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Ca^{2+} Uptake Through Voltage-gated L-type Ca^{2+} Channels by Polarized Enterocytes from Atlantic Cod *Gadus morhua*

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Abstract. The presence and localization of voltage-gated Ca^{2+} channels of L-type were investigated in intestinal cells of the Atlantic cod. Enterocytes were loaded with the fluorescent Ca^{2+} probe, fure-2/AM and changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured, in cell suspensions, in the presence of high potassium levels (100 mM), BAY K-8644 (5 μM), nifedipine (5 μM) or ω-conotoxin (1 μM). L-type Ca^{2+} channels were visualized on intestinal sections using the fluorescent dihydropyridine (-)-STBodipy.

Depolarization of the plasma membrane produced a rapid (within 5 sec) and transient (at basal levels after 21 sec) increase in $[Ca^{2+}]_i$. BAY K-8644 increased the $[Ca^{2+}]_i$ by 7.2%. Cells in a Ca^{2+}-free buffer increased $[Ca^{2+}]_i$ after addition of 10 mM Ca^{2+}, and this increase was abolished by nifedipine in both depolarizing and normal medium but not by ω-conotoxin. Single cell experiments using video microscopy revealed that enterocytes remained polarized several hours after preparation and that the Ca^{2+} entry and extrusion occurred at specific and different regions of the enterocyte outer membrane. Fluorescent staining of L-type Ca^{2+} channels in the intestinal mucosa showed the most intense staining at the brushborder membrane.

These results demonstrate the presence of voltage gated L-type Ca^{2+} channels in enterocytes from the Atlantic cod. The channels are mainly located at the apical side of the cells, and there is a polarized uptake of Ca^{2+} into the enterocytes. This suggests that the L-type Ca^{2+} channels are involved in the transcellular Ca^{2+} entry into the enterocytes.

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Key words: BAY K-8644 — nifedipine — (-)-STBodipy — ω-conotoxin — intestine — teleost

Introduction

The intestine is an important organ for uptake of calcium (Ca^{2+}) from ingested food and water, in order to meet the Ca^{2+} demand of the organism. In freshwater and seawater fish, the intestine contributes to at least 30% of the total body Ca^{2+} uptake (Sundell & Björnsson, 1988; Flik et al., 1990). In the marine teleost, the Atlantic cod (*Gadus morhua*), the intestinal Ca^{2+} absorption consists of a saturable (60%) and a nonsaturable component (40%), reflecting that intestinal Ca^{2+} uptake takes place through both paracellular and transcellular pathways (Sundell & Björnsson, 1988).

The transcellular Ca^{2+} movement through intestinal cells of teleosts can be described in three steps: (i) Ca^{2+} entry across the enterocyte brushborder membrane, (ii) protein-bound Ca^{2+} transport through the cytosol, as indicated by the presence of calcium-binding proteins in intestinal cells from the carp (*Cyprinus carpio;* Ooizumi, Moriuchi & Hosoya, 1970; Chartier Baraduc, 1973), and (iii) extrusion from the cell across the basolateral membrane by Ca^{2+}-ATPases and/or Na^+/Ca^{2+} exchangers (Flik et al., 1990; Schoenmakers & Flik, 1992; Schoenmakers et al., 1993; D. Larsson, P. Lundqvist, A. Linde & K. Sundell, *unpublished*).

Intracellular Ca^{2+} concentrations are normallly in the nM range, and the enterocyte interior is negatively charged compared with the intestinal lumen. This allows Ca^{2+} to move across the brushborder membrane down an electrochemical gradient (Fullmer, 1992; Favus, 1992). For the freshwater tilapia (*Oreochromis mossambicus;* Klaren et al., 1993), as well as for other vertebrates

(Miller & Bronner, 1981; Miller, Li & Bronner, 1982; Takito et al., 1990; Bronner, 1991), the Ca^{2+} uptake into isolated brushborder membrane vesicles is described as a sum of a saturable and a nonsaturable part, suggesting a channel- or carrier-mediated uptake as well as a diffusional uptake. However, the actual molecular mechanisms responsible for the apical Ca^{2+} entry are still unknown.

To examine the nature of the Ca^{2+} entry across the brushborder membrane of the fish intestine, this study was designed to investigate the presence and localization of Ca^{2+} channels in Atlantic cod enterocytes. Furthermore, the present study attempted to characterize the type of Ca^{2+} channels present and discuss their role in transcellular Ca^{2+} transport.

Materials and Methods

Fish and Chemicals

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 days before sacrifice.

Nifedipine was obtained from Sigma (St. Louis, MO). Pluronic F-127 was purchased from Calbiochem (La Jolla, CA). ω-Conotoxin GVIA and BAY K-8644 were obtained from Research Biochemicals International (Natlick, MA). Fura-2/AM was obtained from Fluka (Buchs, Switzerland). The (4,4-diflouro-7-styryl-4-boro-3a,4a-diaza)-3(s-indacene) propionic acid 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethyl-phenyl)-3,5-pyridinecarboxylic acid 2-(aminoethyl)ethyl ester hydrochloride conjugate ((-)-STBodipy) was purchased from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma (St. Louis, MO) and were of analytical grade.

Intestinal Cell Preparation

The intestinal epithelium of the Atlantic cod is homogenous and does not contain crypts. The majority of the cells are lining columnar cells (enterocytes) with occasional mucocytes and endocrine cells (Bishop & Odense, 1966).

The method of intestinal cell isolation was modified from Walters and Weiser (1987). In short, the fish were killed by a blow to the head and the proximal ⅔ of the intestine were dissected out, rinsed with 0.9% NaCl and cut open lengthwise. To reduce the possibility of contamination by excitable cells, the mucosal cell layer was stripped off the circular and longitudinal muscle layers along the submucosa and cut into 1 cm pieces.

