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Sodium-sensitive and -insensitive copper accumulation by isolated intestinal cells of rainbow trout *Oncorhynchus mykiss*

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Summary

The pathway for copper (Cu) uptake across the mucosal membrane into intestinal cells has not been elucidated in fish. Copper accumulation in freshly isolated intestinal cells from rainbow trout *Oncorhynchus mykiss* was measured after exposure to 0–800 μmol l^{-1} $CuSO_4$ for 15 min. With external Cu concentration (Cu_o) of 800 μmol l^{-1}, the rate of Cu accumulation by cells was 1.88±0.52 nmol Cu mg^{-1} cell protein h^{-1} compared to 0.05±0.01 nmol Cu mg^{-1} cell protein h^{-1} with no added Cu_o (means ± S.E.M., N=6). Deduction of a rapid Cu accumulation measured on/in cells at time zero (about 12% of the total Cu uptake when Cu_o was 800 μmol l^{-1}) revealed a saturable uptake curve, which reached a plateau at 400 μmol l^{-1} Cu_o (K_m=216 μmol l^{-1} Cu_o; V_{max}=1.09 nmol Cu mg^{-1} cell protein h^{-1}; 140 mmol l^{-1} NaCl throughout). Incubation of cells at 4°C did not prevent Cu accumulation. Lowering external [Na^+] to 11 mmol l^{-1} (low Na^+_o) generally did not alter the rate of Cu accumulation into the cells over a 15 min period. Under low Na^+_o conditions Cu accumulation was exponential (non-saturable). Na^+-insensitive Cu accumulation dominated (59% of total Cu accumulation) when Cu_o was 400 μmol l^{-1} or less. At high Cu_o (800 μmol l^{-1}), removal of Na^+ caused a 45% increase in Cu accumulation. Pre-incubation of cells with blocking agents of epithelial Na^+ channel (ENaC) for 15 min (normal [NaCl] throughout) caused Cu accumulation rates to increase by 40-fold (100 μmol l^{-1} phenamil), 21-fold (10 μmol l^{-1} CDPC) or 12-fold (2 mmol l^{-1} amiloride) when Cu_o was 800 μmol l^{-1} compared to those in drug-free controls. Lowering the external chloride concentration $[Cl^-]_o$ from 131.6 to 6.6 mmol l^{-1} (replaced by sodium gluconate) caused the rate of Cu accumulation to increase 11-fold when Cu_o was 800 μmol l^{-1}. Application of 0.1 mmol l^{-1} DIDS (normal Cl^-_o) caused a similar effect. Lowering external pH from 7.4 to pH 5.5 produced a 17-fold, saturable, increase in Cu accumulation rate, which was not explained by increased instantaneous Cu accumulation on/in cells at low pH. We conclude that Cu accumulation by intestinal cells is mainly Na^+-insensitive and more characteristic of a pH- and K^+-sensitive Ctr1-like pathway than Cu uptake through ENaCs.

Key words: rainbow trout, *Oncorhynchus mykiss*, dietary copper, sodium, amiloride, phenamil, low pH.

Introduction

Copper (Cu) is an essential nutrient for vertebrates and has numerous functions in cellular biochemistry (Linder, 1991; Huffman and O'Halloran, 2001), such as electron transfer in mitochondria (Moody et al., 1997), acting as a cofactor for more than 30 different enzymes (Linder, 1991), and modulating the neuro-endocrine control of metabolism (Linder, 1991; Handy, 2003). Teleost fish require 1–4 mg Cu kg^{-1} dry mass of food (Murai et al., 1981; Knox et al., 1982; Lanno et al., 1985; Watanabe, 1997), but excess dietary Cu is also toxic to fish (for reviews, see Handy, 1996, 2003; Clearwater et al., 2002), and so Cu uptake across the gut must be carefully regulated (Clearwater et al., 2000, 2002).

In mammals and fish, Cu uptake from the gut lumen to the blood involves (i) electrostatic adsorption of Cu to the surface of the mucosal membrane, (ii) entry into the gut cells by facilitated diffusion, probably through ion channels, (iii) transfer of Cu across the cell by metal chaperones, (iv) export from the cell to the blood against the electrochemical gradient (Linder, 1991; Harrison and Dameron, 1999; Handy et al., 2000, 2002). The latter step involves both exocytosis of vesicular Cu derived from the Golgi complex (Harrison and Dameron, 1999; Huffman and O'Halloran, 2001), and Cu export from the cytoplasm on a serosally located Cu–Cl symporter (Handy et al., 2000). Copper uptake across the gut is also negatively regulated at the intestine, Cu uptake efficiency declining with increasing luminal Cu concentration in isolated perfused catfish intestines (Handy et al., 2000), and dietary Cu bioavailability declining with increasing dietary dose *in vivo* (e.g. trout; Clearwater et al., 2002). Intestinal Cu uptake can also be downregulated by aqueous Cu exposure in trout (Kamunde et al., 2002), implying some degree of systemic control of Cu absorption across the intestine.

However the precise pathway for Cu entry into enterocytes from the gut lumen is uncertain. Current evidence from a variety of epithelia (frog skin, Flonta et al., 1998; rat intestine, Wapnir, 1991; trout gills, Grosell and Wood, 2002; fish intestine, Handy et al., 2002) suggest at least two candidate pathways. These include Cu leak through epithelial Na^+ channels (EnaCs; e.g. fish gills, Grosell and Wood, 2002) and Cu uptake on a Cu-specific carrier, Ctr1 (Lee et al., 2000, 2002a,b). Ctr1 is located in the plasma membrane of cells and appears to be ubiquitously expressed in mammalian tissues (Lee et al., 2000; Klomp et al., 2002). Ctr1 has also been recently identified in zebrafish (Mackenzie et al., 2004).

In the intestine, Cu uptake on Ctr1 is more likely than through ENaCs for several reasons. Firstly, the relatively high external Na^+ concentration (Na^+_o, *circa* 100 mmol l^{-1}) in the gut lumen compared to gills or frog skin in freshwater suggest that Cu will be less able to compete for entry through ENaCs (Handy et al., 2002). Secondly, Cu^{2+} ions bind externally to the alpha subunit of ENaCs rather than going through the channel pore, and Cu exposure does not stop the Na^+ current through ENaCs (frog skin; Flonta et al., 1998). Removal of external Na^+ or Cl^- also tends to lower intestinal Cu uptake rates (rat, Wapnir and Stiel, 1987; catfish, Handy et al., 2000), and this is not consistent with Cu ions competing with Na^+ for entry through ENaCs in the gut mucosa (Handy et al., 2002).

Alternatively, amiloride-dependent depression of Cu uptake by perfused intestines is most easily explained by Cu uptake through ENaCs (Wapnir, 1991; Handy et al., 2002). In Na^+_o removal experiments, the indirect effects of low cell Na^+ on Cl^- balance could also alter Cu status. For example, decreasing intracellular chloride during Na^+_o removal experiments could slow Cu export to the blood on Cu–Cl symport (Handy et al., 2000, 2002), prevent Cu binding to vesicular Cu-ATPase (Davis-Kaplan et al., 1998), and reduce the probability of opening of ENaCs (Kunzelmann et al., 2001; for a discussion, see Handy et al., 2002). These indirect effects would raise whole cell Cu content as Na^+_o declines, giving the impression of apparent Na^+_o-dependent Cu uptake. *In vivo*, there may also be systemic Na^+-dependent modulation of Cu uptake (systemic Na^+ effects on Cu uptake by trout gills; Kamunde et al., 2003; Pyle et al., 2003).

