{Ca}^{2+}]$, during the following 18 s ($t=305$ to 323 s; recovery rate) and the mean final $[\text{Ca}^{2+}]$, value during the last 50 s of the experiment ($t=550$ to 600 s). All rates and values are expressed as % change from mean basal $[\text{Ca}^{2+}]$.

Effects on $[\text{Ca}^{2+}]$

The magnitude of the mean initial decrease in $[\text{Ca}^{2+}]$, the recovery rate of the increase up to a new $[\text{Ca}^{2+}]$, value and the mean final $[\text{Ca}^{2+}]$, value, were all influenced by 25(OH)$_2$D$_3$ in a dose-dependent manner (mean initial decrease: $P<0.05$, $R^2=0.98$, adj$R^2=0.98$; recovery rate: $P<0.05$, $R^2=0.92$, adj$R^2=0.85$; mean final $[\text{Ca}^{2+}]$: $P<0.05$, $R^2=0.98$, adj$R^2=0.98$). The dose-dependent response gave EC$_{50}$ values of 8.5, 17.0 and 18.9 nM for the mean initial decrease, the recovery rate and the mean final $[\text{Ca}^{2+}]$, respectively.

Effects on $\text{Na}^+$/Ca$^{2+}$ exchangers

Figure 5A demonstrates that the initial changes in $[\text{Ca}^{2+}]$, as percentage changes from basal $[\text{Ca}^{2+}]$, during exposure to 10 nM 25(OH)D$_3$ are dependent on the Na$^+$ concentration in the incubation medium. The rapid decrease in $[\text{Ca}^{2+}]$, observed after treatment with 25(OH)D$_3$ could be described by a three-parameter sigmoid function ($P<0.05$; $R^2=0.79$; adj$R^2=0.74$), with an EC$_{50}$ estimated to be 127 ± 5.0 mM Na$^+$. Figure 5B shows initial changes in $[\text{Ca}^{2+}]$, as percentage changes from basal $[\text{Ca}^{2+}]$, during exposure to ethanol, 10 nM 25(OH)D$_3$ and 10 nM 25(OH)D$_3$ together with 10 µM bepridil. Statistical analyses of the three treatment groups showed significant differences in the mean initial change in $[\text{Ca}^{2+}]$, between the vehicle+25(OH)D$_3$-treated enterocytes (initial decrease of 16.24 ± 3.68% of basal $[\text{Ca}^{2+}]$) and the bepridil+ 25(OH)D$_3$ treated cells.
Effects on L-type Ca\(^{2+}\) channels

The [Ca\(^{2+}\)]\(_i\) recovery rate, after exposure to 10 nM 25(OH)D\(_3\), was decreased in the enterocytes preincubated with 5 µM nifedipine, compared with the cells that were preincubated with vehicle (P<0.05; Fig. 6B). No effects were observed on the mean initial decrease in [Ca\(^{2+}\)]\(_i\) (Fig. 6A) or the mean final [Ca\(^{2+}\)]\(_i\) values (Fig. 6C), between nifedipine and control-treated cells, after addition of 10 nM 25(OH)D\(_3\).

Effects on intracellular Ca\(^{2+}\) stores

Addition of 25(OH)D\(_3\) (10 nM) to enterocytes in the absence of extracellular Ca\(^{2+}\) resulted in a decrease in mean initial [Ca\(^{2+}\)]\(_i\) (Fig. 7B). This initial decrease in [Ca\(^{2+}\)]\(_i\) was followed by a recovery and then an increase in [Ca\(^{2+}\)]\(_i\) (Fig. 7C); the increase was maintained until the end of the experiment (Fig. 7D). Thus the same three parameters of [Ca\(^{2+}\)]\(_i\), changes in 25(OH)D\(_3\)-treated enterocytes (mean initial decrease, recovery rate and mean final [Ca\(^{2+}\)]\(_i\)) were calculated as described above and, when compared with the corresponding events in vehicle-treated cells, were all statistically different from their corresponding control groups (P<0.05).