Mucosal segments from 4 fish were pooled, placed in buffer A (in mM): 96 NaCl; 1.5 KCl; 8 KH_2PO_4; 5.6 Na_2HPO_4; 2 citrate; pH 7.3 and vigorously shaken for 10 min. Free cells and the remaining mucosal segments were sedimented by centrifugation at 700 × *g* for 10 min. The pellet was resuspended in buffer B (in mM): 154 NaCl; 10 Na_2HPO_4; 1.5 EDTA; 0.5 dithiothreitol; pH 7.3 and shaken for another 10 min. Free cells were then sedimented by centrifugation at 700 × *g* for 10 min, washed 2 times with buffer C (in mM): 154 NaCl; 10 Na_2HPO_4; pH 7.3 and finally resuspended in buffer D (0.25 M sucrose; 12.5 mM NaCl; 5 mM Hepes; 0.1 mM dithiothreitol; 10 μg/ml imidazole; 0.3 mM phenylmethylsulfonyl fluoride (PMSF); pH 7.3). All preparative steps and centrifugations were performed at 4°C.

Cell viability was determined by trypan blue exclusion in combination with phase contrast microscopy and was found to be greater than 95%.

Loading of FURA-2/AM

The fura-2/AM loading was performed as described by Thomas & Delaville (1991). Briefly, freshly dissected intestinal cells were incubated for 45 min in buffer D with fura-2/AM (5 μM), pluronic F-127 (0.025%) and albumin (0.5%) at 37°C. The cells were washed three times in buffer D by centrifugation at 700 × *g* for 10 min, and finally resuspended in buffer E (in mM): 120 NaCl; 20 Hepes-Tris; 10 Glucose; 4.7 KCl; 1.2 KH_2PO_4; 1.2 $MgSO_4$; 2 $CaCl_2$; pH 7.3.

Measurement of Free Intracellular Ca^{2+} Concentrations ($[Ca^{2+}]_i$)

Fluorescence Spectrophotometry

Measurements of $[Ca^{2+}]_i$ in fura-2 loaded Atlantic cod intestinal cells (10^5 cells/ml), were performed in a Perkin-Elmer LS 50B double beam luminescence spectrometer at a 340/380 nm excitation ratio, with a 510 nm emission wavelength. The cells were placed in a quartz cuvette, slowly stirred at a constant temperature of 10°C. Fluorometric calibrations were made by addition of 10 μl Triton X-100 to lyse the cells and obtain the maximum fluorescence intensity of Ca^{2+}-saturated fura-2/AM, and followed by addition of 15 μl 400 mM EGTA/3 M Tris to measure the intensity of Ca^{2+}-free fura-2/AM.

$[Ca^{2+}]_i$ was calculated using the equation:

$$[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{S_f}{S_b} \quad (1)$$

Where K_d is the dissociation constant for fura-2/AM, 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 triton X-100, 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} (Grynkiewicz, Poenie & Tsien, 1985).

Fluorescence video microscopy

Changes in $[Ca^{2+}]_i$ of single fura-2 loaded enterocytes were recorded at 22°C with a Rainbow excitation wavelength filter wheel (Life Science Resources, Cambridge, England) carrying 340 and 380 nm filters, attached to an inverted Diaphot 300 microscope (Nikon, Tokyo, Japan), equipped with oil immersion and fluorescence objectives. The emitted light was measured at 510 nm using a digital CCD video camera (Optronics, Goltea, CA). Data obtained were processed, and digital images were acquired using a Ca^{2+} image software, Mira*Cal* (Life Science Resources, Cambridge, England). Fluorometric calibrations were performed as described previously (*see* Fluorescence Spectrophotometry). Background fluorescence was measured and subtracted in all recordings.

Experimental Design and Ca^{2+} Channel Characterization

Ca^{2+} binding constant (K_d)

K_d for fura-2/AM was determined in vitro at 37, 22 and 10°C according to Grynkiewicz et al. (1985), using fluorescent spectrophotometry.

Briefly, four fluorometric titrations with increasing total Ca^{2+} concentrations (0, 0.8, 1.54, 2.21, 2.84, 3.41, 3.94, 4.42, 4.87, 5.28 and 5.66 mM) in buffer F (100 mM KCl; 10 mM 3-[N-morpholino]propanesulfonic acid (MOPS); 10 mM EGTA; 1 mM $MgCl_2$; 1 μM fura-2, pentasodium salt; pH 7.2) were performed at each temperature. The free $[Ca^{2+}]$ was determined from known total $[Ca^{2+}]$ according to Schoenmakers et al. (1992), and the K_d of the Ca^{2+}/fura-2 complex was resolved by Hill plots at each temperature.

Ca^{2+} uptake kinetics

Ca^{2+}-dependent Ca^{2+} uptake was investigated in cell suspensions, using fluorescent spectrophotometry. In each experiment, 3 ml of the cell suspension (diluted 1:30 in Ca^{2+}-free buffer E) was placed in a cuvette and acclimated for 5 min to reach a constant temperature of 10°C (n = 4). The $[Ca^{2+}]_i$ was measured after addition of increasing Ca^{2+} concentrations to the cell suspension (0, 0.25, 0.5, 1, 2, 4, 8 and 10 mM), in order to evaluate the kinetics of Ca^{2+} uptake into enterocytes. J_{max} and K_m were calculated according to Michaelis & Menten (1913), using nonlinear regression.

Voltage-dependent Ca^{2+} uptake

Fluorescence video microscopy was used to investigate the voltage-dependent Ca^{2+} uptake into single enterocytes from the Atlantic cod. Forty μl of the cell suspension in buffer E (n = 10) was transferred to, and spread out on a 150 μm thick microslide. The cells were placed in a dark and humid chamber for 45 min in order to allow the cells to attach to the glass surface. The basal $[Ca^{2+}]_i$ was recorded during 30 sec, then 0.5 μl of a 5 M K^+-solution was added and the $[Ca^{2+}]_i$ was continuously recorded for another 60 sec.