In this experiment we resolve some of these controversies for fish, and explore the Na^+_o-dependence of Cu accumulation by isolated intestinal cells from rainbow trout. Freshly isolated intestinal cells have the advantage of retaining the biochemical and metabolic characteristics of the gut mucosa for several hours (Kimmich, 1990). Primary cultures of intestinal cells are not used because they gradually become quiescent as they reach confluence, and for this reason intestinal cell lines derived from hybrids with cancer stem cells are preferred by most researchers for studies on ion transport (Dharmsathaphorn and Madara, 1990). Furthermore, external Cu concentrations as low as 5 μmol l^{-1} may compromise the tight junctions in monolayers of cultured intestinal cells (Caco-2 cells; Ferruzza et al., 1999). Such cell lines are not readily available in rainbow trout, and so in the present study we use freshly isolated intestinal cells. This approach also enables Cu accumulation by the mucosal cells to be measured without contributions from the underlying muscularis or enteric nervous system, and avoids systemic/organ level Cu-dependent endocrine modulation of gut function (Handy, 2003). Putative loss of polarity in cell suspensions is not so problematic for studies on Cu accumulation by intestinal cells, because unlike major electrolytes, Cu appears only to move from the luminal side across the mucosal membrane, through the cell, and out of the cell across the serosal membrane in a variety of experimental conditions (Arredondo et al., 2000; Zerounian et al., 2003; Bauerly et al., 2004). There is no evidence that intestinal cells can secrete accumulated Cu (back flux) across the mucosal membrane (Arredondo et al., 2000), and this is not surprising since the endosomal recycling of Ctr1 back to the mucosal membrane does not colocalise with Golgi Cu stores (Bauerly et al., 2004). Thus Cu-loaded Caco-2 cells retain 40% or more of their Cu for at least 18 h (Zerounian et al., 2003), and the observed Cu efflux from the cells is best explained by vesicular trafficking *via* metal chaperones to the serosal membrane (Arredondo et al., 2000; Zerounian et al., 2003; Bauerly et al., 2004).

Our aims are fourfold: (i) to demonstrate concentration-dependent Cu accumulation in isolated intestinal cells and the effect of Na^+_o removal on Cu uptake; (ii) to critically evaluate the effect of the ENaC blocking agent amiloride compared to other more specific ENaC inhibitors, 6-chloro-3, 5-diamino-2 pyrazinecarboxamide (CDPC) and phenamil (Garvin et al., 1985; Flonta et al., 1998; Reid et al., 2003), on intestinal Cu uptake; (iii) to determine the indirect effects of manipulations of anion transport on Cu accumulation and Na^+ content of intestinal cells; (iv) explore Cu accumulation in the low pH conditions known to stimulate Ctr1 (Lee et al., 2002a).

Materials and methods

Stock animals and tissue collection

Rainbow trout *Oncorhynchus mykiss* Walbaum weighing 316.3±14.5 g (mean ± S.E.M. N=54) were held in an aerated re-circulating aquarium filled with dechlorinated Plymouth tapwater (in mmol l^{-1}: Na^+, 1.5; K^+, 0.3; Ca^{2+}, 0.1; Mg^{2+}, 1.8; total Cu <0.0004; Cl^-, 1.7; pH 7.4; temperature, 14±1°C). Stock fish were fed on commercial trout feed (containing 11 mg Cu kg^{-1} dry mass of feed; Trouw No 2, Northwich, Cheshire, UK), but were not fed for 48 h before experiments to minimise food debris in the gut lumen prior to cell isolations. Fish were humanely killed by a sharp blow to the head, pithed, weighed and total length measured. The intestine was immediately removed by cutting just behind the pyloric caecae and also at the rectum, and then resting gut length was measured. The gut was carefully cleaned of fat and mesentery during dissection, and rinsed with deionised water.

Intestinal cell isolation

Intestinal cells were freshly isolated by a method modified from Kimmich (1990). Briefly, each rinsed intestine was cut

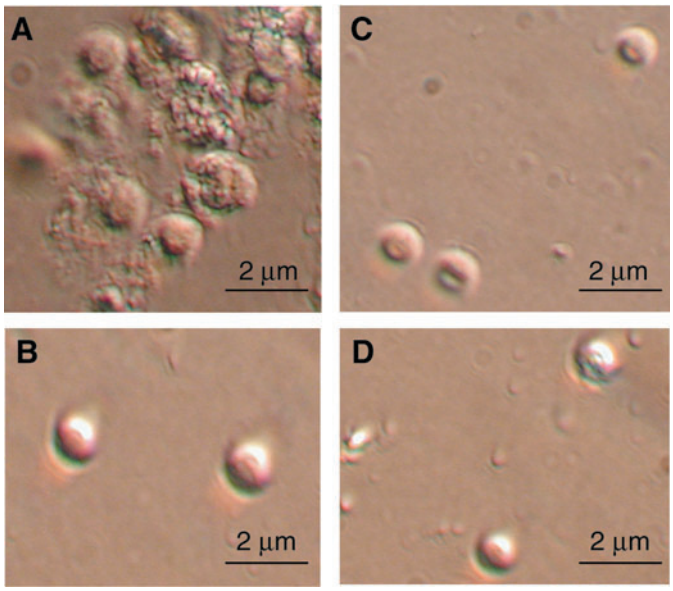

Fig. 1. Examples of isolated trout intestinal cells in normal physiological saline (no added Cu_o) after 15 min (A) and 3 h (B), compared to cells exposed to saline containing 800 μmol l^{-1} Cu_o for 15 min (C) and 3 h (D). Cells were photographed in phase contrast (not fixed or stained) using a Leica DMIRB microscope and a Nikon coolpix digital camera. Scale bar, 2 μm.

open longitudinally and divided into 1 cm^2 pieces, then added to 50 ml of ice-cold isolation medium (in mmol l^{-1}: NaCl, 125; $NaHCO_3$, 10; K_2HPO_4, 3; $MgCl_2$, 1; $CaCl_2$, 1; dithiothreitol, 0.1; glucose, 10; Hepes, 10; Trizma base 10; pH 7.4). The tissue was gently agitated using a Pasteur pipette to detach enterocytes from the mucosa. The resulting cell suspension was then filtered through a 200 μm mesh to remove debris, and then divided between 4× 13 ml tubes and centrifuged (5 min at 200 ***g***, Denley 401 refrigerated centrifuge; Thermo-Denley, Basingstoke, UK). The supernatant was discarded and the pellet of cells in each test tube was resuspended in 850 μl of physiological saline (in mmol l^{-1}: NaCl, 125; $NaHCO_3$, 10; NaH_2PO_4, 1; KCl, 3; $MgSO_4$, 2; $CaCl_2$, 1.8; glucose, 10; adjusted to pH 7.4 with 2 drops of 1 mmol l^{-1} HCl or NaOH). The tubes were then combined, typically providing a total of 3.4 ml of cell suspension per fish intestine (mean ± S.E.M., 1.05±0.005×10^7 cells ml^{-1} and 94.1±1.08% cell viability by Trypan Blue exclusion; *N*=54 separate cell isolations). At least six separate cell isolations were used for each experiment. Cell counts and cell viability were measured immediately before experiments, and only cell isolations showing in excess of 80% viability were used.

