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NaCl and fluid secretion by the intestine of the teleost *Fundulus heteroclitus*: involvement of CFTR

W. S. Marshall*, J. A. Howard, R. R. F. Cozzi and E. M. Lynch

Department of Biology, St Francis Xavier University, Antigonish, Nova Scotia, Canada B2G 2W5

*e-mail: bmarshal@stfx.ca

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Summary

Sections of posterior intestine of the euryhaline killifish *Fundulus heteroclitus* adapted to sea water were stimulated by the calcium ionophore ionomycin (1 μmol l^{-1}) in combination with agents to elevate intracellular cyclic AMP levels, 0.5 mmol l^{-1} dibutyryl-cyclic AMP (db-cAMP) with 0.1 mmol l^{-1} 3-isobutyl-1-methylxanthine (IBMX). Intestinal bag preparations from recently fed animals (but not from overnight unfed animals) changed from fluid absorption (+18.9±8.30 μl cm^{-2} h^{-1} , N=8) in the untreated control period to net fluid secretion after stimulation (–7.43±1.30 μl cm^{-2} h^{-1}, N=8, P<0.01; means ± S.E.M.), indicative of the capacity of teleost intestine to undergo secretion. Posterior intestinal pieces mounted *in vitro* in Ussing-style membrane chambers showed net Cl^- uptake (+2.245±0.633 μequiv cm^{-2} h^{-1}, N=7) that turned to net secretion following stimulation by ionomycin + db-cAMP + IBMX (–3.809±1.22 μequiv cm^{-2} h^{-1}, N=7, P<0.01). Mucosal application of the anion channel blocker 1 mmol l^{-1} diphenylamine-2-carboxylate (DPC) after ionomycin + db-cAMP + IBMX treatment significantly reduced serosal-to-mucosal unidirectional Cl^- flux (P<0.001), net Cl^- flux (P<0.05), short-circuit current (I_{sc}, P<0.001) and tissue conductance (G_t, P<0.001), while 0.1 mmol l^{-1} 4,4′-diisothiocyano-2,2′-stilbene-disulphonic acid (DIDS, a blocker of anion exchange) was without effect. Stimulation by db-cAMP + IBMX (no ionomycin) significantly increased unidirectional fluxes, I_{sc} and G_t but did not produce net Cl^- secretion. Ionomycin alone produced a transient increase in I_{sc} but had no effect on G_t and caused no significant changes in unidirectional or net Cl^- fluxes. Addition of db-cAMP + IBMX after ionomycin treatment produced net secretion of Cl^- and large increases in unidirectional fluxes and G_t. Cystic fibrosis transmembrane conductance regulator (CFTR) was immunocytochemically localized with a monoclonal mouse antibody to the carboxy terminus and found to be present in the cytoplasm and basolateral membranes of all enterocytes and in the brush-border membrane of some cells, whereas NKCC immunofluorescence, demonstrating the presence of the $Na^+/K^+/2Cl^-$ cotransporter, was present in the cytoplasm and brush-border membrane. We conclude that the teleost intestine is capable of salt and fluid secretion only if intracellular Ca^{2+} and cyclic AMP pathways are stimulated together and that this secretion appears to involve activation of CFTR ion channels in the apical membrane of a subpopulation of enterocytes.

Key words: epithelium, ion transport, cystic fibrosis transmembrane conductance regulator, immunocytochemistry, secretory diarrhoea, $Na^+/K^+/2Cl^-$ cotransporter, Na^+/K^+-ATPase, osmoregulation, ionomycin, cyclic AMP.

Introduction

Seawater teleosts drink sea water and absorb ions and fluid across the oesophagus and intestine and in this way obtain water for osmoregulation (for a review, see Loretz, 1995). In seawater teleosts, the uptake of NaCl and K^+ is *via* a $Na^+/K^+/2Cl^-$ cotransporter apparently located in the apical membrane (e.g. in winter flounder *Pleuronectes americanus*) (O'Grady et al., 1987). Ion uptake at the apical membrane is linked to anion channels, K^+ channels and Na^+/K^+-ATPase in the basolateral membrane of the enterocytes (Loretz, 1995). In the herbivorous marine fish *Oreochromis mossambicus* and in seawater-adapted rainbow trout *Oncorhynchus mykiss*, NaCl uptake occurs instead *via* parallel Na^+/H^+ and Cl^-/HCO_3^- ion exchangers (Howard and Ahearn, 1988) that are also functional in acid–base balance and in rainbow trout contribute significantly to total body base excretion (Wilson et al., 1996). Recent studies suggest the co-existence of the $Na^+/K^+/2Cl^-$ cotransporter together with a 4,4′-diisothiocyano-2,2′-stilbene-disulphonic acid (DIDS)-sensitive anion exchange that can account for base secretion being driven by a favourable Cl^- electrochemical gradient through the Cl^-/HCO_3^- exchanger (Grosell and Jensen, 1999). Many studies showed that ion reabsorption in the intestine of the seawater-adapted teleost is accompanied by isotonic fluid reabsorption (Loretz, 1983, 1987b, 1995).

Mammalian intestine can easily be made to secrete ions and fluid by application of agents that increase cyclic AMP levels,

such as cholera toxin and forskolin (Field et al., 1980). Such secretory diarrhoea is associated with common pathological conditions evoked by bacterial endotoxins. This secretion, in turn, involves activation by cyclic AMP of apically located cystic fibrosis transmembrane conductance regulator (CFTR) ion channels, thus implicating CFTR in this pathological condition (Mathews et al., 1999). Our work has demonstrated a high degree of expression of killifish CFTR (kfCFTR) in the posterior intestine (Singer et al., 1998), consistent with this model, but the function(s) of CFTR in the intestine of teleosts is unknown. Previous studies with fish have not observed ion or fluid secretion by the intestine in spite of numerous attempts (Field et al., 1980; Loretz, 1987a,b, 1995). This lack of secretion has been associated with the absence of crypts of Lieberkühn from teleosts (Loretz, 1987a). Killifish live in estuaries and are detritus feeders that are therefore likely to encounter endotoxin-producing bacteria and, in polluted estuaries, xenobiotic toxins. If secretory diarrhoea is of selective advantage in purging the intestine of endotoxin-producing bacteria, then estuarine teleosts (indeed all coelomates with peristaltic alimentary tracts) ought to have this capability.

Killifish are a model for the osmoregulatory physiology of teleost fish, and much is known about their ion regulation by the mitochondria-rich chloride cells of the gill and opercular epithelium (Karnaky, 1998; Wood and Marshall, 1994; Marshall and Bryson, 1998; Singer et al., 1998). Very little is known about intestinal function in ion and osmoregulation by this species. Classical work by Babkin and Bowie (1928) examined enzyme activities and the operation of the intestine and gallbladder during feeding; these workers found that the alimentary canal is stomachless (as is true for all Cyprinodontidae) and that the chyme is at approximately neutral pH. The killifish intestine is known to be involved in the uptake of toxins and in detoxification. The insecticide DDT accumulates in the intestine (Crawford and Guarino, 1976), and the intestine abundantly expresses cytochrome P450 isoforms that are important in detoxification (Oleksiak et al., 2000). The intestine also absorbs bile salts *via* a Na^+-dependent secondary active transport system (Honkanen and Patton, 1987). However, NaCl and fluid transport and their control have not been studied.

We therefore sought to examine ion and fluid transport by killifish intestine by using combinations of pharmaceuticals that might evoke intestinal secretion. Particularly, we used ionomycin, a Ca^{2+} ionophore that is more effective in poikilotherm preparations than is ionophore A23187 (Marshall et al., 1993). Ionomycin has been used previously with teleost intestinal preparations (O'Grady et al., 1988; O'Grady, 1989) but not specifically to test for ion and fluid secretion. Ionophore A23187 has inhibitory effects on ion transport by the goby (*Gillichthys mirabilis*) intestine, but by itself does not produce secretion (Loretz, 1987b).

Materials and methods

Animals

Adult killifish (mummichogs, *Fundulus heteroclitus* L.) of both genders (5–9 g) were captured in Antigonish estuary, transferred to indoor holding facilities and adapted to brackish water (salinity 3 g l^{-1}) at 20–25 °C and ambient photoperiod under artificial light for at least 10 days. Fish were tranferred to full-strength sea water (salinity 32 g l^{-1}) or dechlorinated fresh water and acclimated for at least 2 weeks before experimentation. They were fed marine fish food blend (Nutrafin flakes and tubifex worms; R. C. Hagen, Montreal, Canada) at 1 g 100 g^{-1} body mass day^{-1}, supplemented twice weekly with frozen brine shrimp.

Intestinal preparation

Killifish were anaesthetized in 0.2 g of ethyl 3-aminobenzoate, methane sulphonic acid salt (MS-222) in 1 l of isotonic saline (0.9 g l^{-1} NaCl), with the pH of the anaesthetic solution adjusted to 6.5–7.5, before being killed by decapitation. The posterior portion of the intestine was dissected, i.e. the section from the anus cephalad to the point where the diameter increases just posterior to the first intestinal loop, which approximates the ileum and jejunum together but excludes the duodenum. Some preparations came from fish that had been starved overnight and killed just before the morning feeding, while others had been fed in the hour prior to the experiment. The intestine section was rinsed inside with Cortland's saline, then ligatured at one end around a polyethylene tube (PE50 tubing; i.d. 0.58 mm, o.d. 0.97 mm). A second ligature closed the bag preparation, and the bag was filled by syringe with Cortland's saline to a steady-state hydrostatic pressure, measured as a column of water connected to the tubing, of 10 cmH_2O (approximately 7 mmHg or 0.9 kPa). The tube was then plugged, and the preparation placed in well-stirred and oxygenated Cortland's saline.

