

KINETICS OF CALCIUM FLUXES ACROSS THE INTESTINAL MUCOSA OF THE MARINE TELEOST, *GADUS MORHUA*, MEASURED USING AN *IN VITRO* PERFUSION METHOD

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Accepted 20 April 1988

Summary

An *in vitro* technique for perfusion of the intestinal vasculature and lumen was developed and used to measure calcium (Ca^{2+}) fluxes across the intestinal mucosa of the marine teleost, the Atlantic cod (*Gadus morhua*). Saturable and nonsaturable components of the calcium influx and efflux were determined.

The calcium influx had one passive component and one saturable component, following Michaelis–Menten kinetics with $K_m = 8.41 \text{ mmol l}^{-1}$ and $V_{\max} = 0.604 \mu\text{mol Ca}^{2+} \text{ kg}^{-1} \text{ h}^{-1}$. At physiological Ca^{2+} concentrations in the vascular ($[\text{Ca}^{2+}] = 1.9 \text{ mmol l}^{-1}$) and luminal ($[\text{Ca}^{2+}] = 14.9 \text{ mmol l}^{-1}$) perfusion fluids, the saturable component amounted to 60% of the Ca^{2+} influx. The high-affinity Ca^{2+} -ATPase inhibitor chlorpromazine (CP, $10^{-4} \text{ mol l}^{-1}$) antagonized 45% of the Ca^{2+} influx.

The Ca^{2+} efflux across the intestinal mucosa of the cod was a saturable process, following Michaelis–Menten kinetics with $K_m = 6.15 \text{ mmol l}^{-1}$ and $V_{\max} = 3.79 \mu\text{mol Ca}^{2+} \text{ kg}^{-1} \text{ h}^{-1}$, but insensitive to CP ($10^{-5} \text{ mol l}^{-1}$). The Ca^{2+} efflux was $1.22 \mu\text{mol Ca}^{2+} \text{ kg}^{-1} \text{ h}^{-1}$, representing about 20% of the total calcium excretion and about 50% of the extrarenal excretion of the Atlantic cod *in vivo*.

Introduction

Teleost fish maintain free plasma calcium (Ca^{2+}) at stable levels, between 1.7 and 1.9 mmol l^{-1} (Chan & Chester Jones, 1968; Björnsson & Nilsson, 1985), irrespective of the calcium concentration in their natural environment, which can range from 0.005 – 2.5 mmol l^{-1} in fresh water (FW) to about 10 mmol l^{-1} in sea water (SW) (Urist, 1962; Chan & Chester Jones, 1968; Pang *et al.* 1980; Fenwick & Wendelaar Bonga, 1982).

Studies on the major routes of Ca^{2+} uptake and excretion in teleost fish have to a large extent been focused on the gills (see Simmons, 1971; Simkiss, 1974), where both passive and active fluxes have been demonstrated (Payan *et al.* 1981; Flik *et al.* 1985; Perry & Wood, 1985). In marine teleosts, however, not only the gills but

Key words: Ca^{2+} efflux, Ca^{2+} influx, perfusion, chlorpromazine, Atlantic cod, *in vitro*, intestine.

also the gastrointestinal tract is in close contact with the external environment, as these species drink SW ($2.1\text{--}11.3\text{ ml kg}^{-1}\text{ body mass h}^{-1}$) to compensate for osmotic water loss (Smith, 1930; House & Green, 1965; Skadhauge, 1969; Bentley, 1971). Since the ingested SW contains approximately five times higher Ca^{2+} levels than the blood, marine teleosts are forced to reduce the influx of calcium over the intestine and/or compensate for the diffusive influx by excretion of calcium. From studies on marine teleosts, Smith (1930) concluded that the monovalent ions were primarily absorbed in the gastrointestinal tract, whereas the divalent ions were retained in the intestinal fluid. For a long period it was accepted that the intestine of marine teleosts is relatively impermeable to divalent ions (see Simkiss, 1974). However, more recent *in vivo* studies on euryhaline species adapted to sea water (Hickman, 1968; Shehadeh & Gordon, 1969) as well as on marine species (Fletcher, 1978; Björnsson & Nilsson, 1985) have concluded that magnesium and sulphate ions are retained to a great extent in the intestinal fluid, while 30–70% of the ingested calcium is absorbed. Most of these studies suggest that Ca^{2+} absorption is due to a passive diffusional entry, but no analysis has been performed to confirm this.

The *in vitro* methods most extensively used for studies of intestinal ion fluxes in fish have been static preparations where a closed-off part of the intestine is suspended in an organ bath and fluxes between the luminal and serosal sides are measured. Such preparations may be right-side out or everted, with or without the muscle layers of the intestinal wall stripped off, all procedural differences which may affect the results (Ando & Kobayashi, 1978). For example, experiments were carried out to determine Ca^{2+} fluxes across the intestine of the Japanese eel, and a net absorption of Ca^{2+} was shown (Hasegawa & Hirano, 1984). However, the mechanisms involved in this absorption have not been investigated.

The present study describes a method of assessing intestinal calcium fluxes in the Atlantic cod, *Gadus morhua*, using a perfused *in vitro* system which allows determination of calcium fluxes across the cell layers separating the lumen and the circulation, instead of fluxes across the whole (or stripped) intestinal wall. The aim of the study was to assess intestinal calcium fluxes which may reflect the *in vivo* situation, and to determine the kinetics of the calcium flux components in this marine teleost species.