Preliminary experiments

Several preliminary experiments were performed to explore cell viability and to determine the optimum exposure times/Cu concentrations needed to measure physiological Cu accumulation. In an initial trial the survival of a batch of cells was assessed by Trypan Blue exclusion over 4 h in normal physiological saline without Cu (no added Cu). In this trial, viability was initially 82% and remained at 82% for the first 3 h, then decreased only slightly to 78% by 4 h (4% decline over 4 h). Thus freshly isolated intestinal cells remained intact for at least 4 h on the bench in normal physiological saline. Experiments were then repeated in the presence of 800 μmol l^{-1} Cu_o. Cells were intact and morphology also remained normal for several hours, with or without added Cu (Fig. 1). Other batches of cells were then used to establish whether or not Cu accumulation could be detected in cells exposed to Cu_o for periods lasting several hours. Cells (*N*=6 batches from separate isolations) were treated for 2 h with either nominally Cu-free saline (normal saline + 1 μmol l^{-1} ethylenediaminetetraacetic acid, EDTA), control with no added Cu (normal saline with no EDTA or added Cu), and normal saline containing 10, 50, 100 or 200 μmol l^{-1} Cu. The Cu content of cells was easily detected, and mean values after 2 h were 0.01±0.002 and 0.96±0.09 nmol Cu mg^{-1} cell protein in controls with no added Cu and in saline containing 200 μmol l^{-1} Cu_o, respectively (mean ± S.E.M., *N*=6; significantly different, student's *t*-test, $P<0.05$). We therefore elected to explore Cu accumulation over a much shorter time scale (0–30 min) and with Cu_o up to 800 μmol l^{-1} (Fig. 2A). Cells that were exposed to Cu for 30 min showed a dose-dependent increase in Cu content, with the Cu content of cells reaching a plateau by 10 min at most exposure concentrations, and Cu content remained steady for at least another 20 min (Fig. 2). The leakiness of these cells was assessed by measuring lactate dehydrogenase (LDH) release into the medium (for LDH assay, see Campbell et al., 1999) and cell Na^+ and K^+ content (Table 1). Control cells (no added Cu) showed stable Na^+ and K^+ contents over a 30 min period with no changes in membrane permeability measured by LDH leak (Table 1). Similarly Cu-treated cells did not leak LDH, the release rate of which remained at or below detection limits for all external Cu concentrations (<0.1 μmol LDH min^{-1} ml^{-1} medium, *N*=6). Cu-treated cells also showed stable Na^+ and K^+ contents over 30 min (Table 1). In another series of experiments, cells were loaded with Cu by exposing them to 800 μmol l^{-1} Cu_o for 15 min and then placed in normal saline (no added Cu) to determine whether the accumulated Cu would leak out of the cells. This was not observed (Fig. 2B) over a 15 min period. Cu-loaded cells retained 86±5.1% (mean ± S.E.M., *N*=9) of the accumulated Cu after 15 min recovery in normal saline (not statistically different from the control, Fig. 2B). Even after 1 h in clean saline, cells still retained 50% of the accumulated Cu. Overall, these preliminary experiments demonstrated that freshly isolated intestinal cells could survive physiological concentrations of Cu_o of up to 800 μmol l^{-1} and did not leak LDH, Cu or electrolytes over exposure periods of 15 min. We therefore elected to conduct the main experiments using total external Cu concentrations of between 0–800 μmol l^{-1} (added as $CuSO_4.5H_2O$ to the physiological saline above), for exposures lasting 15 min.

Cu exposure protocol

Copper exposures lasting 15 min were used in the main

experiments, and the protocol was derived from the preliminary experiments outlined above. Briefly, 125 µl of the washed cell suspension was added to 500 µl of the appropriate Cu concentration (final concentrations of 0, 10, 50, 100, 200, 400 or 800 µmol l^{-1} Cu, as $CuSO_4.5H_2O$) in physiological saline (saline as above), and incubated in an Eppendorf tube at room temperature (20°C) for 15 min (in triplicate). Copper concentrations in all solutions were measured prior to experiments, and if necessary solutions were prepared again to meet the exact concentrations indicated above. Controls included incubation in physiological saline with no added Cu (saline only control) and a Cu-free control (saline without Cu, + 1 µmol l^{-1} EDTA). At the end of the exposure period the cells were quickly pelleted (1 min at 13 000 rpm, Sanyo Microcentaur, Fischer Scientific, Loughborough, UK), and the pellet was washed gently (3 times) with 100 µl of 0.1 µmol l^{-1} EDTA. Finally the cells were lysed with 0.5 ml of analytical grade 0.1 mol l^{-1} nitric acid prior to metal analysis (see below). The entire protocol was repeated on at least six separate occasions for each experiment, using gut tissue from a new fish each time.

Each experiment also included a 'time zero' control, where batches of cells were added to Cu_o solutions (<1 min to prepare all tubes), immediately washed in the EDTA solution above, then pelleted. These rapid measurements were used mainly as an additional check within each experiment to confirm that net Cu accumulation was a progressive accumulation during the 15 min incubations rather than spontaneous Cu adsorption/surface binding in the first minute of incubation (Handy and Eddy, 2004). The time zero controls showed a rapid Cu accumulation component, which was maximally (when Cu_o=800 µmol l^{-1}) around 12–16% of the total apparent accumulation (see Results, and Table 2) and at Cu_o <800 µmol l^{-1} this component was <1%. Thus over most of the Cu_o range used in the experiment (0–400 µmol l^{-1}), the instantaneous component was small and was therefore not deducted from the Cu accumulation data after the 15 min incubations.

Copper uptake in normal and low Na^+_o

This series of experiments was conducted to determine dose-dependent net Cu uptake by freshly isolated cells in the presence of normal NaCl (Na^+_o=140 mmol l^{-1}; Handy et al., 2002) using the protocol and saline described above. The experiment was then repeated with Cu incubations on ice at 4°C (normal NaCl, 140 mmol l^{-1} Na^+_o) to determine whether