The intestinal preparation was removed from the incubation solution, blotted dry and weighed every 10 min, and the mass was recorded. Loss of mass indicated fluid absorption, while gain of mass indicated secretion into the lumen. A control period of 1 h established the absorption rate, after which 1 μmol l^{-1} ionomycin + 0.5 mmol l^{-1} dibutyryl-cyclic AMP (db-cAMP) + 0.1 mmol l^{-1} 3-isobutyl-1-methylxanthine (IBMX) was added to the bath, and weighings were continued for a further 2 h. At the end of the experiment, the intestine ligatures were cut off, the preparation was emptied, wet mass was measured and the preparation was cut lengthwise and flattened to measure the surface area. The rate of fluid transport (J_v) was measured as the slope of a least-squares linear regression of mass on time for the control and test periods, each regression comprising at least seven sequential measurements; data were expressed as ml cm^{-2} h^{-1}.

Flat in vitro *preparation*

The intestine was dissected as above and divided into two pieces, and each piece was cut lengthwise using artery scissors and mounted over a 0.125 cm^2 round aperture. The epithelium was pinned out over the aperture with the rim area lightly greased and bevelled to minimize edge damage. The tissue was stretched gently to the extent that the villi were distinct (not

bunched) so that saline could effectively stir the transporting surface. Any adherent mucus was rinsed away, and the intestine was mounted in a modified Ussing chamber so that transmembrane electrophysiological variables could be monitored: V_t, transepithelial potential (in mV; mucosal side ground), G_t, transmembrane conductance (in mS cm^{-2}), and I_{sc}, short-circuit current (in μA cm^{-2}). I_{sc} is expressed as positive for the secretion of anions. Epithelia were clamped to 0 mV except for short periods to record V_t. A current-voltage-clamp (D. Lee Co., Sunnyvale, CA, USA, or WP Instruments DVC 1000) measured epithelial variables.

Radioisotopic fluxes were measured on paired intestinal pieces from a single animal. Radioactive chlorine (^{36}Cl, 0.04 MBq ml^{-1}; NEN Life Science, Boston, MA, USA) was added as the neutral salt and the epithelia were left to equilibrate for 1 h. Samples from the non-radioactive side (225 μl from the 4.0 ml hemichamber) were taken at 20 min intervals, mixed with a scintillation cocktail (Optifluor, United Technologies Packard, Downer's Grove IL, USA) and counted (Packard 2000CA liquid scintillation system) to 1 % error. Samples from the more radioactive side (50 μl) were taken initially and at the end of each hour of the experiment. The control period consisted of three flux periods (of 1 h), after which the test periods (hours 1 and 2) included three flux periods each. Radioisotope fluxes are expressed as μequiv cm^{-2} h^{-1}.

Bathing solutions

A modified Cortland's saline (305 mosmol kg^{-1}, pH 7.8) was used to bathe both membrane surfaces symmetrically; its composition was (in mmol l^{-1}): NaCl, 160.0; KCl, 2.6; $CaCl_2$, 1.6; $MgSO_4$, 0.9; $NaHCO_3$, 17.9; NaH_2PO_4, 3.0; and glucose, 5.6. The saline had a pH of 7.8 when equilibrated with a 99 % O_2/1 % CO_2 gas mixture. Both sides of the membrane in the Ussing chamber were bubbled with the gas mixture, and the outside of the bag preparations was also continuously bubbled.

Immunocytochemistry

The primary antibody to detect CFTR was mouse monoclonal anti-hCFTR (R&D Systems, Minneapolis, MN, USA) with the known epitope of (-dtrl), the carboxy terminus of human CFTR (hCFTR). Killifish CFTR has the same carboxy terminus (Singer et al., 1998) and, thus, is selective for this protein. The primary antibody against the $Na^+/K^+/2Cl^-$ cotransporter was mouse monoclonal anti-human $Na^+/K^+/2Cl^-$ cotransporter (NKCC) (Lytle et al., 1995) (Iowa Hybridoma Bank, University of Iowa, Iowa City, IA, USA), an antibody that reacts readily with the $Na^+/K^+/2Cl^-$ cotransporter from a wide variety of species. The primary antibody for Na^+/K^+-ATPase was mouse polyclonal anti-Na^+/K^+-ATPase α-subunit from chicken (antibody α-5; Iowa Hybridoma Bank, University of Iowa, Iowa City, IA, USA), an antibody with wide applicability in vertebrates and invertebrates (Lebovitz et al., 1989), including teleost fish (Wilson et al., 2000). The secondary antibody was goat polyclonal anti-mouse IgG conjugated to an Oregon Green 488 fluorophore (Molecular Probes, Eugene, OR, USA), chosen because of the stability and reliability of this antibody.

Whole intestines were fixed overnight in a formaldehyde-free 80 % methanol/20 % dimethyl sulphoxide (DMSO) fixative at –20 °C. The methanol was used as a dehydrating agent and the DMSO as a cryoprotective agent. Intestinal pieces were then embedded in embedding medium (Cryogel; S.P.I. Supplies West Chester, PA, USA) and sectioned at a thickness of 10 μm. Sections were fixed for an additional 3 h in 80 % methanol/20 % DMSO at –20 °C and rinsed in rinsing buffer (TPBS) consisting of 0.1 % bovine serum albumin (BSA)/0.05 % Tween 20 in phosphate-buffered saline (PBS, composition in mmol l^{-1}: NaCl, 137; KCl, 2.7; Na_2HPO_4, 4.3; KH_2PO_4, 1.4; pH 7.4). They were then blocked with 5 % normal goat serum (NGS)/0.1 % BSA/0.05 % TPBS, pH 7.4, for 30 min at 25 °C and incubated in the primary antibody (8 μg ml^{-1} in 0.5 % BSA in TPBS) overnight at 4 °C. Sections were rinsed three times and exposed to the secondary antibody (diluted 1:50 in 0.5 % BSA in PBS) for 5 h at 4 °C. They were then rinsed three times and incubated with Mitotracker Red (Molecular Probes, Eugene, OR, USA; 100 nmol l^{-1} in PBS) for 2 h at room temperature. After three final rinses, the sections were mounted in mounting medium (Geltol; Immunon Thermo Shandon, Pittsburgh, PA, USA). Control slides were prepared in a similar manner but had the first or second antibody step eliminated.

Four different animals were used for each antibody. In most cases, paired sections were used in control procedures and the two antibody treatments. Slides were viewed in single blind fashion and images were collected with a laser confocal microscope (Olympus FV300); there was no detectable 'bleed-through' between the red and green confocal fluorescence channels. The false colour for areas that are positive for Mitotracker Red and Oregon Green 488 appears yellow.

Western blots

Intestine and gill filaments, scraped from the arch with a razor blade, were homogenized in ice-cold SEI buffer (300 mmol l^{-1} sucrose, 20 mmol l^{-1} EDTA, 100 mmol l^{-1} imidazole, pH 7.4) using a homogenizer. Homogenates were centrifuged at 2000 ***g*** for 6 min. The pellet was resuspended in 2.4 mmol l^{-1} deoxycholate in SEI buffer and centrifuged a second time at 2000 ***g*** for 6 min. The total protein content of the resulting supernatant was determined using the Bradford method (Bradford, 1976).

Proteins were separated on a 7 % polyacrylamide gel using a Mini-Protean 3 Cell system (Bio-Rad, Mississauga, Ontario, Canada). In total, 20 μg of protein was loaded and run for 30 min at 200 V. Proteins were then transferred to a Immobilon-P membrane (Millipore, Bedford, MA, USA) for 2 h using a Mini-Trans-Blot Cell (Bio-Rad). Blots were dried at 37 °C for 1 h, stained with Ponceau S and destained with 90 % methanol/2 % acetic acid to visualize the lanes and the molecular mass markers. Blots were then blocked in 3 % bovine serum albumin (BSA)/TTBS (0.05 % Tween 20 in Tris-buffered saline: 20 mmol l^{-1} Tris-HCl, 500 mmol l^{-1} NaCl,

5 mmol l^{-1} KCl, pH 7.4) for 2 h at room temperature on a shaker.

The blocking buffer was poured off, and the blots were incubated with the primary antibody solution [anti-hCFTR monoclonal antibody (R&D Systems, Minneapolis, MN, USA), 1 μg ml^{-1} in 1% BSA/TTBS] for 2 h at room temperature. Following a 5 min wash in TTBS buffer, the membranes were incubated with the secondary antibody solution [biotin-SP-conjugated AffiniPure goat anti-mouse IgG (Biochem Scientific, Mississauga, Ontario, Canada), diluted 1:8000 in 1% BSA/TTBS] for 1 h at room temperature. After being washed in TTBS, the blots were incubated for 1 h with an alkaline-phosphatase-conjugated Streptavidin solution (Biochem Scientific) diluted 1:1000 in 1% BSA/TTBS. Bands were visualized by incubating the blots in a BCIP/NBT Blue substrate development solution (Sigma, Oakville, Ontario, Canada).

Pharmaceuticals

Ionomycin (Calbiochem) was dissolved in DMSO and delivered to the serosal bath at a final concentration of 1 μmol l^{-1}. Dibutyryl-cyclic-AMP (db-cAMP; Sigma) and 3-isobutyl-1-methylxanthine (IBMX; Sigma) were dissolved in a minimum of DMSO (1 mg in 30 μl), diluted in saline and added to the serosal side to final concentrations of 0.5 mmol l^{-1} and 0.1 mmol l^{-1}, respectively. Addition of vehicle alone (DMSO, final concentration 0.5% v/v) was without effect (see Fig. 1).