Materials and methods

Fish

Atlantic cod, *Gadus morhua*, were captured off the Swedish west coast and transferred to the laboratory. The experiments were performed at all seasons, except for experiments 5 and 6 which were carried out in late spring and summer (postspawning period). The fish were acclimated in tanks (3.6 m^3) with aerated, recirculated and filtered SW at 10°C , in a 15 m^3 aquarium system, for at least 1 week prior to experiments. The fish were of both sexes and weighed 400–800 g.

Ion composition of intestinal fluid and plasma

Fish were kept unfed in the laboratory for 2 weeks to clear the gut of food residues. At the time of the experiment, the fish were stunned with a blow to the head; blood was sampled by puncture of the caudal vessels using a heparinized (sodium heparin, Kabi Vitrum) syringe, and plasma was obtained by centrifugation. The fish were opened laterally on the right side and the first two-thirds of the intestine was ligated at the anterior and posterior ends and removed. The intestinal contents were carefully emptied into a syringe and filtered through a planktonic net (mesh size = 200 μm) into a test-tube. Plasma and intestinal fluid samples were kept at 4°C for no more than 12 h before being analysed.

Samples of intestinal fluid (200 μl) were ultrafiltered (Amicon MPS-1) to separate free and protein-bound fractions of calcium and magnesium (D'Costa & Cheng, 1983). The levels of protein-bound calcium and magnesium were then calculated as the differences between the levels in intestinal fluid and the levels in corresponding ultrafiltrates. Calcium and magnesium levels in intestinal fluid, ultrafiltrates and plasma were determined by atomic absorption spectroscopy (IL Video 12).

The protein-bound fractions of calcium and magnesium in intestinal fluid ($N = 8$) did not exceed 0.2 % of the total levels; thenceforth only total levels were measured. Plasma and intestinal fluid were also analysed for sodium and potassium by flame emission spectroscopy (Turner 510) using an internal lithium standard; for chloride by amperometric titration (Radiometer CMT 10) and for osmolality by vapour pressure osmometry (Wescor 5100B). Sulphate in plasma and intestinal fluid was measured indirectly by precipitation of sulphate with ^{133}Ba (Miller *et al.* 1963) and phosphate was measured spectrophotometrically (Perkin-Elmer Lambda 3) according to the method used by Dryer *et al.* (1957).

Intestinal perfusion technique

The fish were stunned with a blow to the head and injected with 2500 i.u. heparin kg^{-1} (5000 i.u. ml^{-1}) into the caudal vessels. The fish were opened laterally on the right side to expose the gastrointestinal tract. An inflow catheter (PE 50, Intramedic) was inserted into the coeliac artery, which supplies the first two-thirds of the intestine with blood, and an outflow catheter (PE 90, Intramedic) was inserted into the intestinal vein. Both catheters were secured to their respective vessels and all other vessels to and from the first two-thirds of the intestine were ligated (Fig. 1). The intestinal vasculature was perfused through the inflow catheter with filtered (Pyrex 3; Millipore) cod Ringer's solution containing (in mmol l^{-1}): NaCl, 150.1; NaHCO_3 , 23.8; KCl, 5.2; CaCl_2 , 2.9; NaH_2PO_4 , 2.7; MgSO_4 , 1.8; and glucose, 5.6. The Ringer's solution was gassed continuously with 97% O_2 and 3% CO_2 to maintain pH at 7.3 (Holmgren & Nilsson, 1974). A modified Ringer's solution containing $1.9 \text{ mmol l}^{-1} \text{ Ca}^{2+}$, which corresponds to

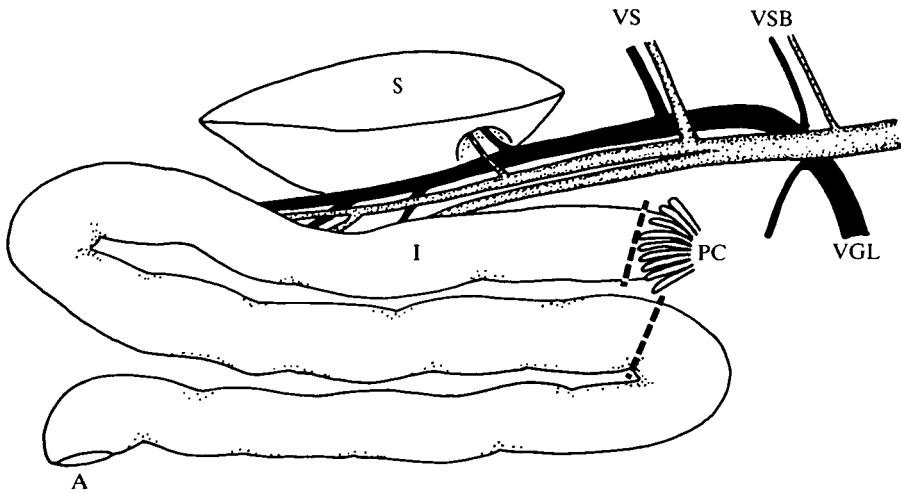


Fig. 1. The intestine (I) and its major vessels in the Atlantic cod *Gadus morhua*. Catheters were inserted into the coeliac artery (dotted) and the portal vein (black). The intestine was cut off (dashed line) just behind the pyloric caeca (PC) and at about two-thirds of the total intestinal length. Plastic tubing was inserted into the ends of the segments and tightly secured. VGL, branches of vessels to the gallbladder and liver; VSB, branches of vessels to the swimbladder; VS, branches of vessels to the stomach; spleen (S) and anus (A).

the free calcium concentration of cod plasma (Björnsson & Nilsson, 1985), was used for experiments on the effect of different luminal calcium concentrations on calcium influx (experiment 5, see below).