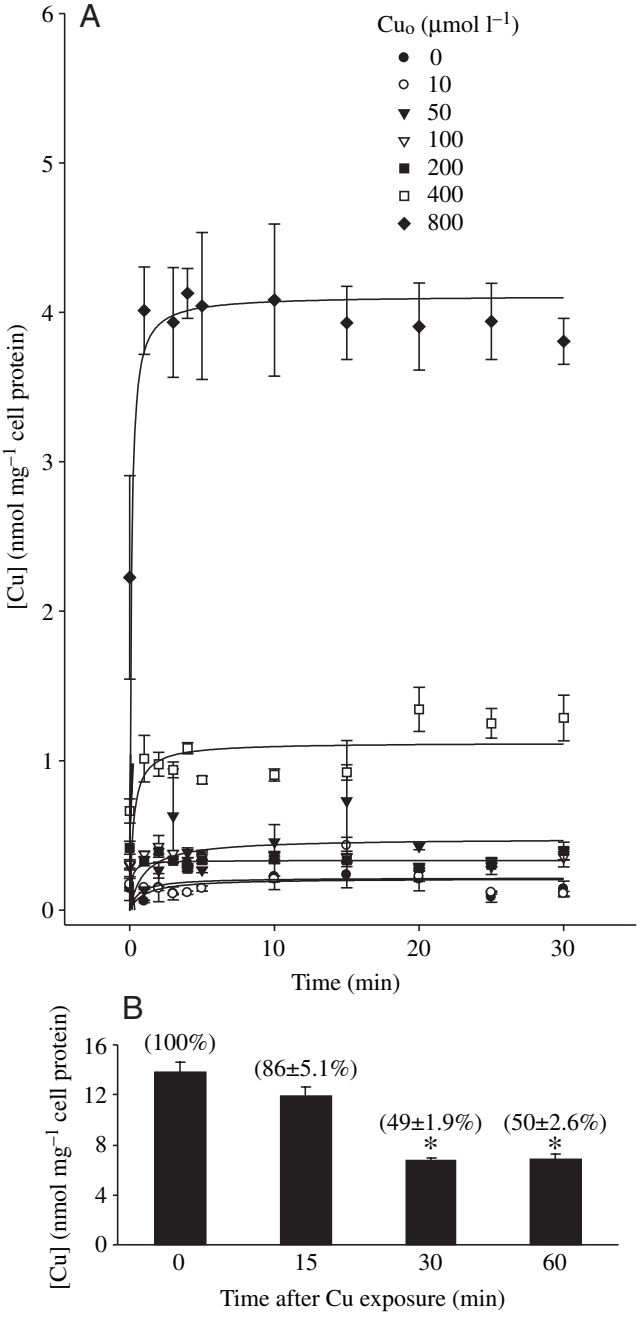

Fig. 2. Preliminary experiment to define (A) the time course of Cu accumulation by freshly isolated intestinal cells, and (B) Cu retention by Cu-loaded cells placed in normal physiological saline. (A) The cells were incubated in external Cu (Cu_o) concentrations of no added Cu (control, filled circles), 10 (open circles), 50 (filled triangles), 100 (open triangles), 200 (filled squares), 400 (open squares), or 800 µmol l^{-1} Cu (filled diamonds) for up to 30 min. Cells were then quickly washed in a 0.1 µmol l^{-1} EDTA washing solution and pelleted prior to determination of cell Cu content (expressed as nmol Cu mg^{-1} cell protein). Values are means ± S.E.M. (N=6 experiments). Rectangular hyperbola are fitted to the raw data using Sigma plot. All cells had reached a stable Cu content by 15 min of exposure time (all significantly higher than the control with no added Cu, Student's t-test, P<0.05, except for the 10 µmol l^{-1} Cu_o treatment). (B) Cells were loaded with Cu by exposing them to 800 µmol l^{-1} Cu for 15 min, and the subsequent loss of accumulated Cu was followed for 1 h by placing cells back into normal physiological saline (no added Cu). Values are means ± S.E.M. (N=9 experiments) and expressed as nmol Cu mg^{-1} cell protein. Values in parentheses are the percent decrease in Cu content (means ± S.E.M.) of the cells relative to the start of the post-exposure period where the Cu content of the Cu-loaded cells is defined as 100%. *Statistically different from time 0 post-exposure by Kruskall–Wallis test (P=0.00005). Note, cells did not leak Cu for at least the first 15 min in normal saline (no statistical difference from time 0 post-exposure control).

Table 1. *LDH release and stability of Na^+ and K^+ contents in freshly isolated trout intestinal cells*

Treatment	Time (min) 0	15	30
Control (no added Cu)			
Rate of LDH release (μmol min^{-1} ml^{-1} saline)	<0.1	<0.1	<0.1
Cell [Na^+] (μmol mg^{-1} cell protein)	11.8±1.0	12.3±2.3	13.0±4.8
Cell [K^+] (μmol mg^{-1} cell protein)	1.0±0.1	2.0±0.3	1.1±0.2
Copper (800 μmol l^{-1})			
Rate of LDH release (μmol min^{-1} ml^{-1} saline)	<0.1	<0.1	<0.1
Cell [Na^+] (μmol mg^{-1} cell protein)	15.4±2.9	14.6±1.3	15.6±1.8
Cell [K^+] (μmol mg^{-1} cell protein)	1.5±0.1	1.2±0.3	0.8±0.1

Values are means ± S.E.M., N=6 cell isolations.

Cells were incubated in physiological saline for 30 min immediately after the cell isolation protocol.

Control; cells in physiological saline without added Cu.

Copper; cells from the same isolations, but incubated in saline containing the highest Cu concentration (800 μmol l^{-1} Cu) used in experiments.

Rate of lactate dehydrogenase (LDH) release from cells to the medium remained below detection limit (<0.1 μmol ml^{-1} saline min^{-1}) throughout in all treatments.

No statistically significant effects of time (within rows) were observed (Kruskal–Wallis test, P>0.05) in any of the electrolyte data. No treatment effects were observed at any time point (Student's t-test, P>0.05) for cell Na^+ or K^+.

Cells remain viable for at least 4 h (see text).

net Cu uptake was energy/temperature-dependent. Then the Na^+_o-dependence of Cu accumulation was explored (at laboratory temperature, 19°C) using a low-Na^+_o version of the physiological saline above for the incubations, where the NaCl was replaced by 125 mmol l^{-1} choline chloride (Na^+_o=11 mmol l^{-1}).

Effect of epithelial Na^+ channel blocking agents on Cu accumulation

This series of experiments explored the effects of epithelial Na^+ channel (ENaC) blocking agents on net Cu uptake when Na^+_o was normal. Drug concentrations were selected to produce complete blockade of ENaC, and included: (i) 2 mmol l^{-1} amiloride (blocks Cu uptake in perfused catfish intestine; Handy et al., 2002), (ii) 10 μmol l^{-1} 6-chloro-3, 5-diamino-2 pyrazinecarboxamide (CDPC; Acros Chemical Co., Morris Plains, NJ, USA), which is an amiloride analogue that does not chelate Cu (Flonta et al., 1998) and alters Cu uptake in perfused catfish intestine (R. D. Handy, unpublished observations), or (iii) 100 μmol l^{-1} phenamil (Sigma Aldrich, Poole, UK), which partly inhibits Cu uptake (Grosell and Wood, 2002) and Na^+ influx (Reid et al., 2003) by trout gills. In order to eliminate theoretical Cu chelation by the drugs, 150 μl of cell suspension was incubated with 200 μl of the appropriate ENaC inhibitor in normal physiological saline for 15 min prior to the addition of Cu (at the final drug concentrations indicated above; drugs dissolved in water, except for phenamil, which was dissolved in DMSO, final concentration <1% DMSO). This also ensured that inhibitors had free access to bind to the epithelium. Cells were then briefly washed in drug-free saline (as above) and resuspended in 500 μl of physiological saline (normal NaCl throughout) containing the appropriate Cu concentration for 15 min (for the remainder of the experiment and replication as above). This series of experiments also included controls with no added Cu + drug, and no drug + DMSO, in addition to those described above.

Effect of manipulating anion transport and external pH on Cu accumulation

Copper uptake is sensitive to removal of external chloride (Cl^-_o) from the gut lumen and the serosal additions of the anion transport inhibitor 4, 4-diisothiocyanato-stilbene-2, 2′-disulfonic acid (DIDS) in perfused catfish intestine (Handy et al., 2000). We therefore conducted similar experiments with isolated trout cells. Cells were exposed to a range of Cu_o concentrations as above, but this time in low Cl^-_o conditions (NaCl replaced by sodium gluconate, Cl^-_o=6.6 mmol l^{-1}), or in the presence of 0.1 mmol l^{-1} DIDS throughout the 15 min incubation with Cu (normal NaCl).

Finally, Lee et al. (2002a) demonstrated that Cu flux through the Cu-specific pathway, Ctr1, was stimulated at pH 5.5 compared to neutral pH. We therefore repeated our first series of Cu-uptake experiments (in normal NaCl) at pH 5.5 (physiological saline acidified with a few drops of 2 mmol l^{-1} HNO_3).

Trace metal analysis

In all experiments, cell lysates were analysed by inductively coupled plasma atomic emission spectrophotometry (ICP-AES, Varian Liberty 200, Walton on Thames, UK) for total Cu and Na^+, and in some experiments for K^+, according to Handy et al. (2002). Cell metal content was normalised per mg of cell protein, following protein assays (in triplicate) on each cell suspension using a modified Lowry method (Handy and Depledge, 1999).

Statistics and calculations

In some preliminary experiments on the time course of Cu accumulation, data are presented as an absolute metal content and normalised for cell protein content (e.g. nmol Cu mg^{-1} cell protein). Similarly electrolyte contents of cells are expressed as μmol mg^{-1} cell protein. In other experiments where data are expressed as a rate of Cu accumulation, the cell Cu content was divided by incubation time to give net accumulation rates in nmol Cu mg^{-1} cell protein h^{-1}. Rapid Cu accumulation in

Table 2. *Relative contribution of rapid or instantaneous Cu accumulation in/on cells at time zero compared to net Cu accumulation over 15 min exposure to 800 μmol l^{-1} Cu*

Treatment	Copper accumulation rate (nmol mg^{-1} cell protein h^{-1})		Instantaneous Cu accumulation (%)
	Time 0 control	After 15 min	
Normal Na^+	0.24±0.14[a]	1.88±0.52*[,a]	12.8
Low Na^+	0.51±0.05[b]	3.04±0.69*[,b]	16.8
Ice	4.63±0.89[c,d]	9.53±4.78[c]	48.5
†Amiloride (2 mmol l^{-1})	3.00±0.38[c,d]	21.76±5.15*[,d]	13.8
†CDPC (10 μmol l^{-1})	6.39±2.01[c]	39.42±16.56*[,d,g,e]	16.2
†Phenamil (100 μmol l^{-1})	6.33±1.36[c]	75.39±13.47*[,e]	8.4
Low Cl^-	2.58±0.47[d]	21.56±2.44*[,d]	12.0
DIDS (100 μmol l^{-1})	2.79±0.33[d]	18.40±0.94*[,f]	15.1
pH 5.5	8.84±2.41[e]	32.65±3.70*[,g]	27.1

Values are means ± S.E.M, N=5–6 experiments.