Effects of 1,25(OH)\(_2\)D\(_3\) on enterocyte Ca\(^{2+}\) homeostasis

The mean initial decrease in [Ca\(^{2+}\)]\(_i\) (Fig. 8B), the recovery rate up to a new [Ca\(^{2+}\)]\(_i\) value (Fig. 8C) and the mean final [Ca\(^{2+}\)]\(_i\) value (Fig. 8D), were all affected by 1,25(OH)\(_2\)D\(_3\) (0.26 pM – 32.5 nM) in a dose-dependent manner (mean initial decrease: P<0.05, \(R^2=0.97, \text{adj} R^2=0.96\); recovery rate: P<0.05, \(R^2=0.99, \text{adj} R^2=0.99\); mean final [Ca\(^{2+}\)]\(_i\): P<0.05, \(R^2=0.99, \text{adj} R^2=0.99\)) with EC\(_{50}\) values of 8.3, 24.5 and 7.7 nM respectively. The observed EC\(_{50}\) values associated with the effects of 1,25(OH)\(_2\)D\(_3\) on [Ca\(^{2+}\)]\(_i\) are well above the concentrations of 1,25(OH)\(_2\)D\(_3\) measured in the circulation of the Atlantic cod (125.1 ± 12.3 pM in the present study; 130 ± 39.9 pM in the study by Sundell et al. (1992)). This suggested that the rapid effects of 1,25(OH)\(_2\)D\(_3\) on [Ca\(^{2+}\)]\(_i\) were non-physiological, and no further attempts were made.
to characterize effects of this seco-steroid on Atlantic cod enterocytes.

**Discussion**

The present study demonstrated that, in physiological concentrations, 24R,25(OH)2D3 and 25(OH)D3 have profound effects on [Ca2+]i regulation in Atlantic cod enterocytes, whereas physiological concentrations of 1,25(OH)2D3 did not affect [Ca2+]i. This strongly suggests that 24R,25(OH)2D3 and 25(OH)D3 have roles in the short-term (seconds to minutes) endocrine control of Ca2+ balance in the Atlantic cod, whereas 1,25(OH)2D3 does not.

**Figure 4** Effects of 25(OH)D3 on Ca2+ uptake on Atlantic cod enterocytes. (A) Representative recordings of [Ca2+]i of intestinal cell suspensions. The arrow denotes addition of 25(OH)D3 (20 nM) or vehicle (control). Effects of 25(OH)D3 (0.002, 0.02, 0.2, 1, 5, 10, 20 and 50 nM): (B) on the mean initial change in [Ca2+]i in enterocytes, 0–5 s after addition of hormone (P<0.05); (C) on the recovery rate of [Ca2+]i during the time interval 5–24 s after treatment with 25(OH)D3 (P<0.05); (D) on the mean final [Ca2+]i after treatment with 25(OH)D3 (P<0.05). n=8 for each treatment; each experiment contained enterocytes from four fish.
The study also demonstrated that 24R,25(OH)2D3 inhibits Ca2+ entry and abolishes an increase in [Ca2+]i evoked by S(-)-BAYK 8644 in enterocytes. L-type Ca2+ channels as a target for 24R,25(OH)2D3 action have previously been reported in ROS 17/2·8 cells (Caffrey & Farach-Carson 1989, Yukihiro et al. 1994, Takeuchi & Guggino 1996) and UMR 106 cells (Li et al. 1997). In ROS 17/2·8 cells, 24R,25(OH)2D3 decreases the entry of calcium-45 and inhibits voltage-gated Ca2+ channels at positive membrane potentials (Caffrey & Farach-Carson 1989, Yukihiro et al. 1994). Furthermore, 24R,25(OH)2D3 antagonizes an increased opening of L-type Ca2+ channels evoked by 1,25(OH)2D3 and testosterone (Takeuchi & Guggino 1996). In UMR 106 cells, Li et al. (1997) reported a dual action of 24R,25(OH)2D3 in modulating the activity of L-type Ca2+ channels. Thus 24R,25(OH)2D3 regulates [Ca2+]i and Ca2+ entry by affecting the activity of L-type Ca2+ channels in both osteoblasts and enterocytes.