L-type Ca^{2+} channels

The presence of L-type Ca^{2+} channels was examined in cell suspensions, using fluorescence spectrophotometry, by the aid of the L-type Ca^{2+} channel agonist BAY K-8644 and the antagonist nifedipine (Triggle & Rampe, 1989). In the first set of experiments, 3 ml of the cell suspension (diluted 1:30 in high-Ca^{2+} buffer E, $[Ca^{2+}]$ = 10 mM) were placed in the cuvette and allowed a 5 min acclimation period to reach a constant temperature of 10°C. Basal $[Ca^{2+}]_i$ was recorded over a 5 min period. Thereafter, 15 μl of BAY K-8644 in buffer E (giving a concentration of 5 μM in the cuvette; n = 13) or 15 μl of the vehicle alone (n = 10) was added directly to the cuvette, after which the $[Ca^{2+}]_i$ was recorded for another 5 min. In the second set of experiments, 3 ml of the cell suspension (diluted 1:30 in Ca^{2+}-free buffer E) were acclimated in the absence (n = 22) or presence (n = 10) of 5 μM nifedipine. Basal $[Ca^{2+}]_i$ was recorded during 5 min, after which 10 mM Ca^{2+} was added directly to the cuvette and the $[Ca^{2+}]_i$ was continuously recorded for 5 min.

N-type Ca^{2+} Channels

Fluorescence spectrophotometry was likewise used to investigate the presence of N-type Ca^{2+} channels in cod enterocytes. The intestinal cells were acclimated in a Ca^{2+}-free buffer E, in the absence (n = 12) or presence (n = 12) of 1 μM ω-conotoxin. The basal $[Ca^{2+}]_i$ was recorded for 5 min, then 10 mM Ca^{2+} was added to the cuvette and the $[Ca^{2+}]_i$ was recorded for another 5 min period.

Significance of L-type Ca^{2+} Channels in Voltage Dependent Ca^{2+} Uptake

Fluorescent spectrophotometry was used to examine the importance of dihydropyridine sensitive Ca^{2+} channels in voltage dependent Ca^{2+} uptake. Cell suspensions of enterocytes were acclimated in a Ca^{2+}-free, K^+-high buffer G (in mM): 20 Hepes-Tris; 10 Glucose; 120 KCl; 1.2 KH_2PO_4; 1.2 $MgSO_4$; pH 7.3 with (n = 6) or without (n = 7) 5 μM nifedipine present in the cuvette. The basal $[Ca^{2+}]_i$ was recorded for 5 min, then 10 mM Ca^{2+} was added to the cuvette and the $[Ca^{2+}]_i$ was recorded for another 5 min period.

Visualization of L-Type Ca^{2+} Channels

The proximal ⅔ of the intestine from Atlantic cod (n = 3) were dissected out, flushed with 0.9% NaCl and cut into 1 cm segments. Three segments from each intestine were mounted in Tissue-Tek (Histolab, Göteborg, Sweden) and immediately frozen in liquid nitrogen. The frozen segments were sectioned in a cryostat and adhered to microslides. The slides were washed and hydrated for 3 × 5 min in buffer H (in mM): 140 NaCl: 10 Hepes-Tris; 5.5 Glucose; 5 KCl; 0.5 $MgCl_2$; 1.2 $CaCl_2$; pH 7.3 prior to incubation. Each section was incubated for 5 min in the presence of 10 nM of the fluorescent (-)-STBodipy dihydropyridine conjugate. Control samples were preincubated with 10 μM nifedipine in the medium for 10 min prior to the addition of (-)-STBodipy. After the incubations, the samples were washed for 3 × 5 min in buffer H, and visualized in a Nikon fluorescence microscope, equipped with 546 nm excitation and 590 emission filters (Knaus et al., 1992).

Statistics

Student's t test was used to test for significant differences between control and treatment groups in each experiment. A one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls posthoc test was performed when comparing a factor with more than two levels. The testing used was two-tailed, and the significance level was set at $P < 0.05$. Data are presented as mean ± SEM.

Results

Ca^{2+} Binding Constant

The dissociation constant (K_d) of the Ca^{2+}/fura-2/AM complex at different temperatures was determined from Hill plots, where the intercept with the abscissa equals the pK_d (Fig. 1). The Hill plot yielded a K_d of 232 nM, 308 nM and 360 nM at 37, 22 and 10°C, respectively. The Hill coefficients were 1.03 (r^2 = 0.99), 1.00 (r^2 = 0.99) and 1.20 (r^2 = 0.98), in accordance with the 1:1 stoichiometry of the Ca^{2+}/fura-2/AM complex at all 3 temperatures.

Ca^{2+} Uptake Kinetics

Figure 2 shows $[Ca^{2+}]_i$ in enterocytes of the Atlantic cod, measured at different Ca^{2+} concentrations in the assay

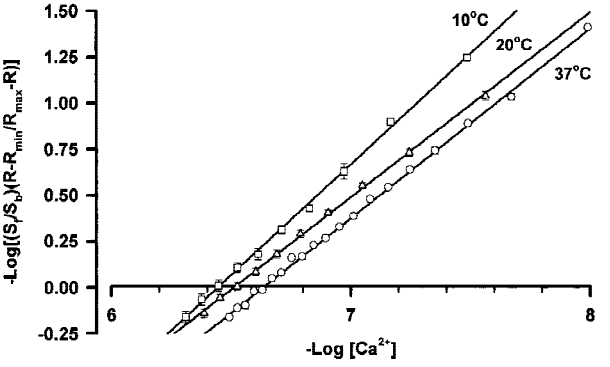

Fig. 1. The pK_d for fura-2/AM binding to Ca^{2+} is determined by the intercept with the x-axis in a Ca^{2+} dependent double log plot. Four separate titrations with 10 different Ca^{2+} concentrations were performed at 37, 22 and 10°C. The K_d was calculated to be 232 nM, 308 nM and 360 nM at 37, 22 and 10°C, respectively.