*Significantly different from time 0 control (within rows) by Student's t-test or Mann–Whitney U-test (P<0.05); values within columns with one or more common superscript letters are not significantly different from each other (Kruskal–Wallis test, P>0.05).

Time 0 control; cells were pipetted into saline containing 800 μmol l^{-1} Cu and then immediately washed in 0.1 μmol l^{-1} EDTA solution.

After 15 min, Cu accumulation rate of cells at the end of a 15 min exposure to saline containing 800 μmol l^{-1} Cu.

% instantaneous Cu accumulation=time 0 control/after 15 min × 100%

†Cells were pre-incubated with the appropriate epithelial Na^+ channel inhibitor for 15 min prior to the addition of Cu.

'time zero' controls was also expressed as nmol Cu mg^{-1} cell protein h^{-1} to allow comparison with data on net accumulation rates over the 15 min period. The rapid component was not deducted from any of the data, except in Fig. 4B where deduction of the fast component reveals a saturable Cu component (see Results). Statistics were performed using Statgraphics 4.0 Plus software. The variance of the data was checked using Bartlett's test. Cu-dose effects within treatment, or the effects of experimental manipulations within Cu concentrations, were compared using one-way ANOVA followed by the least-squares difference (LSD) multiple-range test. Where data were non-parametric, the Kruskal–Wallis test was applied instead. The location of statistical differences from the Kruskal–Wallis test were identified from the *post-hoc* box whisker plots generated by Statgraphics. Where visual differences in overlap in box whisker plots were difficult to discern, the Mann–Whitney U-test was applied to the pairs of data points concerned to confirm the statistical difference. In some experiments where only two treatments were used, data were compared between treatments at fixed Cu concentration using either the Student's t-test or the Mann–Whitney U-test. All statistical analysis used a rejection level of P<0.05 and included Bonferroni correction where appropriate. Curve-fitting was used to describe some of the experimental data, where appropriate, and equations describing the best fits to the raw data were derived using Sigma Plot version 8.0.

Results

Time course of copper accumulation

Preliminary experiments showed that Cu content of cells (normal NaCl throughout) reached a plateau within about 10 min of exposure to Cu at the Cu_o range used here (Fig. 2A), and therefore, all the subsequent experiments were performed after a 15 min exposure, where cell Cu content was maximal at the appropriate external [Cu]. However, Cu accumulation was also detected over the first few minutes of exposure (Fig. 2A) and so additional control experiments were performed to measure rapid Cu accumulation in/onto the cells in the first minute of exposure (Fig. 3, Table 2). In these control experiments cells were resuspended in Cu solutions and then immediately washed in the 0.1 μmol l^{-1} EDTA washing solution (i.e. no incubation, <1 min in Cu solution and 1 min in centrifuge to pellet, see Fig. 3). In normal NaCl conditions, the rapid accumulation component was 0.01 nmol Cu mg^{-1} cell protein h^{-1} or less at Cu_o up to 400 μmol l^{-1}, compared to a net Cu accumulation rate of 1.17±0.18 nmol mg^{-1} cell protein h^{-1} (mean ± S.E.M., N=5) in cells exposed to 400 μmol l^{-1} Cu_o for 15 min (Fig. 4A). Thus over most of the Cu_o range used, the rapid component was 0.8% or less of the total Cu accumulation rate. However, the rapid component of Cu accumulation increased in the presence of 800 μmol l^{-1} Cu_o to 0.24±0.14 nmol Cu mg^{-1} cell protein h^{-1} (Fig. 3, normal NaCl), equivalent to 12.8% of the total Cu accumulation over 15 min (Table 2). Similarly, most other experimental treatments showed a rapid component of about 12–16% of the total Cu accumulation over 15 min at Cu_o=800 μmol l^{-1}. However, the rapid component was a higher proportion of total Cu accumulation at low pH (48%) and in the ice-cold conditions (27%), at Cu_o=800 μmol l^{-1} (Table 2). It was not possible (or the purpose of the experiments) to definitively establish how much of the rapid Cu accumulation at time zero was Cu binding to the exterior of the cells (surface adsorption) and how much was very fast influx into the cells. We therefore elected not to deduct the rapid component from

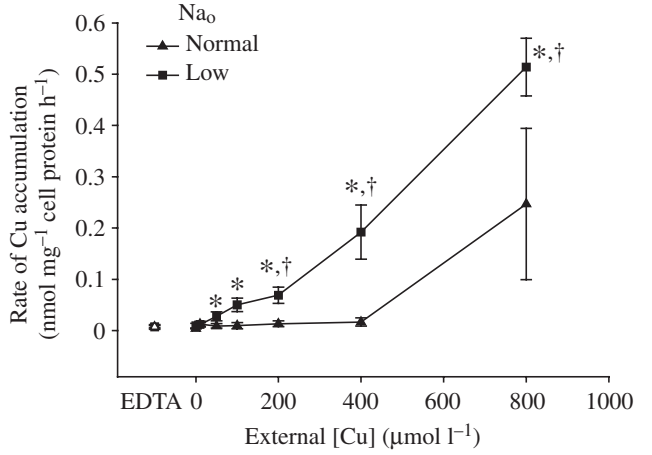

Fig. 3. Rapid or instantaneous Cu accumulation in/on intestinal cells incubated in external Cu (Cu_o) concentrations between 0 (no added Cu control) and 800 μmol l^{-1}. A Cu-free EDTA control is also included (no added Cu + 1 μmol l^{-1} EDTA, open symbol). Cells were exposed to solutions of various Cu_o and then immediately (<1 min to prepare all tubes) washed in a 0.1 μmol l^{-1} EDTA washing solution and pelleted (1 min in a bench centrifuge). These rapid measurements represent a time 0 control for the main Cu exposures, which lasted 15 min (see Figs 4–10 for 15 min exposures), and were performed mainly as an additional check to confirm that spontaneous Cu adsorption/accumulation (Handy and Eddy, 2004) was only a small component of net Cu accumulation over a 15 min period. Fig. 3 shows time 0 rapid Cu accumulation in/on cells in the presence of normal (140 mmol l^{-1}, triangles) and low (11 mmol l^{-1}, squares) external [Na^+] (Na^+_o). Values are means ± S.E.M. of N=5–6 separate experiments using fresh cells from different individual fish. *Significantly different from the control with no added Cu within treatment by ANOVA (P <0.05). †Significantly different from normal Na^+_o at the indicated Cu_o; Student's t-test (P<0.05). Accumulation is normalised mg^{-1} cell protein and h^{-1} to allow comparison between plots in Figs 3 and 4.