Plastic tubes (inner diameter = 2.5 mm) were inserted into the lumen of both ends of the intestinal segment and secured to prevent leakage (Fig. 1). The intestinal lumen was flushed with a balanced intestinal solution (BIS) based on measured ion levels in the intestinal fluid (in mmol l^{-1}): MgSO_4 , 102.7; NaCl , 80.3; K_2SO_4 , 29.8; CaCl_2 , 14.9; NaHCO_3 , 4.8; and NaH_2PO_4 , 0.55. The BIS was gassed continuously with 97% O_2 and 3% CO_2 to maintain pH at 6.5. The intestinal section was then dissected out and submerged in cod Ringer's solution kept at 10°C (Fig. 2). The preparation was completed within 30 min.

Throughout the experiments the intestinal lumen was perfused with BIS at a rate of 3.5 ml h^{-1} , using a peristaltic pump (Ole Dich), simulating estimated *in vivo* intestinal fluid flow in the Atlantic cod (Fletcher, 1978).

The intestinal vascular bed was perfused as described by Nilsson & Grove (1974) with a constant pressure head of 4 kPa, corresponding to the *in vivo* blood pressure in the coeliac artery of the cod (Helgason & Nilsson, 1973). The outflow pressure was kept around zero and the venous outflow (drops min^{-1}) was monitored. The luminal and the vascular perfusates were continuously sampled in 5-min fractions (Fig. 2).

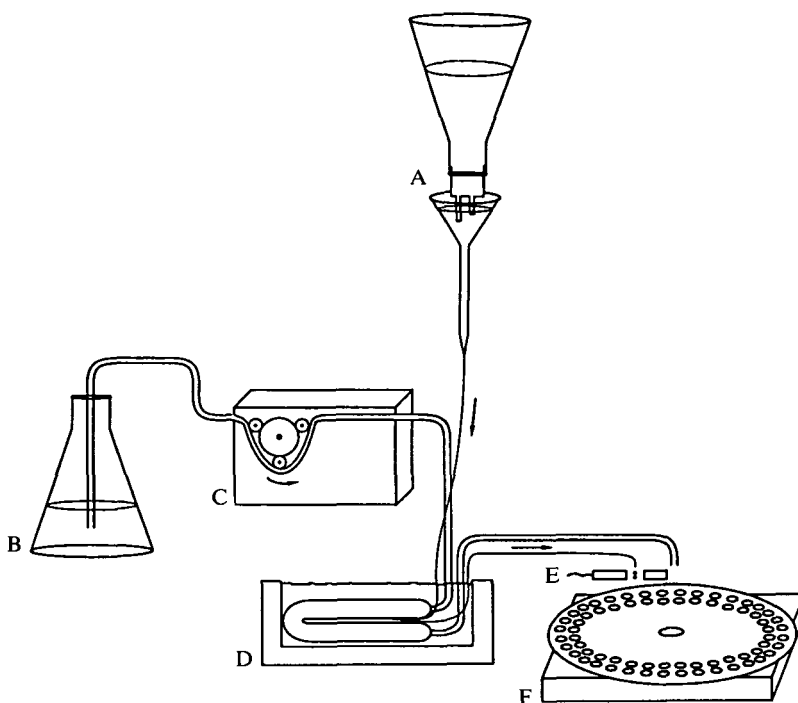


Fig. 2. Experimental set-up for the *in vitro* perfusion technique. A, Cod Ringer's solution for vascular perfusion at a constant perfusion pressure of 4 kPa; C, peristaltic pump perfusing the intestinal lumen at about 3.5 ml h^{-1} , with B, balanced intestinal solution; D, temperature-regulated bath filled with cod Ringer's solution at 10°C , submerging the intestine; E, drop counter registering the venous outflow in drops min^{-1} ; F, fraction collector, collecting 5-min fractions of vascular and luminal perfusate.

Control experiments

Lumen-to-circulation steady state

To determine the time needed to establish a steady flux of ^{45}Ca (Amersham) from the intestinal lumen to the circulation, the intestine was perfused as described above. The intestinal lumen was rapidly filled and thereafter perfused with ^{45}Ca -containing BIS (specific activity $0.2\text{--}0.6 \text{ MBq } \mu\text{mol}^{-1} \text{ Ca}^{2+}$). The vascular perfusate was sampled for 2 h and the amount of ^{45}Ca (disints min^{-1}) in each fraction was determined in a liquid scintillation counter (LKB Wallac, 1217 Rackbeta) with internal standard quench correction using 10 ml of scintillation fluid to 1 ml of sample. A steady flux of ^{45}Ca between the intestinal lumen and the vasculature was reached within 60 min ($N = 7$) (Fig. 3A).

Circulation-to-lumen steady state

To assess the time necessary to reach a steady flux of ^{45}Ca from the vasculature to the intestinal lumen, ^{45}Ca (specific activity $3.0\text{--}6.3 \text{ MBq } \mu\text{mol}^{-1} \text{ Ca}^{2+}$) was

added to the vascular perfusion fluid. The intestinal perfusion fluid was collected for 2 h and the radioactivity measured. A steady flux was reached within 60 min ($N = 4$) (Fig. 3B).