medium. The $[Ca^{2+}]_i$ reaches a new steady level 30 sec after addition of extracellular Ca^{2+} and reflects an increased Ca^{2+} uptake with increasing extracellular Ca^{2+} concentrations. The Ca^{2+} uptake was saturable and followed Michaelis Menten kinetics ($r^2 = 0.53$). J_{max} and K_m are calculated to be 318 ± 21 nmol $Ca^{2+} \cdot sec^{-1}$ and 56.4 ± 1.1 μM Ca^{2+}, respectively.

Voltage Dependent Ca^{2+} Channels

When the cells were depolarized by addition of 100 mM K^+ to the incubation medium ($n = 10$), a biphasic response in the $[Ca^{2+}]_i$ was observed. A rapid increase in $[Ca^{2+}]_i$ (367 nM ± 84 nM, at $t = 5$ sec) was followed by a slower decrease back to basal levels (200 nM ± 11 nM, at $t = 21$ sec; $P < 0.05$). A representative recording of $[Ca^{2+}]_i$ in fura-2/AM loaded cod enterocytes is shown in Fig. 3A. Digital images of a single polarized enterocyte showing the $[Ca^{2+}]$ in different parts of the cell, at 4 different time-points (I-IV), during the K^+-induced membrane depolarization are presented in Fig. 3*B*. At time-point II; 5 sec after administration of K^+, the $[Ca^{2+}]$ has increased at one side of the enterocyte, 10 sec later (at time-point III) the highest $[Ca^{2+}]$ is found associated with the other side of the enterocyte. At the last time-point (IV) the $[Ca^{2+}]$ has reached basal levels again and the image resembles that at the first time-point (I). The enterocytes remained polarized with respect to Ca^{2+} uptake and extrusion several hours after preparation, indicating that the enterocytes are able to keep the composition of the brushborder and the basolateral membranes intact even when they are isolated.

Ca^{2+} Channel Characterization

Representative recordings from the flurorescence spectrophotometric analyses using the L-type Ca^{2+} channel

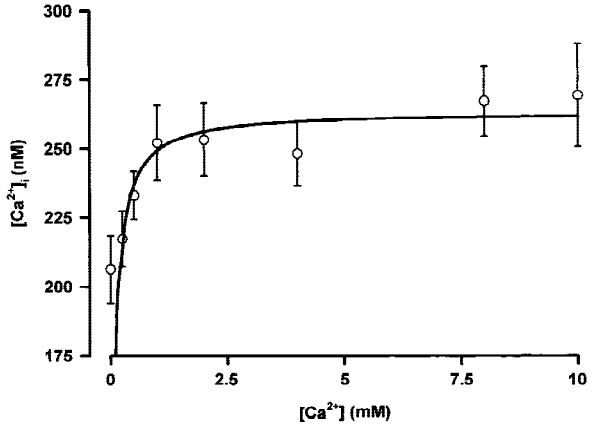

Fig. 2. The Ca^{2+} uptake into enterocytes of the Atlantic cod was measured at different Ca^{2+} concentrations in the assay medium. The $[Ca^{2+}]_i$ was measured 30 sec after addition of 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 10 mM Ca^{2+} in 4 different cell suspensions (300,000 cells · ml^{-1}), each consisting of enterocytes prepared from 4 individuals. Data points were fitted to the Michaelis Menten equation ($r^2 = 0.53$). J_{max} was calculated, using nonlinear regression, to be 318 ± 21 nmol $Ca^{2+} \cdot sec^{-1}$ and K_m to 56.4 ± 1.1 μM Ca^{2+}.

agonist, BAY K-8644 and the L-type and N-type Ca^{2+} channel antagonists, nifedipine and ω-conotoxin, are presented in Fig. 4*A-C*. The addition of BAY K-8644 to intestinal cells in a high-Ca^{2+} buffer increases $[Ca^{2+}]_i$ with 7.2 ± 2.3% compared with the control group ($P < 0.05$; Figs. 4*A* and 5). After pre-exposure of intestinal cells to either of the two antagonists in a Ca^{2+}-free buffer and a 5 min basal recording period, 10 mM Ca^{2+} was added to the cells and changes in $[Ca^{2+}]_i$ were recorded (Fig. 4*B* and *C*). The L-type Ca^{2+} channel antagonist nifedipine suppressed the increase in $[Ca^{2+}]_i$ seen in the control group by approximately 90% ($P < 0.05$; Figs. 4*B* and 5). The N-type Ca^{2+} channel antagonist ω-conotoxin, on the other hand, did not affect the increase in $[Ca^{2+}]_i$ observed after addition of 10 mM Ca^{2+} (Figs. 4*C* and 5).