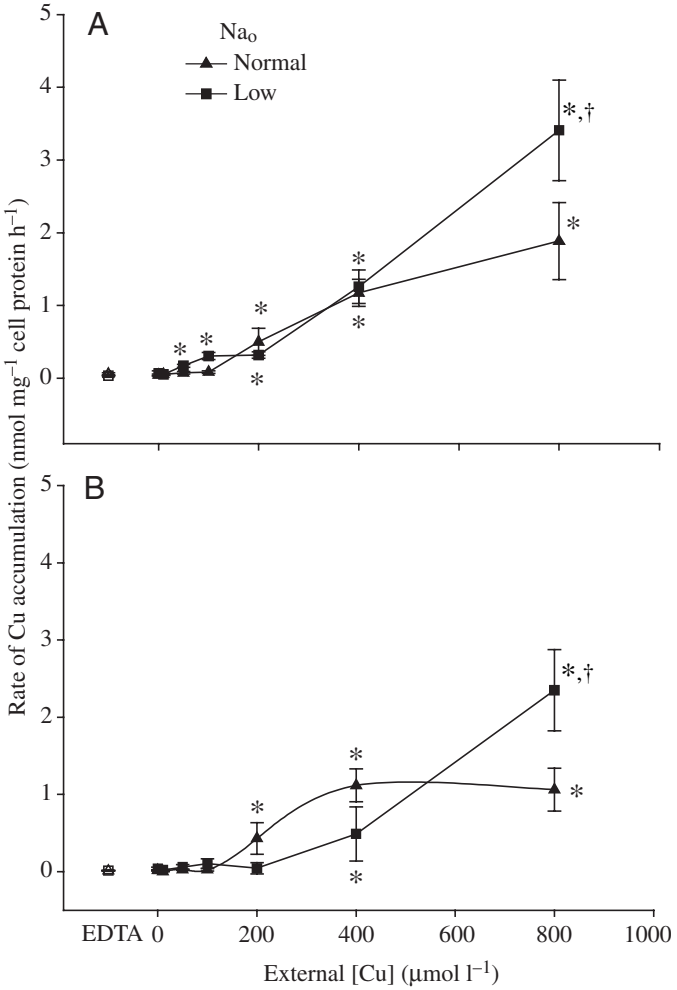

Fig. 4. The effect of external Cu (Cu_o) on Cu accumulation by intestinal cells in the presence of normal (140 mmol l^{-1}, triangles) and low (11 mmol l^{-1}, squares) external [Na^+] (Na^+_o). (A) Total Cu accumulation by intestinal cells during 15 min incubation in Cu-containing saline and (B) net Cu accumulation revealed after rapid Cu accumulation in/on cells at time 0 (Fig. 3) had been deducted from the total Cu accumulation over 15 min. Values are means ± S.E.M. of N=5–6 separate experiments using fresh cells from different individual fish. *Significantly different from the control with no added Cu within treatment; Kruskal–Wallis test (P<0.05). †Significantly different from normal Na^+_o at the indicated Cu_o; Mann–Whitney U-test (P<0.05). Accumulation is normalised mg^{-1} cell protein and h^{-1} to allow comparison between plots, and with Fig. 3.

total Cu accumulation over a 15 min period in all the experiments that followed, except in one part of the normal Na^+_o *versus* low Na^+_o experiment (Fig. 4B only), where deduction of the rapid component also reveals a slower saturable accumulation (see below).

Copper accumulation with normal Na^+_o

Fig. 4A demonstrates the net accumulation of Cu by cells during 15 min exposure to Cu_o in the presence of normal NaCl (rapid Cu accumulation at time zero is not deducted from Fig. 4A). Intestinal cells showed a curvilinear trend in net Cu accumulation for Cu_o up to 800 μmol l^{-1} (Fig. 4A), which reached a maximum Cu accumulation rate of 1.88±0.52 nmol Cu mg^{-1} cell protein h^{-1} compared to 0.05±0.01 nmol Cu mg^{-1} cell protein h^{-1} in controls with no added Cu (mean ± S.E.M., N=6 cell isolations). The Cu accumulation rate in normal NaCl conditions, although beginning to plateau at Cu_o >400 μmol l^{-1}, did not completely saturate. However, when the rapid Cu accumulation component at time zero (Fig. 3) was deducted from the net accumulation data for 15 min incubations in Cu solutions (Fig. 4A), a sigmoidal accumulation response, which saturated at about 400 μmol l^{-1} Cu_o, was revealed (Fig. 4B). The latter accumulation curve (Fig. 4B) had a half maximal constant, K_m, of about 216 μmol l^{-1} Cu_o (derived from x values at 50% of maximum y using Sigma plot) and a maximum accumulation rate of 1.09 nmol Cu mg^{-1} cell protein h^{-1} (best fit, sigmoid to mean values, y=1.0907/1+exp–(x–213.08)/30.870), r^2=0.99, N=36 cells).

Copper accumulation with low Na^+_o

Dose-dependent Cu accumulation by the cells also occurred

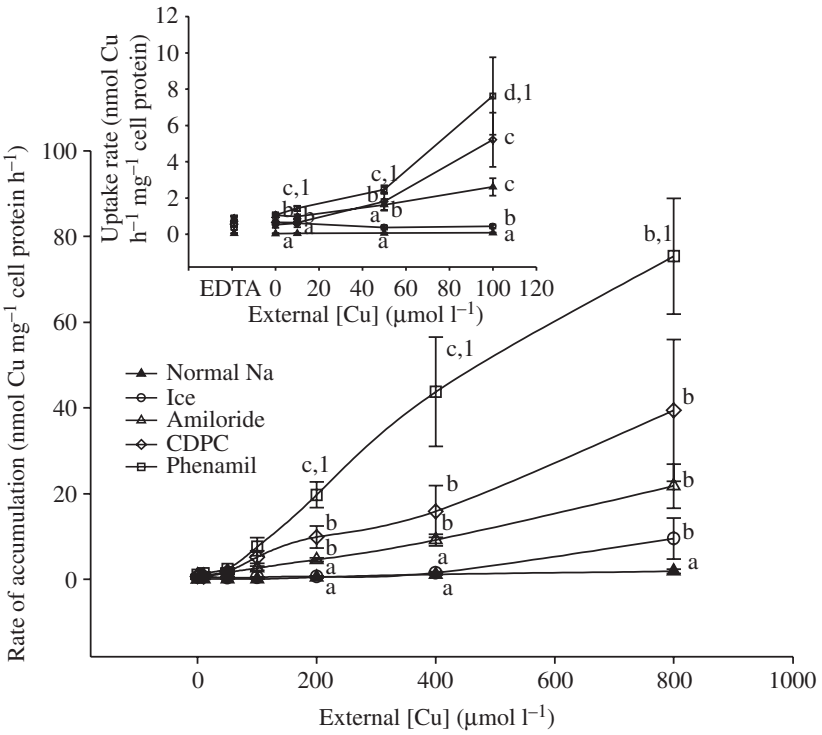

Fig. 5. The effect of pre-incubating intestinal cells with epithelial Na^+ channel (ENaC) inhibitors on rate of Cu accumulation at external [Cu] (Cu_o) ranging from 0–800 μmol l^{-1}. Cells were pre-incubated for 15 min without Cu_o (no added Cu) but with either 100 μmol l^{-1} phenamil (open squares), 10 μmol l^{-1} CDPC (open diamonds), or 2 mmol l^{-1} amiloride (open triangles), compared to controls with no added drug (normal NaCl and no inhibitors, filled triangles) and cells incubated at 4°C without inhibitors (open circles). Drugs were then washed off and cells exposed to 0–800 μmol l^{-1} Cu_o for 15 min. All experiments used normal Na^+_o (140 mmol l^{-1} NaCl) throughout. Rapid Cu accumulation in/on cells at time 0 was not deducted from the data. Values are means ± S.E.M. of N=5–6 separate experiments using fresh cells from different individual fish. Different letters (a, b, c or d) indicate a statistically significant difference between adjacent treatments within Cu_o concentration (looking up at adjacent data points between plots at each Cu_o, Kruskal–Wallis test, P<0.05). [1]Significant effect of phenamil compared to amiloride within Cu_o (Kruskal–Wallis test, P<0.05). For clarity, statistical differences between treatment effects (between plots) for 10, 50 and 100 μmol l^{-1} Cu_o, respectively, are shown on the insert with the axis expanded for the lowest Cu_o concentrations. The insert also shows the Cu-free (+1 μmol l^{-1} EDTA) control (EDTA on the axis label) compared to no added Cu (zero on the axis label). No statistical differences were observed between EDTA and controls with no added Cu (Student's t-tests between EDTA and no added Cu within drug treatment, P>0.05). Cu accumulation rates within drug treatments were all significantly different from the no added Cu control at Cu_o=50 μmol l^{-1} or greater (labels not added for clarity; Kruskal–Wallis test, P<0.05). Cu_o effect within ice-cold treatment was significantly different from no added Cu ice control at Cu_o=800 μmol l^{-1} only (Kruskal–Wallis test, P=0.0035).