Intestinal passage time

To determine the time needed for the intestinal perfusate to pass through the intestinal lumen of the preparation and the attached anal tube, approximately $20 \mu\text{l}$ of Evans blue (1 ng ml^{-1} , Sigma) was injected into the plastic tubing at the oral end of the intestine after the preparation had been perfused for 1 h. The

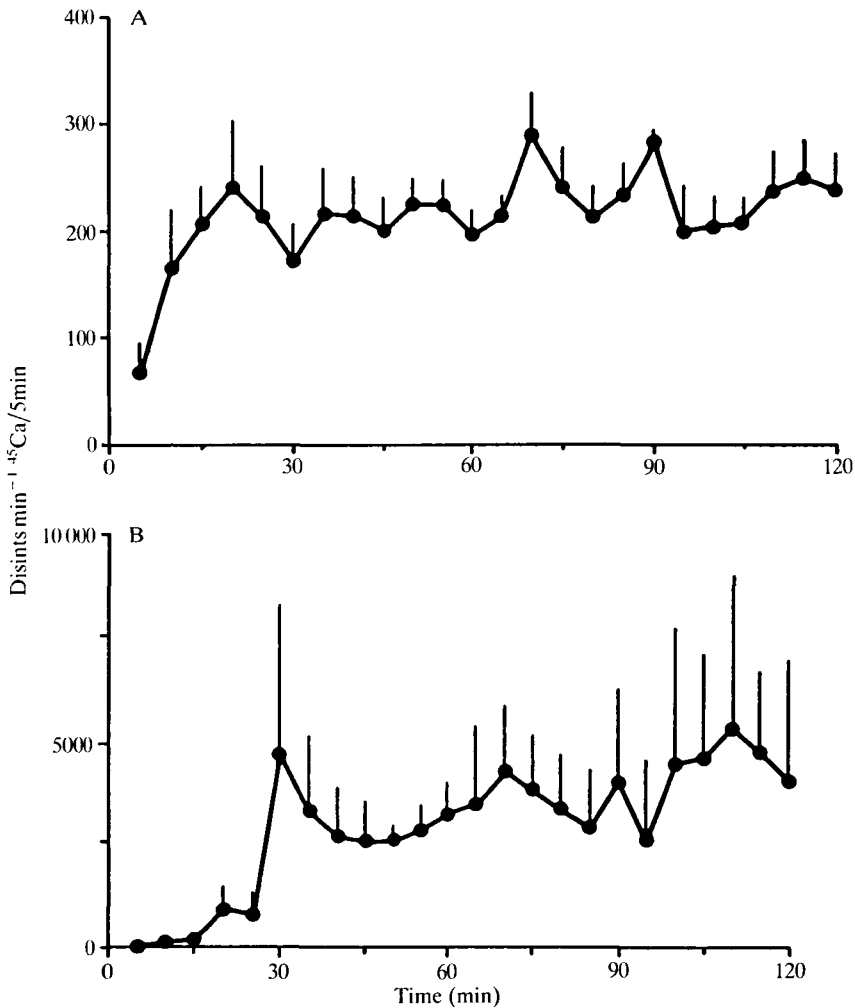


Fig. 3. Time course of ^{45}Ca fluxes across the intestinal mucosa of the Atlantic cod. (A) Amount of ^{45}Ca appearing in the vascular perfusate with time. ^{45}Ca was added to the balanced intestinal solution at time zero ($N = 7$). (B) Amount of ^{45}Ca appearing in the balanced intestinal solution with time. ^{45}Ca was added to the vascular perfusate at time zero ($N = 4$). Data are means \pm S.E.M.

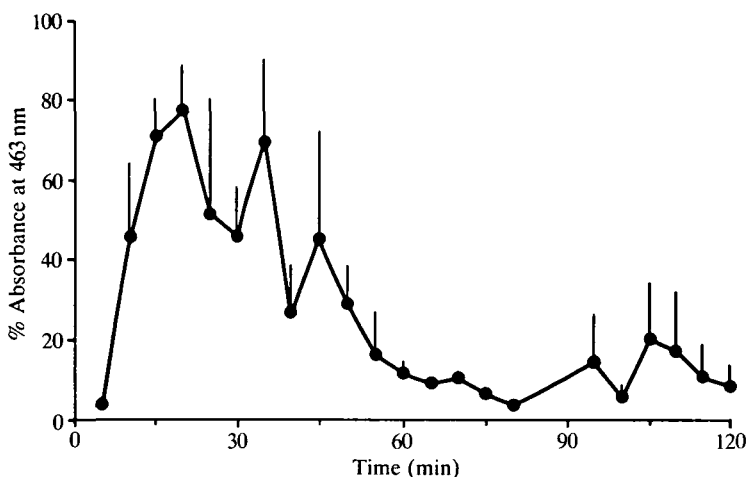


Fig. 4. Absorbance at 463 nm of the luminal perfusate as a function of time. Evans blue (approx. 0.02 ng) was injected into the balanced intestinal solution at the oral end of the intestinal segment at time zero. The absorbance of each fraction is expressed as a percentage of the highest obtained absorbance for each fish. Data are means \pm s.e.m. ($N = 5$).

intestinal perfusate was collected for 2–3 h and each fraction was analysed spectrophotometrically at 463 nm for Evans blue concentration (Perkin–Elmer 310). The highest dye concentration reached the fraction collector within 20 min, and over 80 % of the dye had reached the fraction collector within 1 h of the injection ($N = 5$) (Fig. 4).

Three criteria were set to ensure the physiological and mechanical quality of each perfusion. If any of these criteria were not met, the data were discarded. (1) The venous outflow must have exceeded 1.2 ml min^{-1} , otherwise excessive leakage or abnormal contraction of the preparation was indicated. (2) The intestine must have shown rhythmic activity during the whole experiment, indicating normal muscular activity. (3) The amount of ^{45}Ca in the cod Ringer's solution surrounding the intestine must not have exceeded 1.5 % of the amount of ^{45}Ca in the BIS. Otherwise a significant retrograde ^{45}Ca uptake from the bath to the vasculature occurred (unpublished data).