Significance of L-type Ca^{2+} Channels in Voltage-Dependent Ca^{2+} Uptake

After depolarization of enterocytes with Ca^{2+}-free and K^+-high buffer G, the cells responded to addition of 10 mM extracellular Ca^{2+} by an increase in $[Ca^{2+}]_i$ with 13.2 ± 1.8% compared to basal levels ($P < 0.05$). When nifedipine was present in this system, no difference in $[Ca^{2+}]_i$ was seen after addition of 10 mM extracellular Ca^{2+}. When comparing the changes in $[Ca^{2+}]_i$ for nifedipine, depolarized and BAY K-8644 treated cells in an one factorial ANOVA, with three levels, followed by Student-Newman-Keuls multiple comparison test, two significantly different groups were evident ($P < 0.05$). The nifedipine-treated enterocytes ($\Delta[Ca^{2+}]_i =$

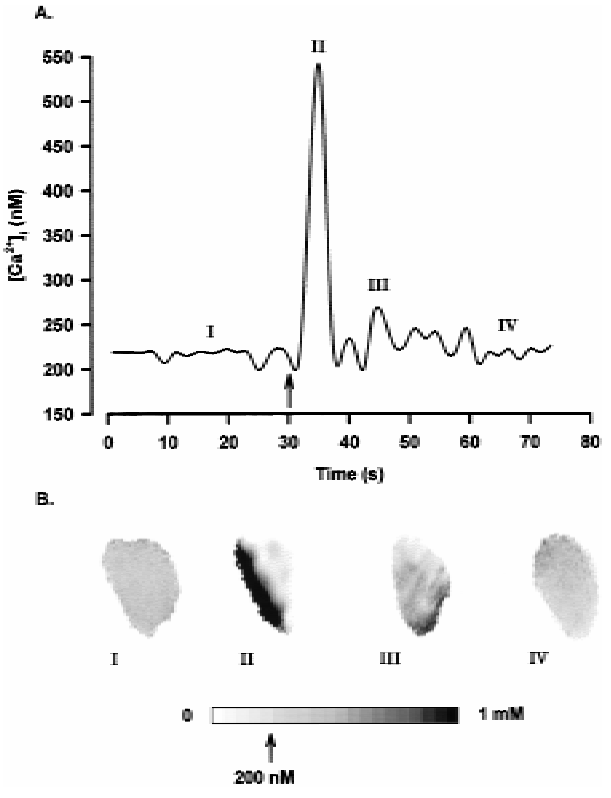

Fig. 3. Effects of 100 mM K^+ on Ca^{2+} uptake in fura-2/AM loaded intestinal cells from the Atlantic cod. (*A*) A representative tracing of changes in $[Ca^{2+}]_i$ during membrane depolarization in a single intestinal cell. The Roman numbers indicate the time-points from which digital images of the cells are acquired. The arrow denotes addition of 100 mM K^+ to the incubation medium. (*B*) Digital images of the $[Ca^{2+}]$ in different parts of the cell, from 4 different time-points, $I = 16$ sec after start of the recording of $[Ca^{2+}]$, II = 5 sec after addition of 100 mM K^+, III = 15 sec after addition of 100 mM K^+ and IV = 55 sec after addition of 100 mM K^+.

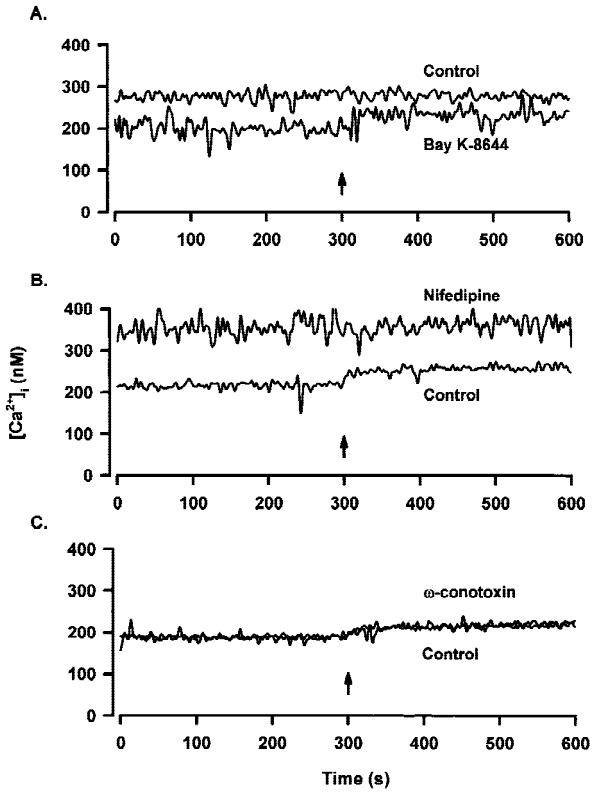

Fig. 4. Representative tracings of changes in $[Ca^{2+}]_i$ after administration of 3 different dihydropyridines. (*A*) Recording of $[Ca^{2+}]_i$ of intestinal cell suspensions in high Ca^{2+} buffer. The arrow denotes addition of vehicle (control) or BAY K-8644 (5 μM). (*B*) Recording of $[Ca^{2+}]_i$ of intestinal cell suspenstions in a calcium-free buffer, with nifedipine (5 μM) or vehicle (control) present from the start of the experiment. The arrow denotes addition of 10 mM Ca^{2+}. (*C*) Recording of $[Ca^{2+}]_i$ of intestinal cell suspensions in a calcium free buffer, with ω-conotoxin (1 μM) or vehicle (control) present from the start of the experiment. The arrow denotes addition of 10 mM Ca^{2+}.

$-0.25 \pm 2.4\%$) in one group and the depolarized ($\Delta[Ca^{2+}]_i = 13.2 \pm 1.8\%$) and BAY K-8644-treated intestinal cells ($\Delta[Ca^{2+}]_i = 7.2 \pm 2.3\%$) in a second group (Fig. 6).

Visualization of L-type Ca^{2+} Channels

Using the fluorescent dihydropyridine conjugate (-)-STBodipy as a probe for L-type Ca^{2+} channels, staining was found in the mucosa and in the muscle layers of the Atlantic cod intestine (Fig. 7*A*). The highest staining intensity was observed in the apical part of the epithelial cells (Fig. 7*B*), in nerve fibers in the myenteric plexa and in nerve fibers and/or blood vessels in the circular muscle layer (Fig. 7*A* and *C*). Preincubation of the intestinal sections with nifedipine resulted in a complete loss of staining (Fig. 7*D*).