when Na^+_o was lowered from 140 to 11 mmol l^{-1} (Fig. 4A, rapid Cu accumulation not deducted). Cu accumulation in low Na^+_o conditions remained linear over the Cu_o range used (linear fit, y=0.0039x, r^2=0.96), unlike the situation with normal Na^+_o. Cu accumulation by the cells was twofold higher in low Na^+_o compared to normal Na^+_o conditions (3.4±0.7 compared to 1.88±0.52 nmol Cu mg^{-1} cell protein h^{-1}, respectively, at 800 μmol l^{-1} Cu_o; significantly different, Mann–Whitney U-test, P=0.045, Fig. 4A; mean ± S.E.M., N=6). However, the rapid Cu accumulation component at time zero also increased twofold when Na^+_o was removed (Fig. 3, Table 2), and reached 0.52±0.05 and 0.24±0.14 nmol Cu mg^{-1} cell protein h^{-1} (mean ± S.E.M., N=6) in low and normal Na^+_o, respectively (significantly different from each other, student's t-test, P<0.05). When the rapid component (Fig. 3) was deducted from the total Cu accumulation over 15 min (Fig. 4A), the low Na^+_o Cu accumulation response showed an exponential rise with Cu_o (exponential fit to means, y=0.0664+1.0045^x, r^2=0.99; Fig. 4B), unlike the saturable profile in the presence of Na^+_o when the rapid Cu accumulation was deducted for net accumulation over 15 min (Fig. 4B).

Effect of epithelial Na^+ channel blocking agents on Cu accumulation

Pre-incubation of intestinal cells with epithelial Na^+ channel (ENaC) inhibitors (normal Na^+_o throughout) generally caused increased rates of Cu accumulation by the cells compared to drug-free controls at the appropriate Cu_o (Fig. 5). The greatest effect of ENaC inhibitors were observed at the highest Cu_o (800 μmol l^{-1}), with cell Cu accumulation rates over a 15 min period of 75.3±13.4, 39.4±16.5 and 21.8±5.1 nmol Cu mg^{-1} cell protein h^{-1} (mean ± S.E.M., N=5–6) for phenamil, CDPC and amiloride, respectively, compared to the drug-free control above (1.88 nmol Cu mg^{-1} cell protein h^{-1}, significantly different from control; see Fig. 5 for details of statistics). In Fig. 5, rapid Cu accumulation at time zero was not deducted from the net Cu accumulation over 15 min. However, although changes in rapid Cu accumulation occurred at time zero, these were not large enough (maximally only 6 nmol mg^{-1} cell protein h^{-1} when Cu_o=800 μmol l^{-1}; Table 2) to explain the observed increases in net Cu accumulation rate over 15 min (20 nmol mg^{-1} cell protein h^{-1} or more in the presence of ENaC inhibitors; Fig. 5 and Table 2). Thus the relative proportions of rapid Cu accumulation to total accumulation over 15 min remained similar to that observed in normal or low NaCl (Table 2) and to drug-free controls (not shown).

Interestingly, in the absence of added Cu_o, intestinal cells contained more Cu after incubation with ENaC inhibitors compared to drug-free controls (also with no added Cu_o). Rates of Cu accumulation were 0.04±0.01, 1.07±0.03, 0.66±0.12,

1.04±0.12 nmol mg^{-1} cell protein h^{-1} (mean ± S.E.M., N=6) for control with no added drug, phenamil, CDPC and amiloride, respectively, in no added Cu_o conditions (drug effect significantly different from control, Kruskal–Wallis test, P<0.05), implying that ENaC inhibitors influence background Cu accumulation even when Cu_o is only at trace nanomolar levels. LDH permeability remained below detection limits for experiments with ENaC inhibitors, including DMSO solvent controls for phenamil (not shown).

Copper accumulation at 4°C

Incubation of cells in an iced water bath caused a small increase in Cu accumulation compared to room temperature controls. The rate of copper accumulation over 15 min in ice-cold conditions was 1.5 nmol Cu mg^{-1} cell protein h^{-1} at Cu_o=400 µmol l^{-1} or less, compared to 1.2± 0.18 nmol Cu mg^{-1} cell protein h^{-1} or less in room temperature controls at the same Cu_o (mean ± S.E.M., N=6; not statistically significant, see Fig. 5). At 800 µmol l^{-1} Cu_o, cells in ice showed a sudden rise in Cu accumulation from the background level of <1.5 nmol Cu mg^{-1} cell protein h^{-1} to 9.5±4.7 nmol Cu mg^{-1} cell protein h^{-1} (significantly different; Kruskal–Wallis test, P<0.05, Fig. 5), indicating a threshold for Cu entry in the cold. However, overall the effects of ice treatment were small compared to the effects of ENaC inhibitors (Fig. 5).

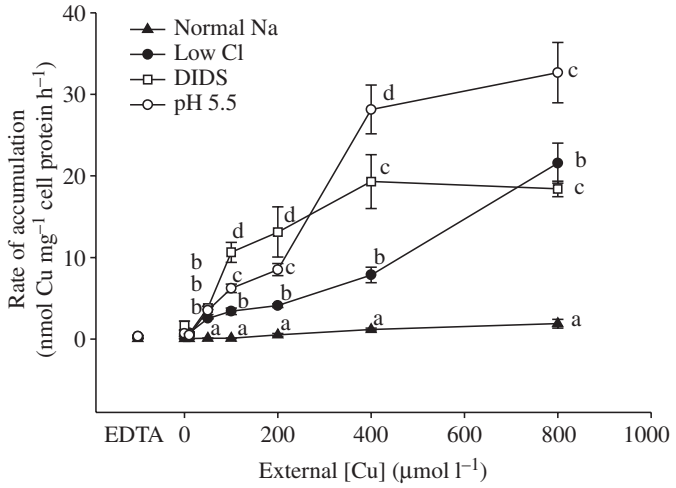

Fig. 6. Copper accumulation by intestinal cells exposed to external [Cu] (Cu_o) ranging from 0 (no added Cu) to 800 µmol l^{-1} for 15 min in normal saline (NaCl=140 mmol l^{-1} at pH 7.4, filled triangles), compared with the effects of either low external chloride (Cl^-_o=6.6 mmol l^{-1}, Cl^-_o replaced by sodium gluconate, filled circles), 0.1 mmol l^{-1} DIDS in normal saline (open squares), or with low external pH (pH 5.5, open circle). Values are means ± S.E.M. of N=5–6 separate experiments using fresh cells from different individual fish. Rapid Cu accumulation in/on cells at time zero was not deducted from the data. Different letters (a, b, c or d) indicate a statistically significant difference between adjacent treatments within Cu_o (looking up at adjacent data points between plots at each Cu_o, Kruskal–Wallis test, P<0.05). For clarity, statistical differences between treatment effects at 10 µmol l^{-1} Cu_o are not shown, but are identical to those at 50 µmol l^{-1} Cu_o. Cu accumulation within treatment (Cu-dose effects within each experiment) were significantly different from the control with no added Cu at Cu_o=50 µmol l^{-1} or greater for the low Cl^-_o and low pH experiments; and at 10 µmol l^{-1} Cu_o or greater for the DIDS experiment (labels not added for clarity, Kruskal–Wallis test, P<0.05). The effect of DIDS reached a plateau at 200 µmol l^{-1} Cu_o because Cu accumulation rates between 200–800 µmol l^{-1} Cu_o were not statistically different from each other within DIDS treatment (from *post-hoc* box whisker plots following Kruskal–Wallis test). Similarly, the low pH effect reached a plateau at 400 µmol l^{-1} Cu_o because Cu accumulation rates between 400–800 µmol l^{-1} Cu_o were not statistically different from each other within the low pH treatment (from *post-hoc* box whisker plots following Kruskal–Wallis test). The above statistics for Cu-dose effect within treatment are not shown for clarity.