Reports concerning the mechanisms behind the ability of 24R,25(OH)2D3 to rapidly modulate the activity of L-type Ca2+ channels are scarce. A specific binding moiety for 24R,25(OH)2D3 has been demonstrated in chick enterocyte basolateral membrane and lysosomes (Nemere et al. 1994, Larsson 1999), chick tibial fracture-healing callus (Seo et al. 1996, Kato et al. 1998) and chondrocytes (Pedrozo et al. 1999), suggesting that the rapid actions of 24R,25(OH)2D3 are mediated through a membrane-associated receptor. In mouse osteoblasts and UMR 106 cells, treatment with 24R,25(OH)2D3 results in a release of Ca2+ from the endoplasmic reticulum and mitochondria (Lieberherr 1987), activation of phospholipase C leading to the formation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Grosse et al. 1993), and activation of protein kinase A and protein kinase C (Li et al. 1996). These second messenger responses reported for osteoblasts correspond well with observations on enterocytes, in which 24R,25(OH)2D3 triggers the release of Ca2+ from intracellular stores and an activation of protein kinase C (Larsson & Nemere 2001). Although current data on non-genomic

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**Figure 5** Effects of 25(OH)D3 on Na+/Ca2+ exchangers in Atlantic cod enterocytes. (A) Sodium-dependent effects on mean initial change in [Ca2+]i, 0–5 s after addition of 10 nM 25(OH)D3, as a percentage change from basal [Ca2+]i, of intestinal cell suspensions pre-exposed to 30, 60, 90 or 120 mM Na+. The observed effects could be fitted to a three-parameter sigmoid function (P<0.05; Y=a(1+e^-(x-x0/b))+c). (B) Mean initial change in [Ca2+]i, 0–5 s after addition of 10 nM 25(OH)D3 or vehicle, in intestinal cell suspensions during exposure to bepridil (10 µM) or methanol (5 µl/3 ml). Data are presented as means±S.E.M. Different letter indicates significant difference between groups (P<0.05). n=8 for each treatment; each experiment contained enterocytes from four fish.
Effects of 24R,25(OH)2D3 and 25(OH)D3 on enterocyte Ca2+ homeostasis

Effects of 24R,25(OH)2D3 are derived from different species and Ca2+ regulatory organs, a consistent feature is its ability to simultaneously regulate the activity of L-type Ca2+ channels and Ca2+ release from intracellular stores. This can be interpreted as an indication that the hormone binds to a membrane-associated receptor and increases IP3 and DAG concentrations, which trigger intracellular Ca2+ release. This could decrease the activity of voltage-gated Ca2+ channels by an increase in [Ca2+]i or through an activation of protein kinase C, or both (Plant et al. 1983). An alternative explanation is that these two effects constitute two independent signaling pathways in enterocytes, mediated through separate receptors or the same receptor associated with different membrane environments.

The physiological effects of 25(OH)D3 and lack of effects of 1,25(OH)2D3 in physiological concentrations seen in the present study are consistent with results from in vitro perfused Atlantic cod intestines, in which 25(OH)D3 increases the intestinal uptake of calcium-45, whereas 1,25(OH)2D3 is without effect (Sundell & Björnsson 1990, Larsson et al. 1995). Thus the combined data from intestinal perfusions, together with the data presented in this study, suggest that 25(OH)D3 increases the intestinal mucosa-to-serosa transport by increasing Ca2+ extrusion through Na+/Ca2+ exchangers across the basolateral membrane, concurrent with an increase in Ca2+ uptake through L-type Ca2+ channels.