Discussion

The present study provides evidence that Ca^{2+} channels are present in intestinal cells of the marine teleost, the Atlantic cod. The $[Ca^{2+}]$ in the cytosol of single enterocytes changed in a sequential fashion during depolarization by potassium. Addition of dihydropyridines affects the Ca^{2+} uptake of enterocytes in both depolarizing and normal medium, whereas ω-conotoxin was without effect, indicating that the Ca^{2+} channels are voltage sensitive and of L-type. Fluorescent microscopy reveals that the highest density of L-type Ca^{2+} channels is associated with the brushborder membrane of the enterocytes.

Voltage-sensitive Ca^{2+} channels have been demonstrated in excitable cells from various vertebrates. The use of channel-specific agonists and antagonists, such as dihydropyridines, phenylalkylamines and ω-conotoxin, have been important tools for Ca^{2+} channel characteriza-

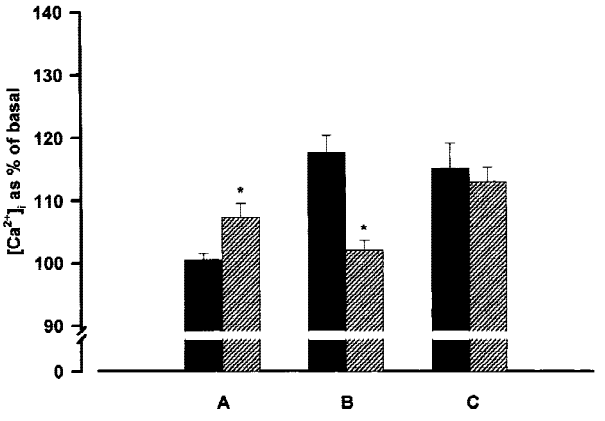

Fig. 5. Changes in $[Ca^{2+}]_i$, as a percentage of basal $[Ca^{2+}]_i$, of intestinal cell suspensions after treatment (striped bars) with (*A*) BAY K-8644 (5 μM), (*B*) nifedipine (5 μM) and (*C*) ω-conotoxin GVIA (1 μM) compared with vehicle treated (black bars) controls. Data are presented as means ± SEM (n = 10–22). *Indicate statistical differences from control ($P < 0.05$).

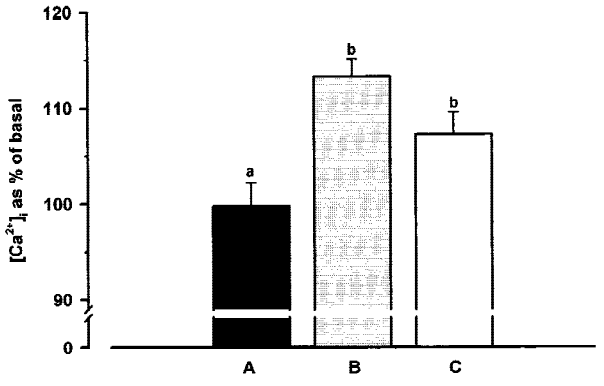

Fig. 6. Changes in $[Ca^{2+}]_i$ after addition of 10 mM Ca^{2+}, as a percentage of basal $[Ca^{2+}]_i$, of intestinal cell suspensions during exposure to (*A*) nifedipine (5 μM) and 120 mM K^+, (*B*) 120 mM K^+. Changes in $[Ca^{2+}]_i$ after addition of (*C*) BAY K-8644 (5 μM), as a percentage of basal $[Ca^{2+}]_i$ of intestinal cell suspensions. Data are presented as means ± SEM (n = 6–13). Different letter indicates significant difference between groups using one-factorial ANOVA with 3 levels followed by Student-Newman-Keuls multiple comparison test ($P < 0.05$).

tion (Tibbits, Hove-Madsen & Bers, 1991; Horne et al., 1993; Sierra et al., 1995; Tokumaru et al., 1995; Grabner et al., 1996). Among teleosts, so far two types of voltage-gated Ca^{2+} channels have been identified. ω-Conotoxin-sensitive N-type Ca^{2+} channels, which regulate transmittor release have been identified in the electric organ of the freshwater teleost, *Gymnotus carapo* (Sierra et al., 1995), and L-type Ca^{2+} channels sensitive to dihydropyridines have been found in the skeletal muscle of the carp and in pituitary cells of the goldfish, *Carassius auratus* (Grabner et al., 1991; Jobin, Neumann & Chang, 1996). The mammalian L-type channel α1 subunit, which forms the channel pore, shows several highly invariant regions compared with the carp α1 subunit, including the dihydropyridine-sensitive part (Grabner et al., 1991; Grabner et al., 1996). Thus, it seems likely that the L-type Ca^{2+} channels are highly conserved among different vertebrate groups and that they are characterized by their unique sensitivity to dihydropyridines (Triggle & Rampe, 1989; Grabner et al., 1996). The fact that the Ca^{2+} channels in the Atlantic cod enterocytes are sensitive to dihydropyridines in a commonly used concentration range (Lieberherr, 1987; Triggle & Rampe, 1989; Massheimer, Boland & de Boland, 1994; Meszaros et al., 1996), lead us to conclude that they are true L-type Ca^{2+} channels.