Statistical analyses

Data are expressed as the mean ± 1 S.E.M. The minimum level of significance is $P<0.05$ on a two-tailed test. Fluid transport data slopes (=flux rates) were established by least-squares linear regression of fluid flow against time. The fluid flux rates were analyzed by two-way analysis of variance (ANOVA) followed by a Bonferroni *post-hoc* test. Ion flux data were analyzed by one-way ANOVA followed by a Bonferroni *post-hoc* test. Anterior *versus* posterior section data of electrophysiology in the two portions of the intestine were analyzed by paired two-tailed *t*-test.

Results

Fluid absorption and secretion

Killifish intestine has a thin musculature (measured in frozen histological sections to be 100–200 μm fully contracted), so whole tissue bag preparations operate very well without stripping of the musculature. The least disturbance was also gained by not inverting the intestine; hence, experiments proceeded with non-inverted bag preparations with identical Cortland's saline on both sides. Preliminary experiments with dilute solutions in the lumen produced a non-linear time course of fluid absorption, presumably because of a change in the osmotic driving force over time. Preparations with saline on both sides had a linear and easily reproducible rate of fluid absorption. Control tissues with Cortland's saline on both sides showed net absorption of fluid and established a steady-state trend line of loss of mass from the preparation in the first hour of incubation; this rate was unchanged over the 3 h incubation (Fig. 1A). The addition of ionomycin + db-cAMP + IBMX on the serosal side produced net fluid secretion in the preparations ($P<0.01$; Bonferroni *post-hoc* test following one-way ANOVA, $P=0.005$, $N=8$), while addition of drug vehicle to the contol membranes had no effect (Fig. 1). This secretion was against the small transmembrane hydrostatic pressure gradient of approximately 7 mmHg (0.9 kPa), the steady-state pressure needed to hold saline in the intestinal lumen.

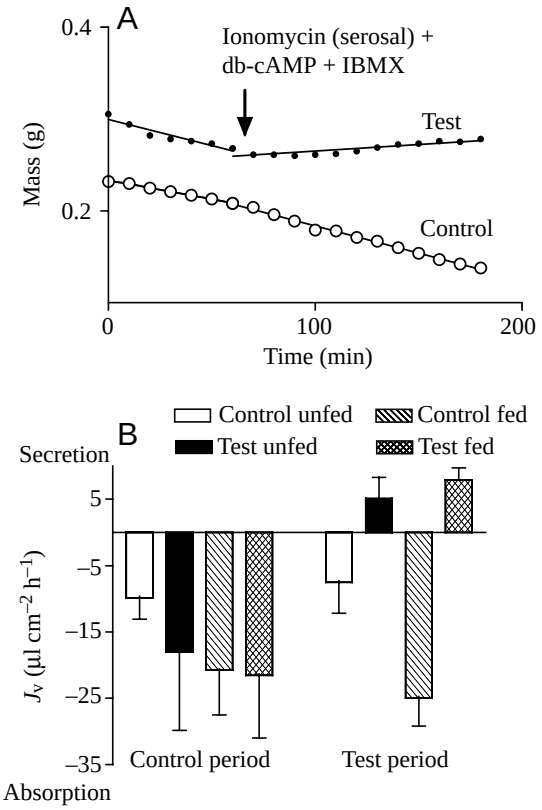

Fig. 1. Gravimetric measurement of fluid flow (J_v) across *Fundulus heteroclitus* intestine for time controls (treated with drug vehicle alone) and drug-treated preparations (B) (test; 0.1 mmol l^{-1} IBMX + 0.5 mmol l^{-1} db-cAMP + 1 μmol l^{-1} ionomycin, added at 60 min; arrow in A) from animals before (unfed) or after (fed) daily feeding. Results for a typical experiment appear in A and include control (open symbols) and test (filled symbols) membranes from fed animals. The lines in A are least-squares linear regressions of mass on time. The combined treatment reversed the normal fluid absorption to net fluid secretion in fed animals ($P<0.01$, Bonferroni *post-hoc* test following one-way analysis of variance, $P=0.005$, $N=8$). In the unfed animals, there was a non-significant trend towards secretion ($P>0.05$). Values are means + S.E.M., $N=8$.

Electrophysiology and ion fluxes

Because the effects of the drug were most clear in fed animals (Fig. 1), ion fluxes were measured on intestinal preparations from fed animals, and because of similar

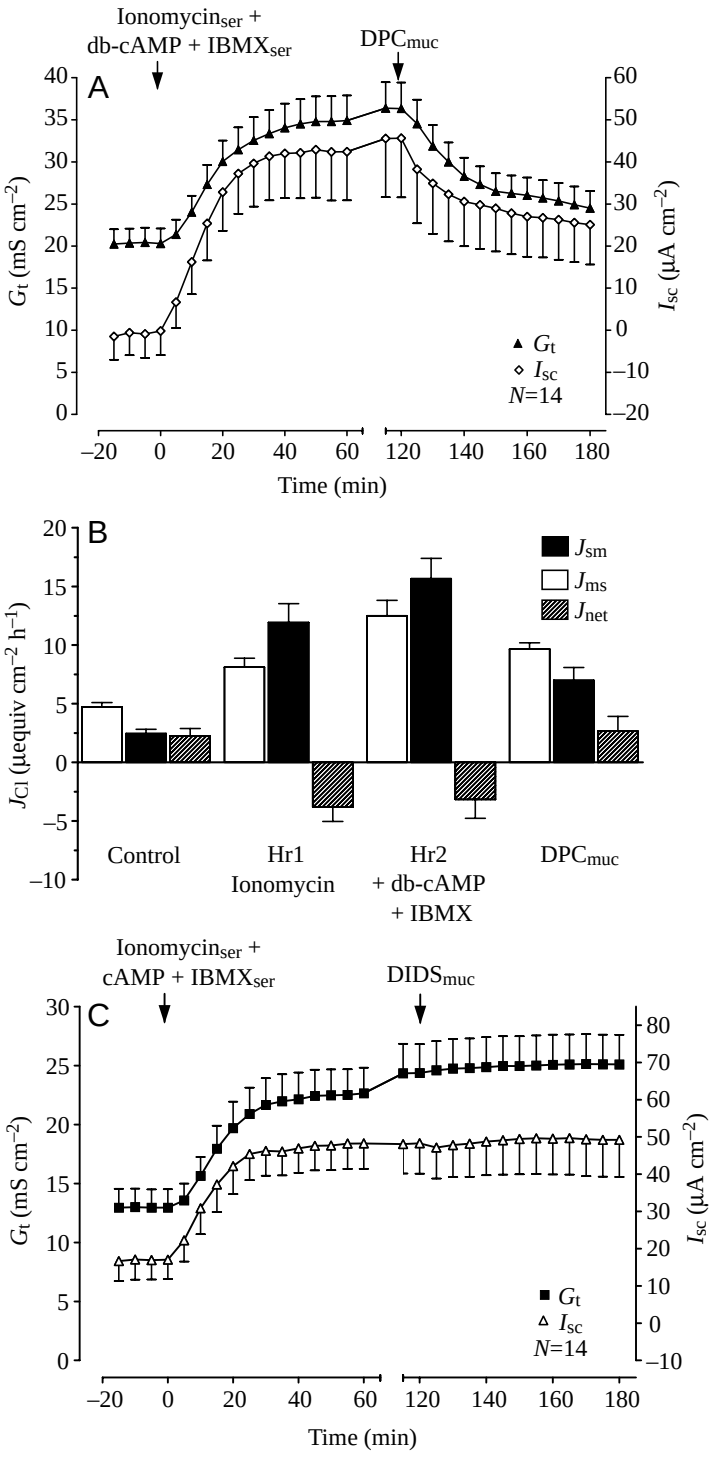

Fig. 2. Effects of treatment with 0.1 mmol l^{-1} IBMX + 0.5 mmol l^{-1} db-cAMP + 1 μmol l^{-1} ionomycin on tissue conductance (G_t), unidirectional Cl^- fluxes (J_{Cl}) and short-circuit current (I_{sc}). (A) Treatment (at time zero) significantly increased tissue G_t and I_{sc}. The effect was reversed by mucosal application of the anion channel blocker diphenylamine-2-carboxylate (DPC; 1 mmol l^{-1}). (B) Drug treatment significantly increased both unidirectional Cl^- fluxes and reversed ion uptake to net Cl^- secretion (P<0.005, paired t-test compared with control period, N=7). J_{sm}, serosal-to-mucosal Cl^- flux; J_{ms}, mucosal-to-serosal Cl^- flux. Apical DPC significantly decreased serosal-to-mucosal unidirectional flux (P<0.02, paired t-test compared with previous period, N=7) and restored Cl^- uptake to control levels. (C) Similar post treatment (i.e. after db-cAMP + IBMX + ionomycin) with 100 μmol l^{-1} DIDS was without effect on the stimulated I_{sc} and G_t. Subscripts ser and muc indicate application of drug to the serosal and mucosal sides, respectively. Values are means ± S.E.M.

responses from anterior and posterior sections of intestine (see below, Fig. 5), the two sections are considered together. The combination ionomycin + db-cAMP + IBMX increased G_t and I_{sc} within 10 min, and new steady-state values were reached in approximately 45 min (Fig. 2A). These levels were maintained during the second hour of flux data collection (not shown in Fig. 2A). There were significant increases in unidirectional fluxes of Cl^- (P<0.05, hour 1 *versus* control; P<0.001, hour 2 *versus* control, Bonferroni *post-hoc* test following one-way ANOVA, P<0.0001, N=7), but the net flux, which was 2.25±0.63 μequiv cm^{-2} h^{-1} in the absorptive direction in the control period, changed to secretion of –3.81±1.22 μequiv cm^{-2} h^{-1} and –3.15±1.62 μequiv cm^{-2} h^{-1} for the first (P<0.05 compared with control) and second (P<0.05 compared with control) hours of drug treatment, respectively. Addition of the anion channel inhibitor DPC (1 mmol l^{-1}) to the apical side significantly reduced G_t, I_{sc} and Cl^- efflux (P<0.001, Bonferroni test, N=7 for fluxes, N=14 for electrophysiology) compared with the previous period, reduced Cl^- net flux (P<0.05, Bonferroni test) and restored the absorptive Cl^- net flux to previous levels 2.67±1.25 μequiv cm^{-2} h^{-1} (Fig. 2B, P>0.05, DPC period *versus* control, Bonferroni test). G_t returned to control levels, while I_{sc} was still significantly elevated (P<0.001, Bonferroni test, N=14) compared with the control period.