The observations that 25(OH)D3, but not 1,25(OH)2D3, mediates a rapid, non-genomic increase in intestinal Ca2+ uptake in Atlantic cod differ from what has been reported for freshwater fish, birds and mammals. In the freshwater-adapted teleosts, European eel (Chartier et al. 1979) and Tilapia (Flik et al. 1982), 1,25(OH)2D3 treatment results in an increased intestinal Ca2+ uptake. Similar results have been obtained for the perfused chick duodenum and rat intestinal cells (Nemere et al. 1984, Yoshimoto & Norman 1986, Massheimer et al. 1994), in which physiological concentrations of 1,25(OH)2D3 stimulate intestinal Ca2+ uptake and increase [Ca2+]i in isolated intestinal cells. Both in chicken and in rat (Olson & DeLuca 1969, Walling et al. 1974, Yoshimoto & Norman 1986), 25(OH)D3 increased intestinal Ca2+ uptake, whereas the effects of 25(OH)D3 on intestinal Ca2+ uptake in freshwater fish have not been elucidated. However, previous studies on European eel (Chartier et al. 1979), American eel (Anguilla rostrata; Fenwick et al. 1984) and goldfish (Carrassius auratus; Fenwick 1984) show that the less polar vitamin D metabolite, vitamin D3, is...
Figure 7 Effects of 25(OH)D₃ on intracellular Ca²⁺ release in enterocytes from Atlantic cod. (A) Representative tracings of changes in [Ca²⁺]ᵢ in enterocytes incubated with 2 mM EGTA. The arrow denotes addition of 25(OH)D₃ to reach a final concentration of 10 nM, or vehicle (control) to the cell suspension. (B) Effects on the mean initial [Ca²⁺]ᵢ in enterocytes, during 5 s of exposure to 10 nM 25(OH)D₃ or vehicle (control). (C) Effects on the recovery rate of [Ca²⁺]ᵢ (% change from basal [Ca²⁺]ᵢ min⁻¹) in enterocytes in a Ca²⁺-free buffer, 5–24 s after addition of 25(OH)D₃ (final concentration 10 nM) or vehicle (control). (D) The mean final [Ca²⁺]ᵢ (% change from basal [Ca²⁺]ᵢ) in enterocytes in a Ca²⁺-free buffer were compared between intestinal cells exposed to 10 nM 25(OH)D₃ and vehicle (control). Data are presented as means±S.E.M. *Statistically significant difference from control (*P<0·05). n=8 for each treatment; each experiment contained enterocytes from four fish.
equivalent to 1,25(OH)$_2$D$_3$ in stimulating intestinal Ca$^{2+}$ uptake and whole-animal Ca$^{2+}$ turnover in freshwater fish. Together, these findings suggest that calcitropic effects of the vitamin D endocrine system may have changed from less polar (vitamin D$_3$ and 25(OH)D$_3$) to more polar metabolites (1,25(OH)$_2$D$_3$) as active stimulators of Ca$^{2+}$ entry during vertebrate evolution.

Figure 8 Effects of 1,25(OH)$_2$D$_3$ on Ca$^{2+}$ uptake were investigated in enterocytes from Atlantic cod. (A) Representative recordings of [Ca$^{2+}$]$_i$ in intestinal cell suspensions. The arrow denotes addition of 1,25(OH)$_2$D$_3$ (to reach a final concentration of 13 nM) or vehicle (control). Effects of 1,25(OH)$_2$D$_3$ (0.26, 26, 130, 650, 6500, 13 000 and 32 500 pM): (B) on the mean initial [Ca$^{2+}$]$_i$ in enterocytes, 0–5 s after addition of hormone (P<0.05); (C) on the recovery rate of [Ca$^{2+}$]$_i$, 5–24 s after hormone treatment (P<0.05); (D) on the mean final [Ca$^{2+}$]$_i$ (P<0.05). n=8 for each treatment; each experiment contained enterocytes from four fish.
Marine teleosts are known to have a high hepatic vitamin D content, which has been suggested to be due to the teleost liver being the major site for hydroxylation of 25(OH)D₃ to more polar metabolites (Kobayashi et al. 1996). A second plausible explanation for the high hepatic vitamin D content of marine teleosts may be that 25(OH)D₃ is stored in the liver, preventing its rapid effect on calcium uptake.