Incubation of intestinal sections from the Atlantic cod with the fluorescent dihydropyridine (-)-STBodipy revealed that the most intense staining was localized in the brushborder membrane of the epithelial cells. These results suggest that the L-type Ca^{2+} channels are unevenly distributed in enterocyte cell membranes, and indicate that they are mainly located in the brushborder membrane. The single cell measurements demonstrated an uptake of Ca^{2+} along one side of the cell and extrusion of Ca^{2+} along a different side of the cell during depolarization. Taken together these two results indicate that L-type Ca^{2+} channels are involved in the uptake of Ca^{2+} into the enterocytes across the brushborder membrane. This is well in agreement with the gradient of Ca^{2+} being directed from the mucosal side into the enterocyte (Fullmer, 1992; Favus, 1992), and the previous results of Ca^{2+} uptake into intestinal brushborder membrane vesicles from fish (Klaren et al., 1993), where the Ca^{2+} permeability was higher than could be explained by simple diffusion across the lipid bilayer (Klaren et al., 1993; Klaren, Wendelaar Bonga & Flik, 1997). In mammals and birds, there are indications for voltage-gated Ca^{2+} channels both in brushborder and basolateral membranes of enterocytes. High and low affinity binding sites for Ca^{2+} have been demonstrated in the brushborder membrane of the rat duodenum (Miller & Bronner, 1981; Miller, Li & Bronner, 1982), and it has been suggested that these binding sites represent Ca^{2+} channels and/or carriers (Bronner, 1991; Favus, 1992). Homaidan et al. (1989) demonstrated voltage gated L-type Ca^{2+} channels in the basolateral membrane of rabbit enterocytes. Both dihydropyridines and phenylalkylamines bound to high-affinity sites on basolateral membrane vesicles and affected the Ca^{2+} uptake in a dose-dependent manner (Homaidan et al., 1989). Furthermore, in vivo perfusions of the chick intestine with depolarizing buffer or dihydropyridines in the vascular perfusate were found to affect intestinal Ca^{2+} uptake (de Boland & Norman, 1990; de Boland, Nemere & Norman, 1990). Also in Atlantic cod Ca^{2+} channels are present in both brushborder and basolateral membranes but in much higher densities on the apical side. It is therefore questionable, if the chan-

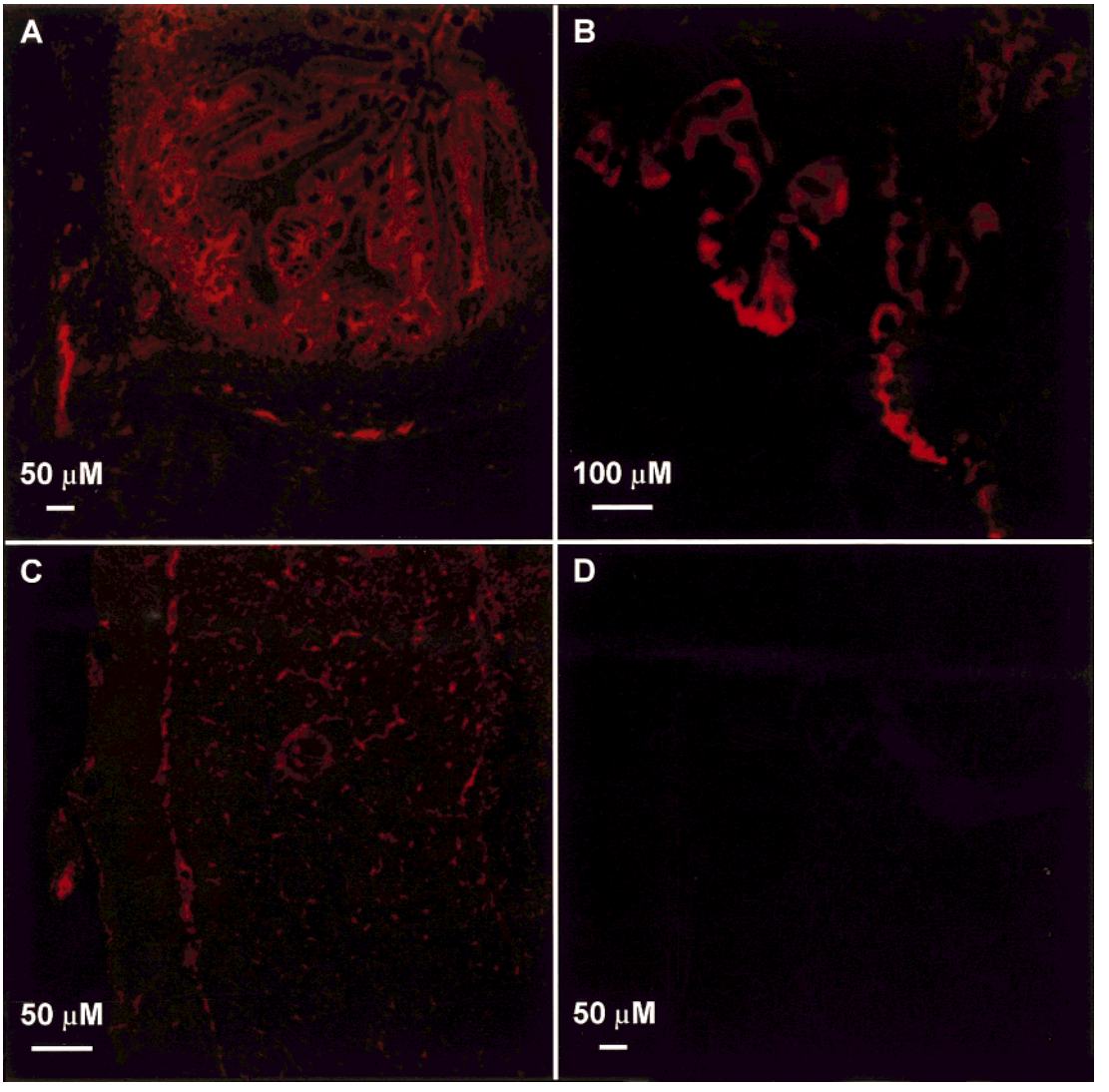

Fig. 7. Incubation of the fluorescent dihydropyridine, (-)-STBodipy, to intestinal sections of the Atlantic cod. (*A*) Columnar cells in the intestinal mucosa and nerve fibers in the circular muscle layer showing staining for L-type Ca^{2+} channels (×40). (*B*) Enterocytes in the intestinal epithelium showing a dense staining for L-type Ca^{2+} channels in the brushborder membrane (×160). (*C*) Nerve fibers in the myenteric plexa and nerve fibers and/or blood vessels in the circular muscle layer showing staining for L-type Ca^{2+} channels (×80). (*D*) Preincubation of intestinal sections with nifedipine abolished all staining for L-type Ca^{2+} channels (×40).