A second series of membranes was exposed to ionomycin +db-cAMP + IBMX, as before, but instead of DPC, 100 μmol l^{-1} DIDS (a blocker of anion exchange and ClC-type anion channels but not of CFTR-like channels) was added to the mucosal bath (Fig. 2C). In this case, there was no reversal of I_{sc} or G_t by DIDS, the unidirectional efflux of Cl^- was unchanged (P>0.05, Bonferroni test after ANOVA, P<0.001, N=7 for fluxes, N=14 for electrophysiology) and the secretory net flux of Cl^- was maintained (P>0.05, control *versus* DIDS period, Bonferroni test, N=7).

In an attempt to distinguish the roles of cyclic AMP from that of intracellular Ca^{2+}, a third series was initiated with the addition of only db-cAMP + IBMX (no ionomycin) (Fig. 3). In this case, drug treatment increased in G_t and I_{sc} as before and in a manner essentially indistinguishable from the combined treatment (compare Fig. 3A and Fig. 2A). However, db-cAMP + IBMX tended to increase net Cl^- reabsorption, and absorption was 5.39±1.11 μequiv cm^{-2} h^{-1} after 2 h (as opposed to the net secretion observed in Fig. 2B). The increase in Cl^- efflux was present (P<0.001 compared with control, Bonferroni test) but was less marked than in the first series (compare Fig. 2B and Fig. 3B), suggesting that the Ca^{2+}

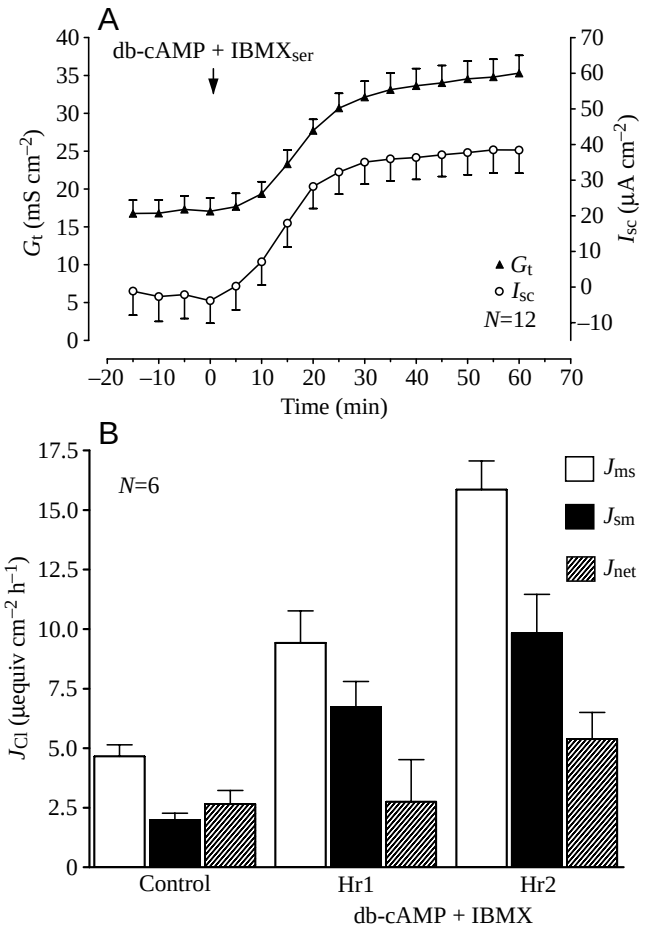

Fig. 3. Effects of 0.1 mmol l^{-1} IBMX + 0.5 mmol l^{-1} db-cAMP (no ionomycin) on short-circuit current (I_{sc}) and tissue conductance (G_t) (A) and serosal-to-mucosal (J_{sm}) and mucosal-to-serosal (J_{ms}) Cl^- fluxes. Values are means ± S.E.M. Hr1, hour 1; Hr2, hour 2. Other details as in Fig. 2. J_{net}, net Cl^- flux.

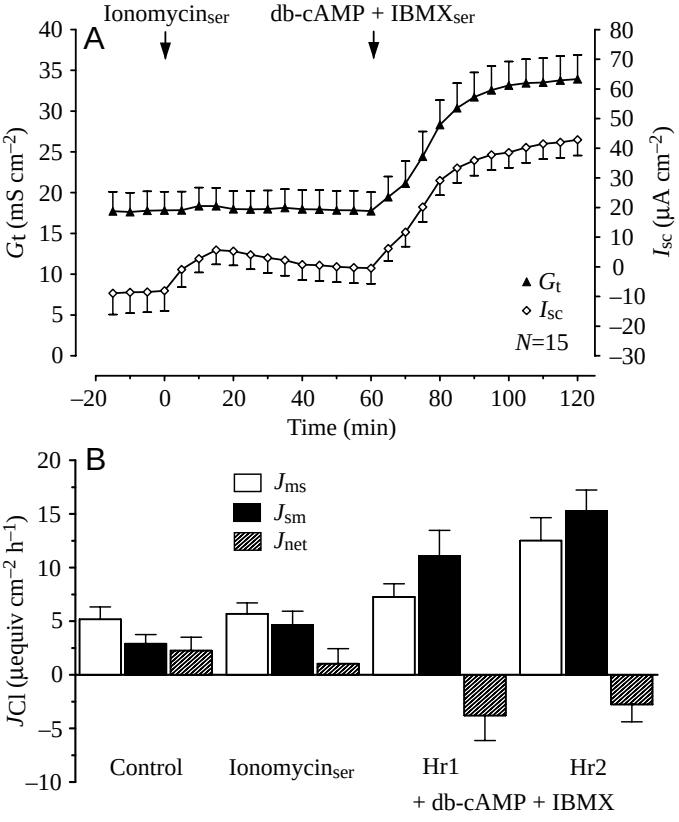

Fig. 4. (A) Effects of 1 μmol l^{-1} ionomycin alone on short-circuit current (I_{sc}) and tissue conductance (G_t). When post-treated with 0.1 mmol l^{-1} IBMX + 0.5 mmol l^{-1} db-cAMP, G_t and I_{sc} increased markedly. (B) Effects of 1 μmol l^{-1} ionomycin alone and subsequent addition of 0.1 mmol l^{-1} IBMX + 0.5 mmol l^{-1} db-cAMP on unidirectional Cl^- fluxes (J_{sm}, serosal-to-mucosal flux; J_{ms}, mucosal-to-serosal flux) and net Cl^- secretion (J_{net}). Hr1, hour 1; Hr2, hour 2. Values are means ± S.E.M. Other details as in Fig. 2.

ionophore may have been responsible for some of the increase in Cl^- efflux and the production of net ion and fluid secretion.

In a fourth series, the addition of ionomycin alone produced no change in G_t and only a transient increase in I_{sc} (Fig. 4A) and V_t (data not shown). There were also no significant changes in the unidirectional and net Cl^- fluxes with ionomycin alone; there was instead a small absorptive Cl^- net flux (Fig. 4B). Subsequent addition of db-cAMP + IBMX rapidly increased G_t and I_{sc} (Fig. 4A) in association with large increases in unidirectional Cl^- fluxes (P<0.01 for J_{ms} and J_{sm} in hour 2 of treatment compared with the ionomycin period, Bonferroni test following one-way ANOVA, P<0.001, N=7) and a net secretion of Cl^-, as seen in the first series (Fig. 4B).

In the flux experiments, we recorded which preparation came from the most posterior section of the intestine compared with the adjacent section immediately anterior and randomized the assignment of unidirectional fluxes between the two sections (Fig. 5). There was no significant difference between the two regions in the I_{sc} of control or ionomycin + db-cAMP + IBMX stimulated membranes (measured in each series and one indicative of combined ion transport; P>0.05, paired two-tailed t-test, N=7), indicating that the two sections were apparently similar in their basal rates of transport and in their reactions to the drugs. For this reason, unidirectional fluxes were combined for the two sections.

Regression of unidirectional Cl^- fluxes on G_t (Fig. 6) for the same tissues gave a linear relationship (r^2=0.669, slope 0.345±0.033 μequiv mS^{-1} h^{-1}, mean ± S.E.M., N=55, P<0.001) and a y-intercept that was not significantly different from zero (–0.91±0.97 μequiv cm^{-2} h^{-1}, mean ± S.E.M., 95 % confidence interval –2.85 to +1.03 μequiv cm^{-2} h^{-1}). The regression included data from control membranes and from membranes that were stimulated by ionomycin + db-cAMP + IBMX. The linear relationship and zero intercept suggest that most of the ion flux across the tissue was conductive and not *via* electrically silent transport systems.