Experimental groups

Calcium influx (experiment 1)

^{45}Ca was added to the BIS (specific activity $0.5\text{--}3.6 \text{ MBq } \mu\text{mol}^{-1} \text{ Ca}^{2+}$; $^{45}\text{Ca}_{\text{sp}}$) in concentrations ranging between 0.008 and 0.06 % of the 'cold' calcium concentration in the BIS. The intestinal lumen was rapidly filled with this solution and then perfused at 3.5 ml h^{-1} . After 1 h, 5-min fractions of luminal and vascular perfusate were collected for 1–2 h. The amount of ^{45}Ca appearing in each fraction

of the vascular perfusion fluid ($^{45}\text{Ca}_f$) was assessed and the influx of calcium was calculated according to the equation:

$$J_{\text{in}}^{\text{Ca}} = \frac{\sum (^{45}\text{Ca}_f \times V)}{^{45}\text{Ca}_{\text{sp}}} \times \frac{1000}{X} \times \frac{60}{Y}, \quad (1)$$

where V is the volume of vascular perfusion fluid in each fraction in ml; X is body mass in g; and Y is the total sampling period in min.

The $J_{\text{in}}^{\text{Ca}}$ is thus the sum of the influx and the retrograde efflux. However, owing to the vascular perfusion flow rate, ^{45}Ca appearing in the circulation is effectively flushed away, so the retrograde flux is assessed as negligible.

Calcium efflux (experiment 2)

The intestinal lumen was rapidly filled with BIS and then perfused at 3.5 ml h^{-1} . ^{45}Ca was added to the vascular perfusate at concentrations of 0.02–0.04 % of the initial 'cold' calcium concentration (specific activity $1.8\text{--}4.4 \text{ MBq } \mu\text{mol}^{-1} \text{ Ca}^{2+}$; $^{45}\text{Ca}_{\text{sp}}$) and [^3H]inulin (specific activity 5.2 GBq g^{-1} inulin; Amersham) at a concentration of 9–17 MBq per ml of vascular perfusion fluid was added as a leakage marker. Collection of 5-min fractions of the vascular and luminal perfusates was started after 80 min of perfusion and continued for 1–2 h. The amounts of ^{45}Ca and [^3H]inulin in each fraction of the luminal perfusate ($^{45}\text{Ca}_f$ and $^3\text{H}_f$, respectively) were assessed. The Rackbeta scintillation counter (see above) was used in dual-isotope mode, in which quench curves were established for both isotopes in both energy windows (8–90 and 90–175 meV for ^3H and ^{45}Ca , respectively) prior to sample assays. Within each sample series, the quenching variation (% CV) was $<2\%$, and the spillover interference between the measurements of the two isotopes was $<5\%$.

The mechanical leakage of ^{45}Ca from the vasculature to the lumen (for example, through damaged capillaries) was assessed by the appearance of [^3H]inulin in the luminal perfusate. The efflux of calcium was calculated according to the equation:

$$J_{\text{out}}^{\text{Ca}} = \frac{\sum [(^{45}\text{Ca}_f - ^{45}\text{Ca}_i \times ^3\text{H}_f / ^3\text{H}_i) \times V]}{^{45}\text{Ca}_{\text{sp}}} \times \frac{1000}{X} \times \frac{60}{Y}, \quad (2)$$

where $^{45}\text{Ca}_i$ is initial disintegrations min^{-1} of ^{45}Ca per ml of the fluid perfusing the intestinal vasculature; $^3\text{H}_i$ is disintegrations min^{-1} of [^3H]inulin per ml of initial vascular perfusion fluid. The $J_{\text{out}}^{\text{Ca}}$ is thus also a gross flux including both the efflux and a negligible retrograde influx (see experiment 1).

Effects of chlorpromazine on calcium influx (experiment 3)

To determine the role of Ca^{2+} -ATPases in the calcium influx over the intestinal mucosa, the $J_{\text{in}}^{\text{Ca}}$ was measured for 1 h in eight preparations as in experiment 1 (specific activity of $^{45}\text{Ca} = 0.7\text{--}1.9 \text{ MBq } \mu\text{mol}^{-1} \text{ Ca}^{2+}$). Chlorpromazine (CP; Hibernall, Leo Rhodia; $10^{-4} \text{ mol l}^{-1}$) (Flik *et al.* 1983) was then added to the BIS and the calcium influx measured for another hour during constant luminal

perfusion with BIS containing both ^{45}Ca and CP. The calcium influx before and during treatment with CP was calculated using equation 1.

Effects of chlorpromazine on calcium efflux (experiment 4)

The role of Ca^{2+} -ATPases in the calcium efflux over the intestinal mucosa was studied in eight preparations. The efflux was measured for 1 h as described for experiment 2 (specific activities $^{45}\text{Ca} = 2.3\text{--}4.4 \text{ MBq } \mu\text{mol}^{-1} \text{ Ca}^{2+}$; $[^3\text{H}]\text{inulin} = 5.2 \text{ GBq g}^{-1} \text{ inulin}$), and $10^{-5} \text{ mol l}^{-1}$ CP was then added to the vascular perfusion fluid. After 20 min the calcium efflux was measured for 1 h and the $J_{\text{out}}^{\text{Ca}}$ before and during CP treatment were calculated using equation 2.