The recovery rate of \([Ca^{2+}]_i\) after 25(OH)D₃ treatment of Atlantic cod enterocytes was sensitive to nifedipine, suggesting a hormone-activated opening of L-type Ca²⁺ channels in these cells. This is in line with the findings of previous studies and seems to indicate a general phenomenon for cells in different Ca²⁺ regulatory organs. In the chicken (deBoland & Norman 1990) and rat (Massheimer et al. 1994), 1,25(OH)₂D₃ caused a rapid increase in transcellular Ca²⁺ transport and an increased \([Ca^{2+}]_i\), respectively. Both events were associated with opening of L-type Ca²⁺ channels. The ability of 1,25(OH)₂D₃ and 25(OH)D₃ to increase the activity of L-type Ca²⁺ channels has also been demonstrated in osteoblasts (Caffrey & Farach-Carson 1989, Yukihiro et al. 1994, Takeuchi & Guggino 1996, Li et al. 1997), in which the two metabolites increase the period during which L-type Ca²⁺ channels stay open, and leading to an increased influx of extracellular Ca²⁺. The sodium- and bepridil-sensitive decrease in \([Ca^{2+}]_i\) observed within 10 s of exposure to 25(OH)D₃ indicates another pathway for non-genomic effects of the metabolite, through which 25(OH)D₃ can rapidly regulate the activities of Na⁺/Ca²⁺ exchangers, in addition to its regulation of L-type Ca²⁺ channels. A similar cellular response has been observed in Goby (Gillichthys mirabilis) enterocytes (Loretz & Assad 1986), in which urotensin II causes an immediate decrease in \([Ca^{2+}]_i\). This rapid decrease in \([Ca^{2+}]_i\) is dependent on the sodium concentration in the medium, which, in parallel with the present study, indicates a hormonal regulation of the Na⁺/Ca²⁺ exchange activity in fish enterocytes. Thus the present data suggest that 25(OH)D₃ can increase intestinal Ca²⁺ uptake in the Atlantic cod through a two-step mechanism: in the first step, the Na⁺/Ca²⁺ exchangers are activated and thus the Ca²⁺ efflux is increased, leading to a rapid decrease in \([Ca^{2+}]_i\), which could trigger the second step – a release of Ca²⁺ from intracellular stores or an opening of L-type Ca²⁺ channels (or both), as suggested by Plant et al. (1983), or alternatively, 25(OH)D₃ may trigger another cellular signal that affects the activity of the L-type Ca²⁺ channels. The combined effects of increased Na⁺/Ca²⁺ exchanger activity in the basolateral membrane and L-type Ca²⁺ channels in the brush border membrane would be a net increase in the enterocyte transcellular Ca²⁺ transport.

The present study demonstrates that 24R,25(OH)₂D₃ and 25(OH)D₃ have rapid, antagonistic effects on Ca²⁺ homeostasis in Atlantic cod enterocytes. Similar antagonistic effects of 24R,25(OH)₂D₃ and 1,25(OH)₂D₃ on Ca²⁺ fluxes across L-type Ca²⁺ channels have previously been reported in osteoblasts (Takeuchi & Guggino 1996). The physiological relevance of rapid, non-genomic effects on intestinal Ca²⁺ transport and on mineralization processes in bone may be to enable a rapid regulation of free Ca²⁺ concentrations in the blood and extracellular fluids. Marine species, such as Atlantic cod, living in a high Ca²⁺ environment, are at a constant risk of becoming hypercalcemic. Freshwater fish, birds and mammals, in contrast, face a situation in which a constant active uptake of Ca²⁺ is necessary to avoid hypocalcemia. Thus the differences in the rapid actions of the vitamin D₃ metabolites in marine fish compared with freshwater fish, birds and mammals may be due to an adaptation of the vitamin D₃ endocrine system to the different calcium environments of different animal species, or a divergent evolution of the vitamin D₃ endocrine system selected on the environmental availability of Ca²⁺, or both (Larsson et al. 1995, Sundell et al. 1996, Larsson 1999).

In conclusion, the present study has demonstrated that 24R,25(OH)₂D₃ and 25(OH)D₃ are active regulators of intestinal Ca²⁺ uptake at physiological concentrations. 24R,25(OH)₂D₃ decreases Ca²⁺ uptake through L-type Ca²⁺ channels, whereas 25(OH)D₃ increases it, concurrent with an increase in Ca²⁺ extrusion by Na⁺/Ca²⁺ exchangers, in enterocytes from Atlantic cod.

Acknowledgements
The authors gratefully acknowledge Dr N Taniguchi and Dr A Kato from Kureha Chemical Industry Co., Tokyo, Japan, for the generous gift of 24R,25(OH)₂D₃. This work was supported by
the Swedish Natural Science Research Council, Nordisk Forskerudanningsakademi (NorFA), Helge Axson Johnsons Foundation and the Hierta-Retzius Foundation. All fish handling and experimental procedures were approved by the Göteborg University Ethical Committee on Animal Research.

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Received in final form 28 July 2001
Accepted 13 September 2001