Immunocytochemistry

For immunocytochemistry of 108 intestine cryosections from 15 fish, we used the mouse monoclonal anti-hCFTR antibody.

This antibody has an epitope at the C-terminal end (-dtrl) that is the same in hCFTR and kfCFTR. Sections treated with anti-CFTR and goat anti-mouse IgG-Oregon Green 488 had positive fluorescence localized to the basal portion of the cells and not in the brush-border membrane of most enterocytes (Fig. 7A,B), but in approximately 20 % of sections and in some enterocytes interspersed among the majority there was CFTR-positive fluorescence in the brush border as well as the basal portion of the cell (Fig. 7C,D). We did not observe a difference in apical CFTR immunofluorescence between the tips of villi or in the grooves between villi (analogous to the location of crypts).

We treated 18 intestinal preparations with IBMX and ionomycin for 2 h prior to fixation, but did not observe a change in the cellular distribution of CFTR compared with 18 paired control intestines incubated without drug. This included tightly matched preparations in which the frozen sections of test and control intestine pieces came from the same cut end of the same intestine. The red fluorescence is Mitotracker Red, which, when added after fixation, provided a general (relatively non-specific) counterstain that included mitochondria but also stained brush-border membrane (Fig. 7, see also Figs 9, 10). Western analysis confirmed that the anti-CFTR antibody cross-reacts specifically with discrete protein bands in gill and intestinal tissue but is absent from heart (Fig. 8). The band at 175 kDa is approximately the size expected for CFTR protein. The lower-molecular-mass bands may be splice variants since northern analysis of gill tissue also indicates lower-mass hybridizing bands (Singer et al., 1998).

In 24 intestinal cryosections from five fish stained with mouse anti-human NKCC, with goat anti-mouse IgG Oregon Green 488 as secondary antibody, staining was distributed across the cytoplasm but not in the nuclei or in mucous cells (Fig. 9A,B). Unlike CFTR immunostaining, in most cases (approximately 80 % of sections) there also was significant staining of the brush-border region of the majority of enterocytes (Fig. 9C,D).

Immunofluorescence of nine cryosections from three fish using a polyclonal antibody to Na^+/K^+-ATPase α-subunit indicated positive reactions outside the nuclei of the enterocytes and in the basal approximately two-thirds of the cells. There was no significant staining of the dermal portions of the villi or of the apical portion of the cytoplasm and the brush-border membrane of enterocytes (Fig. 9E,F). The observed distribution of transporters is consistent with an absorptive mode of transport with Na^+/K^+-ATPase in the basolateral membrane, NKCC at the apical membrane (in part) and CFTR anion channels at the basal and lateral membranes in most enterocytes. A second group of enterocytes has CFTR in the apical membrane and, in combination with basolateral NKCC and Na^+/K^+-ATPase, could secrete Cl^- (and fluid).

Control sections stained with Mitotracker Red and the second antibody but lacking the primary antibody had no green fluorescence, indicating the absence of non-specific binding of the second antibody and the absence of 'bleed-through' from the red channel (Fig. 10). Control sections treated with the primary antibody but without the secondary antibody also demonstrated no detectable fluorescence (not shown).

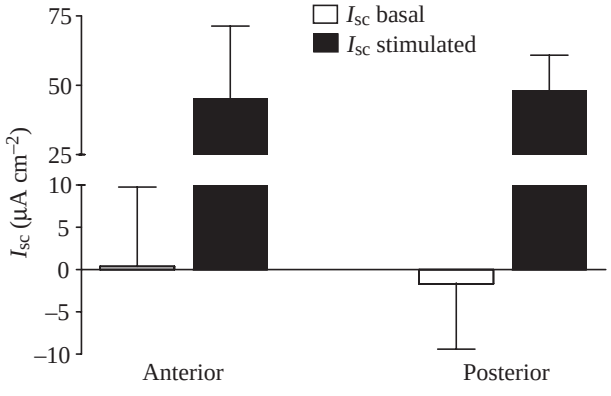

Fig. 5. There was no apparent difference between the posterior and anterior portions of intestine in the basal or stimulated (ionomycin+db-cAMP+IBMX) short-circuit current (I_{sc}), suggesting that the two sections of the intestine responded in a similar manner to stimulation. Values are means ± S.E.M., N=7.

Discussion

Induction of intestinal secretion

Normally, the intestine of marine teleosts absorbs NaCl and fluid (for a review, see Loretz, 1995), and there is progressive reabsorption along the intestine of Na^+, Cl^- and K^+ but not of Mg^{2+} or SO_4^{2-}. The evidence suggests ion uptake either by $Na^+/K^+/2Cl^-$ cotransport in the apical membrane, as in seawater-adapted flounder (*Platichthys flesus*) (for a review, see O'Grady et al., 1987), or possibly *via* Na^+/Cl^- cotransport, again as in flounder (Halm et al., 1985; Grosell and Jensen, 1999), or by parallel antiporter mechanisms, as in *Oreochromis mossambicus* (Howard and Ahearn, 1988). Ca^{2+}-activated K^+

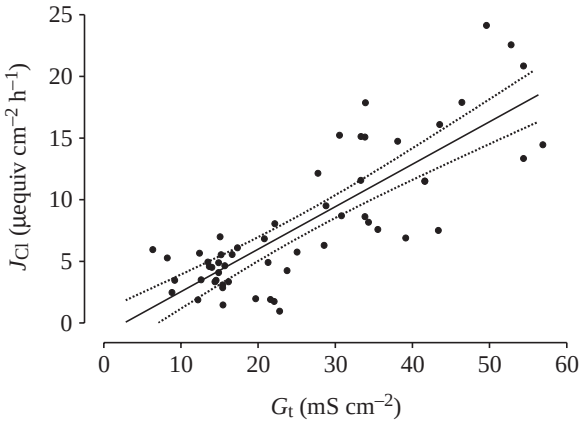

Fig. 6. Plot of spontaneous variation in tissue conductance (G_t) *versus* unidirectional Cl^- flux (J_{Cl}). There was a linear relationship with r^2=0.6692, d.f.=54, a slope of 0.345±0.132 μequiv mS^{-1} h^{-1} and a y-intercept of –0.91±1.94 μequiv cm^{-2} h^{-1} (mean ± 95 % confidence limit) that was not significantly different from zero; hence, all Cl^- flux would appear to be conductive. The solid line is the regression equation and the dotted lines are the 95 % confidence limits.