Effects of luminal calcium levels on the calcium influx (experiment 5)

Luminal calcium concentrations of 2, 4, 8, 15 and 32 mmol l^{-1} were tested on six preparations each, measuring calcium influx as in experiment 1. The calcium concentration in the vascular perfusion fluid was 1.9 mmol l^{-1} in this experiment. The different luminal calcium concentrations were obtained by adjusting the amount of CaCl_2 added to the BIS. The amount of ^{45}Ca added to the BIS was the same for all preparations, not exceeding 0.06% of the lowest calcium concentration (2 mmol l^{-1}), and the specific activity ranged between 0.4 and $6.3 \text{ MBq } \mu\text{mol}^{-1} \text{ Ca}^{2+}$.

Effects of circulating calcium levels on the calcium efflux (experiment 6)

The calcium efflux was measured in the same way as in experiment 2, but with vascular calcium concentrations of 0.5, 1, 2, 4 and 6 mmol l^{-1} . Six preparations were tested at each concentration. The different calcium concentrations were obtained by adjusting the amounts of CaCl_2 in the vascular perfusion fluid. The amounts of ^{45}Ca and $[^3\text{H}]\text{inulin}$ added to the vascular perfusion fluid were the same for all preparations. The levels of added ^{45}Ca did not exceed 0.02% of the lowest calcium concentration (0.5 mmol l^{-1}) and the amount of $[^3\text{H}]\text{inulin}$ was $9\text{--}17 \text{ MBq ml}^{-1}$ of vascular perfusion fluid. The specific activities for ^{45}Ca and $[^3\text{H}]\text{inulin}$ were $0.9\text{--}15.9 \text{ MBq } \mu\text{mol}^{-1} \text{ Ca}^{2+}$ and $5.2 \text{ GBq g}^{-1} \text{ inulin}$, respectively.

Statistical analysis

Two-way analysis of variance was used for testing the effects of the season and sex on calcium influx. The effects of CP on net calcium influx and efflux were tested using the Wilcoxon matched-pairs signed-ranks test. Nonlinear regression analysis, based on Michaelis–Menten kinetics and linear regression analysis, was performed to determine the correlations between calcium influx and luminal concentration as well as the correlation between calcium efflux and circulating calcium concentrations. Nonlinear regression was also used to calculate K_m and V_{max} for the saturable curves (Wilkinson, 1961). Data are presented as means \pm S.E.M.

Results

Ion composition of intestinal fluid and plasma

Ion compositions of plasma ($N = 10$), intestinal fluid ($N = 10$) and sea water are presented in Table 1. Compared with sea water, the intestinal fluid levels of monovalent ions were lower, whereas the divalent ion levels were higher. Sulphate ion concentrations were about seven times the seawater level, whereas $[Mg^{2+}]$ and $[Ca^{2+}]$ were 2 and 1.5 times greater, respectively.

Experiments 1 and 2

The calcium influx rate (J_{in}^{Ca}) was $2.64 \pm 0.57 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$ ($N = 24$), and the efflux rate (J_{out}^{Ca}) was $1.22 \pm 0.27 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$ ($N = 15$). To examine whether sexual maturation affected J_{in}^{Ca} , the fish from experiments 1 and 3 were divided into prespawning and postspawning groups, on the basis of a March spawning (Woodhead, 1968; Eliassen & Vahl, 1982). The J_{in}^{Ca} in the prespawning group, $5.79 \pm 0.76 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$ ($N = 30$), was higher ($P < 0.0001$) than that of the postspawning group, $1.23 \pm 0.2 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$ ($N = 6$), whereas no difference in J_{in}^{Ca} was seen between the sexes ($P > 0.05$).

Experiments 3 and 4

Chlorpromazine ($10^{-4} \text{ mol l}^{-1}$) decreased the J_{in}^{Ca} ($P < 0.02$) from 1.76 ± 0.53 to $0.98 \pm 0.32 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$ ($N = 8$), but did not affect the J_{out}^{Ca} ($P > 0.05$) which was $1.08 \pm 0.37 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$ before and $1.49 \pm 0.63 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$ during treatment with $10^{-5} \text{ mol l}^{-1}$ CP ($N = 8$).

Experiment 5

The J_{in}^{Ca} increased linearly with BIS calcium concentrations above 8 mmol l^{-1} ($y = 0.426 + 0.0181x$; $r = 0.999$; $P < 0.0001$). A parallel shift of this line through the

Table 1. *Ion composition of sea water, plasma and intestinal fluid of the Atlantic cod*

	Sea water ($N = 5$)	Plasma ($N = 10$)	Intestinal fluid ($N = 10$)
Sodium (mmol l^{-1})	$392 \pm 2.0\ddagger$	170 ± 11.0	70.8 ± 6.5
Potassium (mmol l^{-1})	$11.9 \pm 0.1\ddagger$	4.4 ± 0.3	6.0 ± 0.8
Calcium (mmol l^{-1})	$10.9 \pm 0.1\ddagger$	$2.3 \pm 0.1^*$	$14.9 \pm 1.7^*$
Magnesium (mmol l^{-1})	$49.3 \pm 0.4\ddagger$	$1.3 \pm 0.2^*$	$102.6 \pm 6.9^*$
Chloride (mmol l^{-1})	$452 \pm 0.9\ddagger$	133 ± 1.6	100 ± 8.1
Inorganic phosphate (mmol l^{-1})	$0.5 \pm 0.02\ddagger$	1.2 ± 0.1	0.55 ± 0.1
Inorganic sulphate (mmol l^{-1})	$25.2\ddagger$	2.0 ± 0.4	198 ± 31.8
Osmolality (mosmol kg^{-1})	$879 \pm 1.8\ddagger$	329 ± 3.2	583 ± 25.3
pH	—	7.2 ± 0.02	6.5 ± 0.02

Values are means \pm S.E.M.

* Total concentrations; \ddagger data from Björnsson & Nilsson (1985); \ddagger data from Burton (1975).