nels are of same importance for the Ca^{2+} transport across the basolateral membranes as across the brushborder membrane in Atlantic cod. Depolarization of enterocytes evoked a significant Ca^{2+} uptake across one part of the cell membrane. The voltage dependent Ca^{2+} uptake is to a large extent mediated through L-type Ca^{2+} channels. Nifedipine abolished the depolarization induced Ca^{2+} uptake and there was no difference in magnitude of the Ca^{2+} uptake between cells treated with BAY K-8644 and cells in depolarizing medium. Thus, our results imply that L-type Ca^{2+} channels in the brushborder membrane are important for uptake of Ca^{2+} into the enterocyte, and probably also for the transcellular absorption of Ca^{2+} across the fish intestine. On the other hand, at the basolateral side of the cell L-type Ca^{2+} channels may be important for vesicle fusion to the basolateral membrane as suggested by Wassermann et al. (1992), even though they are present in relatively low densities.

Ca^{2+} uptake by Atlantic cod enterocytes exhibited saturation kinetics and was correlated to the calcum concentration in the assay medium. Similar kinetics for Ca^{2+} uptake have been observed in brushborder membrane vesicles from the tilapia intestine (Klaren et al., 1993), rat small intestine (Miller & Bronner, 1981) and chick duodenum (Takito et al., 1990). Furthermore, Ca^{2+} uptake across the teleost gill is mainly a transcellular transport, where the Ca^{2+} entry across the brushborder membrane likewise follows saturable kinetics and

thus, is believed to be mediated by Ca^{2+} channels and/or carriers (Flik & Verbost, 1994). Inhibition of Ca^{2+} and Cd^{2+} entry into branchial Ca^{2+} transporting cells by La^{3+} in both rainbow trout (*Oncorhynchus mykiss;* Perry & Flik, 1988) and zebrafish (*Brachydanio rerio;* Wicklund Glynn, Norrgren & Müssener, 1994) indicates that Ca^{2+} channels are present in branchial tissue. However, the type of Ca^{2+} channel remains to be elucidated as the L-type Ca^{2+} channel antagonist verapamil inhibited Cd^{2+} uptake in the zebrafish (Wicklund Glynn et al., 1994), but another L-type Ca^{2+} channel antagonist, nifedipine, was without effect on Ca^{2+} uptake in rainbow trout branchial cells (Perry & Flik, 1988). Thus, the two organs important for Ca^{2+} uptake from the environment in teleosts, gills and intestine, seem to possess Ca^{2+} channels. Ca^{2+} entry across the brushborder membrane of the enterocyte is mediated by L-type Ca^{2+} channels, whereas the Ca^{2+} channels governing the Ca^{2+} entry in branchial tissue are of unknown nature.

To determine intracellular Ca^{2+} concentrations accurately, it is crucial to determine K_d of fura-2 for the actual temperatures, pH, ionic strength and buffer system used in the fluorometric measurements. Our data show that use of previously published K_d values (224 nM at 37°C) in experiments at room temperature (22°C) or at physiological temperatures for the Atlantic cod (10°C) would result in an error of 27 and 38% for the calculated $[Ca^{2+}]_i$ respectively. This is in accordance with the report of Shuttleworth & Thompson, (1991), showing an increased K_d with decreasing temperature.

As in mammals and birds, the intestinal Ca^{2+} absorption in teleost fish is under hormonal control (Flik & Verbost, 1993; Sundell, Larsson & Björnsson, 1996). In the Atlantic cod, the intestinal Ca^{2+} uptake is regulated by 25-hydroxyvitamin D_3, 1,25- and 24,25-dihydroxyvitamin D_3 (Sundell & Björnsson, 1990; Sundell, Norman & Björnsson, 1993; Larsson, Björnsson & Sundell, 1995), and a possible target for these vitamin D_3 metabolites may be the L-type Ca^{2+} channels in the enterocytes. Treatment with the L-type Ca^{2+} channel antagonists verapamil and nifedipine suppressed the increased intestinal Ca^{2+} absorption normally seen after treatment with 1,25-dihydroxyvitamin D_3 in both rat enterocytes (Massheimer et al., 1994) and chick intestines (de Boland & Norman, 1990), suggesting that 1,25-dihydroxyvitamin D_3 may regulate the L-type Ca^{2+} channels of mammals and birds. Furthermore, studies on osteoblasts have demonstrated that both expression (Meszaros et al., 1996) and activity (Lieberherr, 1987) of L-type Ca^{2+} channels are governed by several vitamin D_3 metabolites.

In conclusion, L-type voltage-gated Ca^{2+} channels are present in enterocytes of the Atlantic cod. A major part of these Ca^{2+} channels is located in the enterocyte brushborder membrane. Furthermore, it is concluded that the Ca^{2+} uptake and extrusion occur at sections of the enterocytic plasma membrane, separate from each other. Expression of voltage-gated Ca^{2+} channels, mainly in the brushborder membrane of intestinal cells, may form a mechanism to control Ca^{2+} entry into the enterocytes and the subsequent transcellular Ca^{2+} uptake. These channels may provide a regulatory site for hormones controlling Ca^{2+} homeostasis of the organism.

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