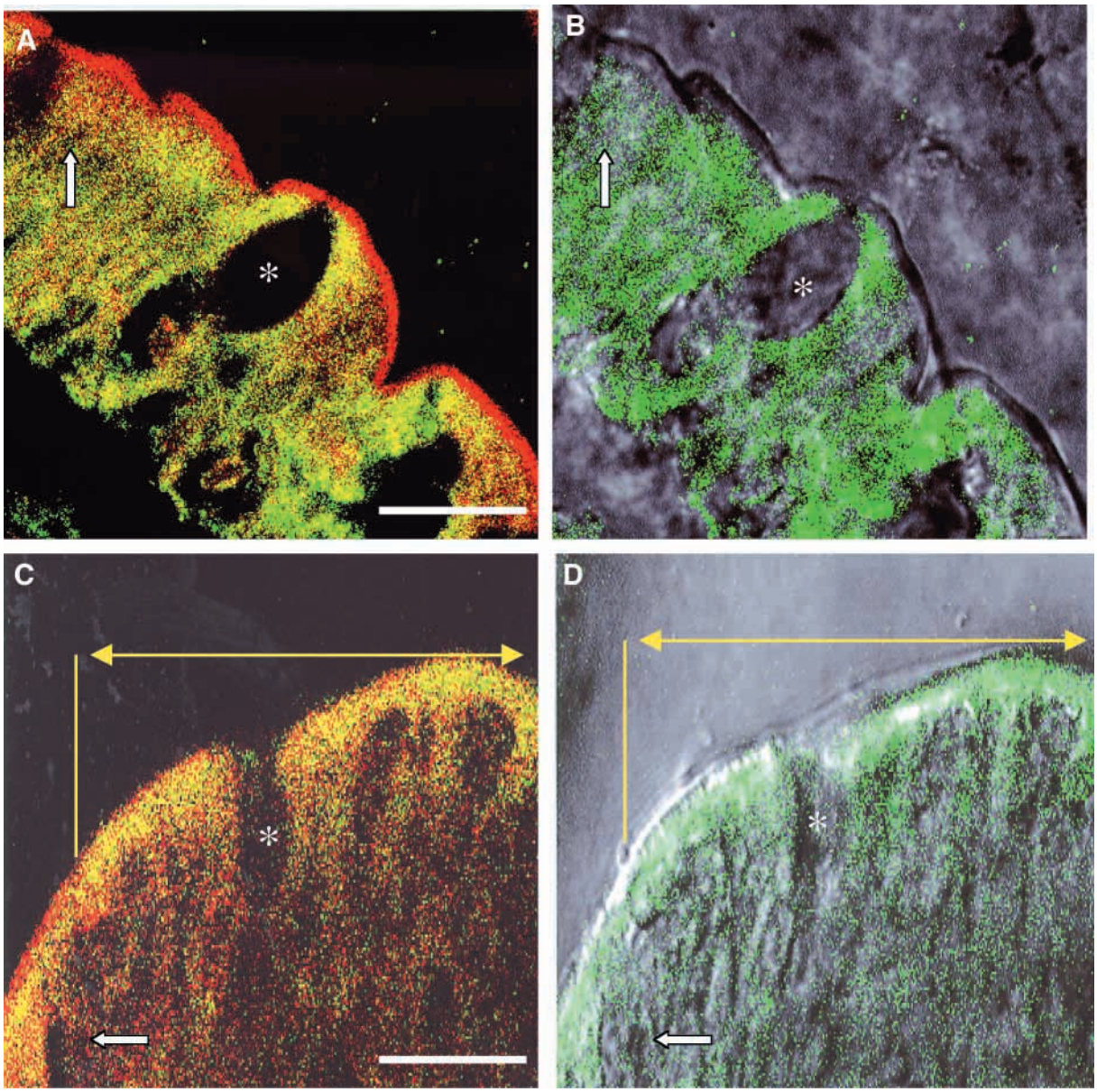

Fig. 7. Confocal laser scanning images of killifish cystic fibrosis transmembrane conductance regulator (kfCFTR) immunofluorescence of mouse anti-human CFTR and goat anti-mouse IgG Oregon Green 488 in seawater-adapted killifish posterior intestine showing the distribution of kfCFTR (green fluorescence) in the enterocytes of an intestinal villus. (A) Red and green fluorescence overlaid. In approximately 80 % of sections, kfCFTR immunofluorescence is evenly distributed throughout enterocytes but is absent from goblet cells (asterisk) and present at low levels only over enterocyte nuclei (white arrow). Brush-border microvilli stain (non-specifically) with Mitotracker Red. (B) Same image as A but with bright field overlaid with green fluorescence. (C) In a minority (approximately 20 %) of sections, kfCFTR immunofluorescence was also present in the apical membrane (area between arrows). (D) Same image as C but with bright field overlaid with green fluorescence. Scale bars, 20 μm.

channels and a voltage-sensitive anion channel (with properties clearly different from those of CFTR) are located in the basolateral membrane of teleost intestine (Loretz and Fourtner, 1988). The anion channel is the likely pathway for Cl^- exit during NaCl absorption (Loretz, 1995).

In teleost intestine, it appears that, for all species studied, ion reabsorption mechanisms have been universally observed. Loretz (1987a) suggests that teleosts have secondarily lost the ability to secrete NaCl and fluid while tetrapods and elasmobranchs retain this secretory ability. Tetrapods secrete ions and fluid intestinally, associated with the crypts of Lieberkühn, while elasmobranchs secrete ions into the intestinal lumen *via* the rectal gland (an outpocket of the posterior intestine). In elasmobranchs and tetrapods, ion secretion is initiated by cyclic AMP and agents that increase cyclic AMP levels (Loretz, 1987b; Valverde et al., 2000). Our results indicate that the killifish intestine can indeed be made to secrete ions with the additional stimulus of an efficient Ca^{2+}

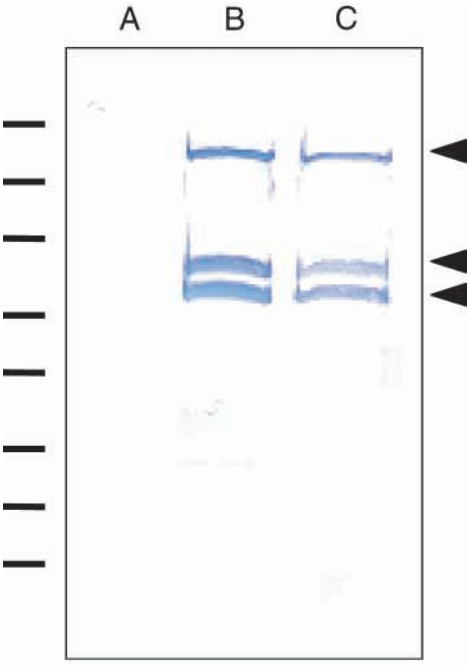

Fig. 8. Western blot analysis of cystic fibrosis transmembrane conductance regulator (CFTR) in intestine and gill tissue from seawater-adapted killifish (20 μg total protein per lane). Proteins were separated on a 7% polyacrylamide gel, transferred onto an Immobilon-P membrane and probed with anti-human CFTR monoclonal antibody (1 μg ml^{-1}). Proteins were visualized following incubation in BCIP/NBT Blue substrate development solution. Lane A, heart tissue (negative control); lane B, gill tissue; lane C, posterior intestine. In gill and intestine, the CFTR antibody cross-reacts with a 175 kDa protein and two lighter bands at 90.3 and 94.2 kDa (*N*=3). Molecular mass markers (kDa) (on left): 205, 116, 97.4, 84, 66, 55, 45 and 33 (from top to bottom).

ionophore. Hence, it is clear that the intestines of lower vertebrates, including those of teleost fish, can be secretory (contrary to previous information) and do not rely upon crypts or specific outpocketing structures to produce the response because killifish intestine lacks these structures. One explanation is that the crypts of Lieberkühn in mammals, which are associated with intestinal secretion, harbour enterocytes capable of secretion, but in teleosts the secretory enterocytes, which possess apical CFTR channels, are interspersed among other absorptive cells with similar microscopic appearance.

Electrophysiology

The killifish intestine has a small negative transepithelial potential (–0.4 to –0.6 mV) and a low transepithelial resistance of 50–100 Ω cm^2 typical of many marine teleosts (see Table I in Loretz, 1995) but unlike the high-resistance goby intestine or the positive potentials of freshwater salmon intestine. On the basis of I_{sc} measurements of resting and stimulated tissues, we found no difference between the most posterior sections of the intestine in the last 3–4 cm of the intestine; however, there is recognized functional zonation of anterior *versus* posterior intestine (Loretz, 1995). We did not examine more anterior portions of the killifish intestine, but instead restricted our examination to the zone in which we had previously demonstrated CFTR expression (Singer et al., 1998). The fluid absorption rates we observed under control conditions (+18.9±8.3 μl cm^{-2} h^{-1} *N*=8, Fig. 1) were lower than those observed previously in seawater eel (*Anguilla japonica*) intestine: 92.4±7.8 μl cm^{-2} h^{-1} (Uesaka et al., 1994) and 42.9±3.1 μl cm^{-2} h^{-1} (Ando, 1983). The eel intestine has a larger transepithelial potential and transepithelial resistance but approximately similar Cl^- net flux compared with seawater killifish intestine (killifish, control periods in Figs 2B, 3B, 4B; eel, 2.51±0.12 μmol cm^{-2} h^{-1}, *N*=7) (Uesaka et al., 1994). The difference in fluid transport rates could be ascribed to species differences, since eel and killifish are evolutionarily distant from each other, or to different measurement techniques (e.g. the thicker eel intestine requires stripping of the muscularis).

The linear relationship between Cl^- fluxes and conductance suggests that most of the ion flux across the tissue was conductive and not *via* electrically silent transport systems. It is likely that some of the Cl^- flux is transcellular because, in goby posterior intestine, regression of radiochloride efflux on tritiated mannitol flux (a paracellular pathway tracer) has a positive intercept (Mooney and Loretz, 1987). These facts point indirectly to the involvement of Cl^- channels in transepithelial Cl^- transport in the intestine and support a role for CFTR.

Ca^{2+} effects

Teleost intestine possesses a Ca^{2+} calmodulin inhibition of NaCl absorption (Loretz, 1987b) that can be initiated by the Ca^{2+} ionophore A23187 (1 μmol l^{-1}) and blocked by the calmodulin antagonists trifluoperazine and calmidazolium (R24571, 0.1 mmol l^{-1}). However, the inhibitory effect of IBMX was also present (Loretz, 1987b). It is likely that the ionomycin response observed here was connected with intracellular Ca^{2+} mediation in that ionomycin has been shown not to elevate intracellular cyclic AMP or cGMP levels in flounder intestine (O'Grady, 1989) and the ionophore inhibits Na^+ and Cl^- absorption by a pathway independent of cyclic nucleotides (O'Grady et al., 1988). The killifish intestine exposed to ionomycin showed a non-significant trend towards a decrease in Cl^- absorption in the first hour of treatment. It is clear that Ca^{2+} stimulation alone cannot eliminate absorption completely or produce net secretion; this is consistent with the traditional view that Ca^{2+} signalling in the vertebrate intestine is 'antiabsorptive' (Powell, 1986).