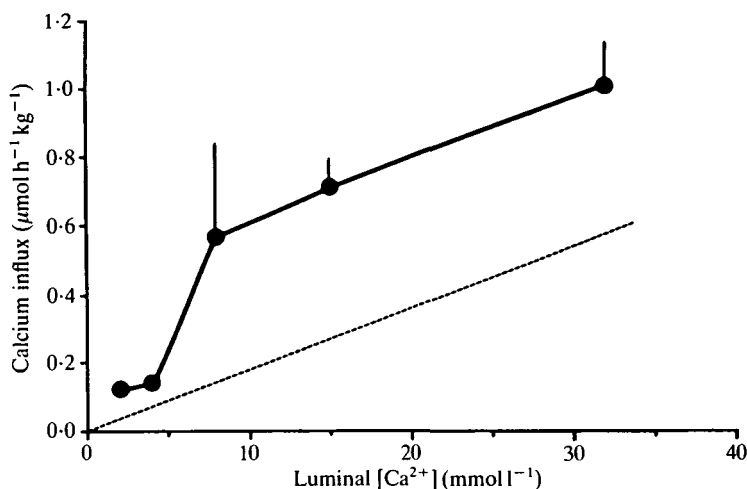


Fig. 5. Calcium influx (solid line) across the intestinal mucosa of the cod, as a function of different luminal calcium concentrations. Broken line indicates the estimated passive diffusion entry ($y = 0 + 0.0181x$). Data are means \pm S.E.M.

origin (Sepulveda & Robinson, 1975) ($y = 0.0181x$) gives an estimate of the passive diffusional entry (dashed line, Fig. 5). Subtracting this passive component from the influx leaves a saturable uptake component following Michaelis–Menten kinetics ($P < 0.01$). $K_m = 8.41 \text{ mmol l}^{-1}$ and $V_{\max} = 0.604 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$ were calculated for this saturable component (Fig. 5).

Experiment 6

The $J_{\text{out}}^{\text{Ca}}$ increased curvilinearly with vascular Ca^{2+} concentration (Fig. 6). Maximal efflux rate of the system could not be reached, as Ca^{2+} precipitated as CaCO_3 in the vascular perfusion fluid at concentrations above $6 \text{ mmol l}^{-1} \text{ Ca}^{2+}$. The available data were interpreted in terms of Michaelis–Menten kinetics ($P < 0.01$), and a $K_m = 6.15 \text{ mmol l}^{-1}$ and $V_{\max} = 3.79 \mu\text{mol Ca}^{2+} \text{ kg}^{-1} \text{ h}^{-1}$ were calculated (Fig. 6).

Discussion

As marine teleosts drink sea water, a large concentration gradient of calcium is created between the intestinal lumen and the vasculature. Thus the calcium influx across the intestinal mucosa has been suggested to be passive (Shehadeh & Gordon, 1969), whereas the efflux was consequently expected to be an active process. In the present study, however, the calcium influx is shown to contain both a passive component and an active, saturable component, the latter amounting to 60% of the calcium influx at physiological calcium concentrations of the intestinal fluid (14.9 mmol l^{-1}). A similar two-component Ca^{2+} influx has been described in different parts of the mammalian gastrointestinal tract (Pansu *et al.* 1981; see

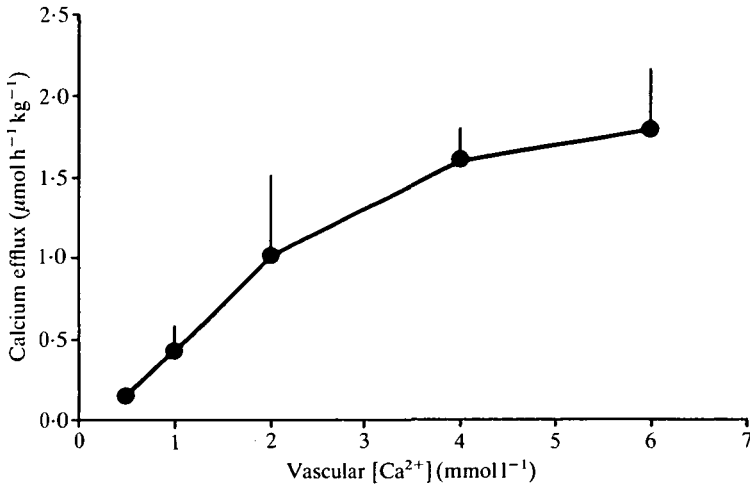


Fig. 6. Calcium efflux across the intestinal mucosa of the cod, as a function of different vascular calcium concentrations. The curve is drawn through the values obtained in the experiment. These values follow Michaelis–Menten kinetics ($P < 0.01$) with $K_m = 6.15 \text{ mmol l}^{-1}$ and $V_{\max} = 3.79 \text{ } \mu\text{mol Ca}^{2+} \text{ kg}^{-1} \text{ h}^{-1}$. Data are means \pm s.e.m.

Favus, 1985; Karbach *et al.* 1986).

The present study cannot unequivocally establish whether the saturable part of the calcium influx is an active process. The inhibitory actions of chlorpromazine (CP), a relatively potent inhibitor of high-affinity Ca^{2+} -ATPases (Ghisjen *et al.* 1980; Gietzen *et al.* 1980; Flik *et al.* 1983, 1984), indicate that the flux may be linked to such an active transport system. A similar inhibitory effect (38%) of CP on the calcium influx is also found in the intestine of the freshwater teleost tilapia (Flik *et al.* 1982). However, the observed actions of CP are possibly not due to specific effects on Ca^{2+} -ATPases, but to nonspecific side-effects (Bowman & Rand, 1984). Thus the saturable calcium influx may be a result of a facilitated diffusion which can be carrier-mediated and/or linked to a selective ion channel.