The value of intestinal secretion

The phenomenon of intestinal secretion in certain pathological conditions is presumably of selective advantage to the animal in purging the intestinal lumen of some or all of an infectious or toxic agent. No previous researchers have investigated the possibility of combined Ca^{2+} and cyclic AMP signals being required to elicit intestinal secretion. The combination of ionomycin + db-cAMP + IBMX evokes

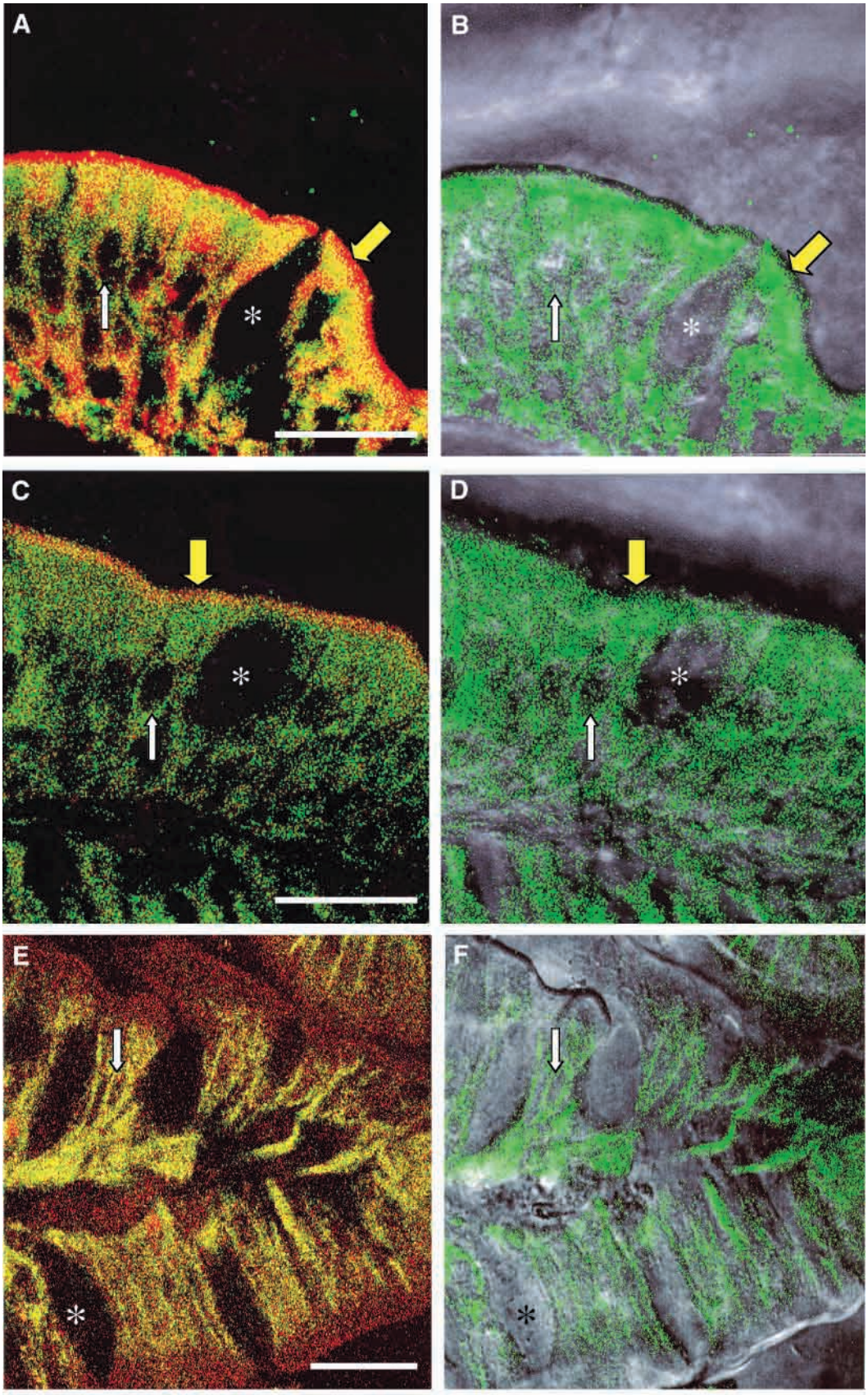

Fig. 9

Fig. 9. (A,B) Immunofluorescence of mouse anti-human $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and goat anti-mouse IgG Oregon Green 488 in seawater-adapted killifish posterior intestine shows the distribution of $Na^+/K^+/2Cl^-$ cotransporter (green fluorescence) in an intestinal villus. (A) In approximately 20 % of sections, NKCC immunofluorescence is evenly distributed throughout the enterocyte cytoplasm but is absent from the brush border and nuclei (white arrow) and from goblet cells (asterisk). In some cells, NKCC immunofluorescence is seen in the brush border (broad yellow arrow). (B) Same section, bright field overlaid with green fluorescence. (C) NKCC immunofluorescence in most sections (approximately 80 %) is present in the cytoplasm and in the brush border (yellow arrow) and is absent from nuclei (white arrow) and goblet cells (asterisk). (D) Same section but with bright field overlaid with green fluorescence. (E,F) Immunofluorescence of mouse anti-chicken Na^+/K^+-ATPase and goat anti-mouse IgG Oregon Green 488 in seawater-adapted killifish posterior intestine showing the distribution of Na^+/K^+-ATPase (green fluorescence) in an intestinal villus. (E) Na^+/K^+-ATPase immunofluorescence is distributed throughout the basal two-thirds of the enterocytes but not in the nuclei (white arrow), the goblet cells (asterisk) or the brush border, which stains (non-specifically) with Mitotracker Red. (F) Na^+/K^+-ATPase immunofluorescence overlaid with bright field. Scale bars, 20 μm.

secretion of Cl^- and fluid that develops in the first hour of treatment and is sustained thereafter. Ionomycin alone and db-cAMP + IBMX alone were ineffective in producing net Cl^- efflux or net fluid secretion. The main function of cyclic AMP seems to be to increase total tissue conductance since ionomycin had no effect on this variable. The present data are consistent with the suggestion that cyclic AMP opens paracellular pathways in the intestine (Bakker et al., 1993). However, in the absence of ionomycin, cyclic AMP increased unidirectional Cl^- fluxes and G_t but did not change the net flux, so ionomycin in the combined response appears to mediate the rapid increase specifically in the unidirectional Cl^- efflux. Hence, the combination of Ca^{2+}-stimulated transcellular Cl^- efflux linked with cyclic-AMP-mediated increases in paracellular conductance may allow transcellular ion (NaCl) secretion to drag solute osmotically, resulting in the observed fluid secretion.

Role for CFTR

The inhibition of Cl^- efflux by DPC (8.7 μequiv cm^{-2} h^{-1}) is larger than the decrease noted in the corresponding Cl^- influx (2.8 μequiv cm^{-2} h^{-1}) and is accompanied by a large (12 mS cm^{-2}) decrease in G_t, consistent with an action of DPC on anion channels in the apical membrane. DIDS, a blocker of the anion exchanger and of some volume-sensitive anion channels (but not CFTR), had no effect on G_t, I_{sc} or the net Cl^- flux. In the chloride cells of the opercular epithelium, CFTR-like channels are activated by cyclic AMP (Marshall et al., 1995). kfCFTR is expressed in killifish intestine (Singer et al., 1998) (northern blot analysis). The results are compatible with the idea that CFTR-like anion channels in the apical membrane of the intestine are activated by cyclic AMP and are responsible for the increase in Cl^- efflux and the development of net Cl^- secretion.

The presence of CFTR immunofluorescence in the apical membrane of some enterocytes and the basal distribution of NKCC and Na^+/K^+-ATPase in most enterocytes is a pattern

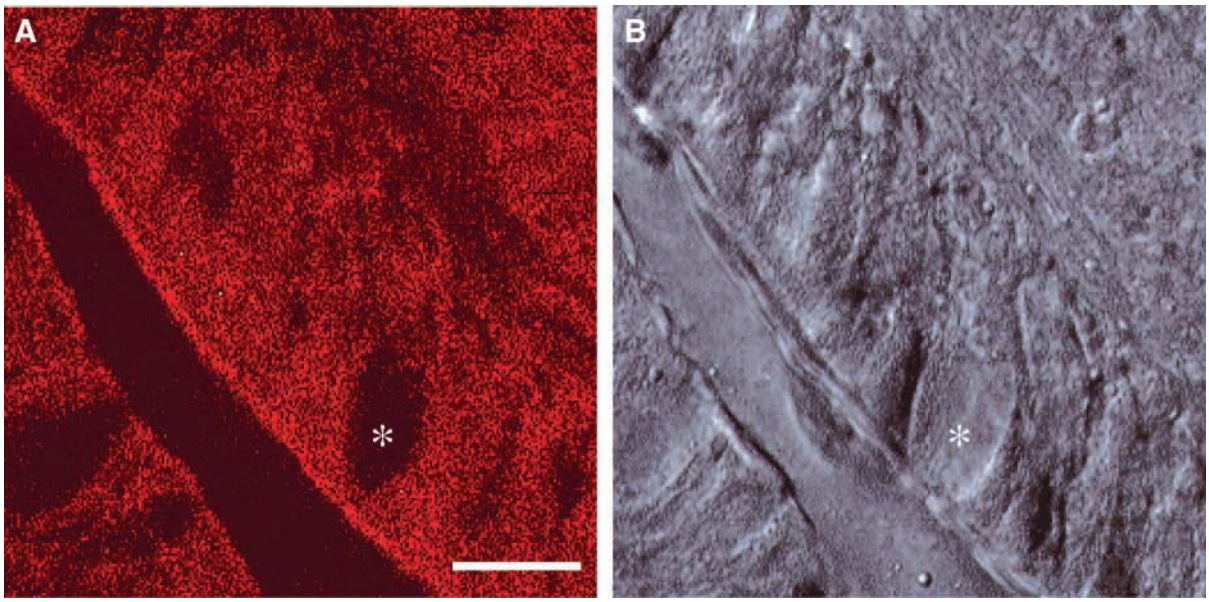

Fig. 10. Control images of seawater-adapted killifish posterior intestine, where the primary antibody (to killifish cystic fibrosis transmembrane conductance regulator, kfCFTR, in this case) was omitted, showing the absence of the secondary antibody goat anti-mouse IgG Oregon Green 488 signal (green fluorescence). Scale bar, 20 μm. (A) Negative control of kfCFTR. Note the sole presence of the Mitotracker Red stain in the enterocytes and its absence from the goblet cells (asterisk). (B) Green channel fluorescence overlaid with bright field has no detectable green signal from the secondary antibody's fluorescence, indicating a lack of non-specific binding of the second antibody and of 'bleed-through' from the red channel.

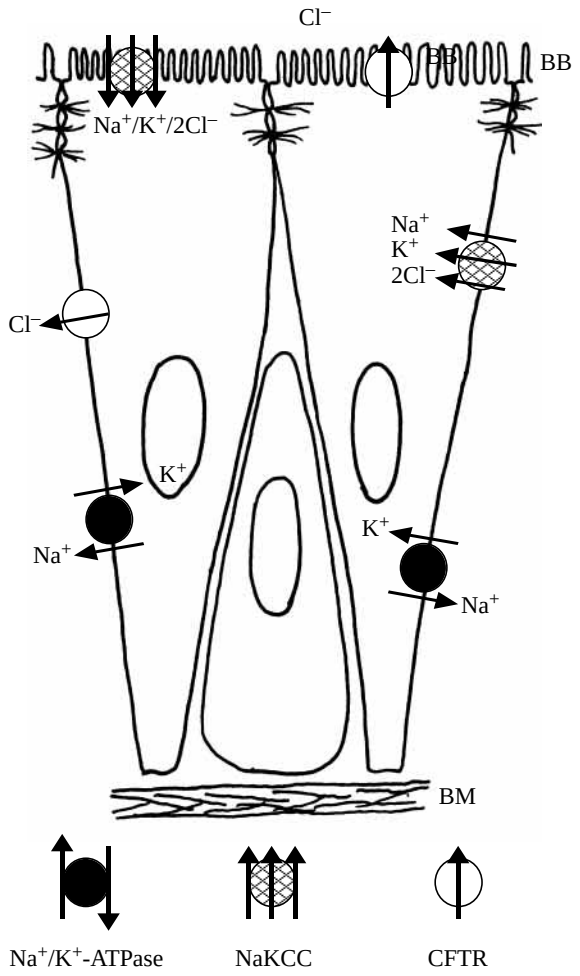

Fig. 11. Model of transporter distribution in enterocytes of killifish intestinal epithelium, showing brush border (BB) on the luminal side and cells overlying the basement membrane (BM) on the serosal side. In the model, all cells have Na^+/K^+-ATPase only in the basolateral membrane. The cell on the left is modelled for uptake of ions and fluid, while that on the right is modelled for secretion. The $Na^+/K^+/2Cl^-$ cotransporter (NKCC) is present in the basal portion of all enterocytes and, in most cells (left), also in the apical brush-border membrane. Cystic fibrosis transmembrane conductance regulator (CFTR) immunofluorescence is present in the basal portion of most cells (left) and in the apical membrane of some enterocytes (right).

expected for cells contributing to NaCl secretion (Fig. 11). From the sections observed, it seems that perhaps 10–20 % of sections have apical staining for CFTR, and the distribution appears patchy. The distribution is similar to the pattern seen in mitochondria-rich cells of the gills of the mudskipper *Periophthalmodon schlosseri* (Wilson et al., 2000). The presence of NKCC immunofluorescence in the basal and apical portions of many enterocytes implies that, functionally, NKCC may be activated differentially to produce the effect of uptake (apical NKCC activated) or secretion (basal NKCC activated) (Fig. 11). Because CFTR appears to be present in the apical membrane of some cells, there may be a subpopulation of enterocytes responsible for ion (and fluid) secretion. The rapidity of the change to fluid secretion observed in the intestine is consistent with previous observations of rapid cyclic AMP activation of CFTR in opercular membranes (Marshall et al., 1995) and by CFTR expressed in amphibian oocytes (Singer et al., 1998).

The present data, i.e. the apparent lack of redistribution of CFTR immunofluorescence in response to treatment with IBMX and ionomycin, also confirm the observations that cyclic AMP (*via* protein kinase A and phosphorylation) activates CFTR already *in situ* in plasma membranes, rather than acting by regulating trafficking of CFTR to the membrane (Moyer et al., 1998; Loffing et al., 1998). There remains the possibility of the involvement of other anion channels in Cl^- uptake, including members of the ClC family known to be involved in Cl^- secretion in mammalian intestine (Gyomorey et al., 2000) and the voltage-sensitive anion channel in teleosts (Loretz and Fourtner, 1988); hence, CFTR appears to contribute to Cl^- uptake and secretion in the teleost intestine.

Composition of secreted fluid

The measured absorption of ions and fluid allows the approximate concentration of the transported fluid to be calculated. If one averages the observed control period Cl^- net flux values (from Figs 2B, 3B and 4B: 2.5, 2.7 and 2.3 μequiv cm^{-2} h^{-1}) and divides by the fluid transport rate (control period, Fig. 1, 18.4 and 18.3 μl cm^{-2} h^{-1}), the calculated concentration is 2.5 μequiv cm^{-2} h^{-1}/8.4 μl cm^{-2} h^{-1}= 136 mmol l^{-1} Cl^-. Given that plasma [Cl^-] for seawater *Fundulus heteroclitus* is approximately 165 mmol l^{-1}, then the absorbate is approximately at the isoionic/iso-osmotic level. Performing a similar calculation for the secreted fluid (net ion fluxes from drug treatment periods of Figs 2B and 4B; 3.8, 3.3, 3.80 and 2.8 μequiv cm^{-2} h^{-1} and fluid secretion of 7.4 μl cm^{-2} h^{-1} in Fig. 1) produces an effective concentration of the secreted fluid of 3.4 μequiv cm^{-2} h^{-1}/7.4 μl cm^{-2} h^{-1}= 460 mmol l^{-1} Cl^-, which is decidedly hypertonic because Na^+ will follow the secretion of Cl^- to maintain electroneutrality. This suggests that marine fish that drink full-strength sea water could absorb ions and fluid in the anterior intestine and (pathophysiologically) secrete a fluid of similar composition to sea water in the posterior intestine. The estimate also suggests that the ion secretion activated by the drug treatment opens a pathway for Cl^- secretion that is transcellular and poorly connected with the osmotic permeability of the whole system; hence, the secreted fluid appears to be hypertonic. The notion of transcellular Cl^- secretion fits our data in that the activation of apical membrane ion channels for ion secretion in a membrane lacking water channels could initiate ion secretion without efficient osmotic water flux, thus producing secretion of a concentrated fluid, as observed.

Function and endogenous regulation of secretion

The function of intestinal secretion in fish is unknown, but it presumably aids in purging toxic intestinal bacterial flora. If such intestinal secretion occurs normally or pathophysiologically in fish, estuarine animals would not

suffer the dire dehydration and demineralization that terrestrial tetrapods do. Indeed, it would be reasonable for estuarine animals to respond to this osmoregulatory stress simply by behaviourally seeking iso-osmotic environments (sea water or brackish water) that could be consumed and absorbed in the anterior sections of the gut to replace both fluid and ions in appropriate amounts during bouts of intestinal secretion. In this way, intestinal secretion could be of selective advantage, particularly in estuarine fish.

The question remains open as to whether a combination of endogenous hormones might evoke intestinal secretion in teleosts. It is curious that secretion was evoked more clearly in fed animals, suggestive of a hormonal priming of solute transporters by feeding. Atrial natriuretic peptide (ANP), mediated by cyclic GMP, inhibits $Na^+/K^+/2Cl^-$ cotransport in the flounder intestine (O'Grady et al., 1985). Acetylcholine and serotonin also inhibit NaCl and water reabsorption (Uesaka et al., 1994; Trischitta et al., 1999), an effect antagonized by somatostatin-related peptides. In turn, the actions of somatostatin and analogues in the goby intestine appear to be *via* a natural receptor that more readily accepts another endogenous hormone, urotensin II, which is somatostatin-like (Loretz et al., 1983; Loretz, 1990). In goldfish (*Carassius auratus*) intestine, vasoactive intestinal polypeptide (VIP) and serotonin may inhibit NaCl reabsorption by an action reducing tight-junctional selectivity (Bakker et al., 1993). This hypothesis was supported by the action of cyclic AMP on tight junctions in eel (*Anguilla anguilla*) intestine (Trischitta et al., 1996). Serotonin inhibits NaCl reabsorption, and its action is mediated *via* adenyl cyclase (Bakker et al., 1993). Whereas ANP in flounder intestine increases intracellular cGMP levels (O'Grady, 1989), VIP apparently acts *via* cyclic AMP in flounder (O'Grady, 1989) and in goldfish (Bakker et al., 1993) intestine. ANP and VIP were hypothesized to be released by enteric nerves and to act locally (O'Grady, 1989), and this localization has been definitively demonstrated by fluorescence immunocytochemistry (Loretz et al., 1997).

Urotensin II stimulates NaCl reabsorption in goby intestine *via* a lowering of intracellular Ca^{2+} levels (Loretz and Assad, 1986). The Ca^{2+} ionophore A23187 produces a marginally significant (Loretz, 1987a,b) or no effect on intestinal preparations (Bakker et al., 1993), but this lack of a clear response may be ascribed to the low efficiency of this ionophore compared with ionomycin. For instance, killifish opercular epithelium Cl^- secretion is inhibited by ionomycin but not by the Ca^{2+} ionophore A23187 (Marshall et al., 1993). The high affinity of adrenergic receptors in goldfish intestine for clonidine and blockade by yohimbine indicate the presence of α_2-adrenergic receptors (Bakker et al., 1993) and, if the receptors are similar to those in the opercular membrane (Marshall et al., 1993), they too may be mediated by Ca^{2+} and could activate this pathway in the intestine. Taken together, the endogenous hormones that might produce intestinal secretion would be a combination of serotonin or VIP to activate adenylate cyclase and an α_2-adrenergic agonist to activate intracellular Ca^{2+}.

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