The saturable calcium efflux was not affected by $10^{-5} \text{ mol l}^{-1}$ CP. This could be either because the carrier is not sensitive to CP or because the CP concentration was too low, although, $10^{-5} \text{ mol l}^{-1}$ CP has been demonstrated to antagonize high-affinity Ca^{2+} -ATPase activity in other biological systems (Ghisjen *et al.* 1980; Flik *et al.* 1983, 1984). Higher CP concentrations could not be used in the vascular perfusate since, in most cases, they abolished peristaltic movements and/or reduced venous outflow in a way that suggested tissue damage.

No quantitative intestinal calcium efflux measurements have been carried out previously in teleost fish, although a qualitative indication of intestinal calcium efflux has been reported in the crucian carp (*Carassius carassius*), where intramuscular injections of ^{45}Ca resulted in a time-dependent appearance of ^{45}Ca in the intestinal lumen (Mashiko & Jozuka, 1964). Previous *in vivo* experiments on the Atlantic cod determined renal and extrarenal excretion of calcium to be 4.2

and $2.0 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$, respectively (Björnsson & Nilsson, 1985). If the measured *in vitro* intestinal calcium efflux ($1.22 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$) in the present study can be taken as an estimate of the flux *in vivo*, the intestine would account for about 20 % of the total calcium excretion by the cod. Excretion from the intestine would thus represent approximately half the extrarenal calcium excretion.

It is probable that large variations in measured calcium fluxes *in vitro* are due to different techniques, and comparisons with *in vivo* situations may be questionable. However, this study presents a model in which *in vivo* conditions have been imitated to a large degree, with vascular and luminal perfusions of physiological composition, flow rate, pressure and temperature.

The calcium influx rate was $2.64 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$, which is about 13 % of the value calculated in previous studies of the whole gastrointestinal tract *in vivo* (approx. $20 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$) in the same species (Björnsson & Nilsson, 1985). This may be due partly to the difference in surface area between the intestinal section used in the present experiments and the total oesophagus–stomach–intestine–rectum surface area, and partly to the probable underestimation of the calculated calcium influx, as about 20 % of the amount of ^{45}Ca perfusing the intestinal lumen was found to be trapped in the mucus on the mucosal side and did not, therefore, reach the exchange area. Further, as in most *in vitro* organ perfusions, oedema was found in the preparation during the perfusion, although the ^{45}Ca in the oedema only accounted for about 1 % of the total amount of ^{45}Ca in the perfusion fluid. However, because the experimental technique is similar in all influx experiments, the present study provides a representative picture of the way in which calcium moves from the intestinal mucosa to the circulation in the Atlantic cod, even though the absolute values of the influx rate are probably underestimated.

Thus, the gastrointestinal tract seems to play a significant role in the calcium balance of the Atlantic cod. Whole-body influx rates in marine teleost species have been determined as 48, 56 and $62 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$ for *Serranus seriba*, *Fundulus heteroclitus* and *Mugil capito*, respectively (Pang *et al.* 1980; Mayer-Gostan *et al.* 1983), and previous *in vivo* measurements have shown calcium influx rates over the whole gastrointestinal tract of 17, 20 and $29.2 \mu\text{mol Ca}^{2+} \text{ h}^{-1} \text{ kg}^{-1}$ for *Salmo gairdneri*, *Gadus morhua* and *Paralichthys lethostigma*, respectively (Shehadeh & Gordon, 1969; Hickman, 1968; Björnsson & Nilsson, 1985). The gastrointestinal calcium absorption thus corresponds to about 30 % of the whole-body influx and would seem to be of importance for the calcium balance of marine teleosts.

The $J_{\text{in}}^{\text{Ca}}$ was found to be higher before than after spawning in both sexes. This may indicate a regulatory role of the intestine during sexual maturation of the Atlantic cod, during which plasma calcium levels increase in both sexes (Woodhead & Woodhead, 1965; Woodhead, 1968). Although it is unclear why plasma calcium levels increase in male cod during sexual maturation, the increase of plasma calcium levels in females during this period probably reflects increased

levels of vitellogenin-bound calcium. Thus, the intestine may be involved in the mobilization of calcium from the environment of marine teleosts during sexual maturation.

Measurements of the ion concentrations of the intestinal fluid were consistent with the process of water uptake by marine teleosts, involving a net uptake of monovalent ions which allows solute-linked water transport in the gastrointestinal tract (Hirano *et al.* 1975; Pang *et al.* 1980). In the present study calcium was concentrated to a lesser degree than magnesium and sulphate; this is in agreement with previous studies of ion level modifications along the gastrointestinal tract of marine teleosts (Hickman, 1968; Shehadeh & Gordon, 1969; Fletcher, 1978). This indicates a net absorption of calcium across the gastrointestinal tract of the Atlantic cod.

It is concluded that the absorption of calcium consists of a passive diffusional component and a saturable component, whereas the excretion of calcium is a saturable flux. The saturable calcium excretion across the intestinal mucosa represents an important extrarenal excretion route for calcium.

We thank Professor Howard A. Bern and Professor Tetsuya Hirano for critical reading of this manuscript. We also thank Professor Stefan Nilsson and Dr Anna-Lena Ungell for valuable discussions on various parts of this study and Mr Per Jonsson for excellent help in preparing the figures. This work was supported by grants from the Lars Hiertas Foundation, the Icelandic Science Foundation and the Swedish Natural Science Research Council.

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