Effect of manipulating anion transport and external pH on Cu accumulation

Lowering external [chloride] (Cl^-_o) from 131.6 to

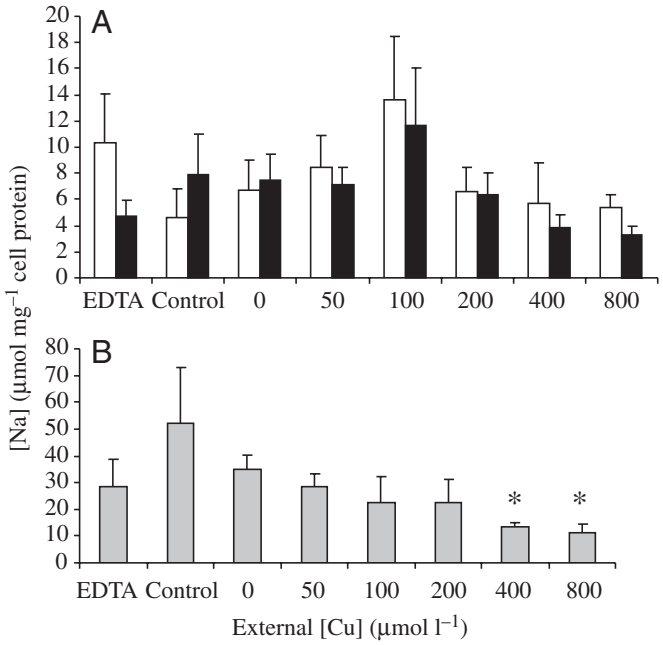

Fig. 7. (A) The effect of external Cu (Cu_o) on the Na^+ content of intestinal cells in the presence of normal (140 mmol l^{-1}, open bars) and low (11 mmol l^{-1}, black bars) external Na^+ (Na^+_o) after a 15 min incubation in Cu-containing saline at room temperature. (B) Na^+ content of intestinal cells incubated at the same Cu_o range but at 4°C and with normal Na^+_o throughout. Values are means ± S.E.M. of N=5–6 separate experiments using fresh cells from different individual fish. *Significantly different from control with no added Cu within treatment by Kruskal–Wallis test (P <0.05). No differences were observed in cell Na^+ content between low and normal Na^+_o within each Cu_o at room temperature (Student's t-tests, P>0.05). Note the larger y-axis scale in B, all values at 4°C were significantly different from the respective room temperature control within Cu_o treatment (comparison of normal NaCl in A with B, Student's t-tests, P>0.05; data not labelled for clarity). There were no differences between Cu_o-free (EDTA, no added Cu but + 1 µmol l^{-1} EDTA) and controls with no added Cu within treatments (Student's t-test, P>0.05).

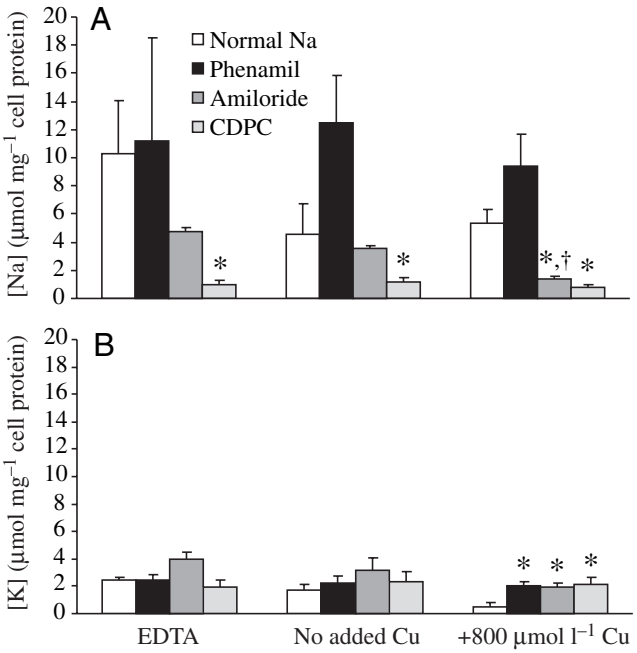

Fig. 8. The effect of pre-incubation of intestinal cells with epithelial Na^+ channel inhibitors on (A) cell Na^+ content or (B) cell K^+ content in Cu-free conditions (+ 1 μmol l^{-1} EDTA), with no added Cu, or after a 15 min incubation in 800 μmol l^{-1} Cu_o in the presence of either 100 μmol l^{-1} phenamil (black bars), 2 mmol l^{-1} amiloride (light grey bars), or 10 μmol l^{-1} CDPC (cross-hatched bars), compared to no added drug controls (white bars). All experiments were at normal Na^+_o and room temperature throughout. Values are means ± S.E.M. of N=5–6 separate experiments using fresh cells from different individual fish. *Significant difference from control without drug within Cu_o treatment (Kruskal–Wallis test, P<0.05). †Significant difference from the control with no added Cu within drug treatment (Mann–Whitney U-test, P<0.05).

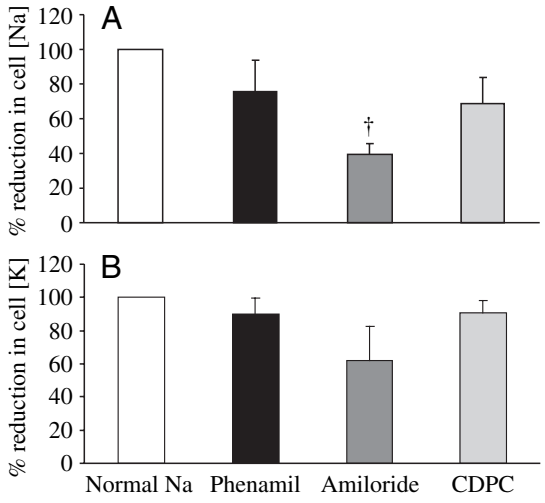

Fig. 9. Relative reduction in (A) cell Na^+ and (B) cell K^+ content in 800 μmol l^{-1} Cu_o, normalised, against the Na^+ content of cells treated with the appropriate drug with no added Cu. 100 μmol l^{-1} phenamil (black bars), 2 mmol l^{-1} amiloride (grey bars), or 10 μmol l^{-1} CDPC (hatched bars), compared to drug containing controls without Cu (white bars, normalised to 100%). Values are means ± S.E.M. of N=5–6 separate experiments using fresh cells from different individual fish. †Significant modulatory effect of Cu_o on the ability of the drug to reduce cell electrolyte content (Kruskal–Wallis test, P=<0.05). Amiloride is relatively more effective than other ENaC inhibitors at reducing cell Na^+ content in the presence of Cu_o. No Cu_o-dependent modulation of drug effects on cell K^+ occurred.

6.6 mmol l^{-1} by replacing NaCl with sodium gluconate caused Cu accumulation rates to increase progressively in intestinal cells with increasing Cu_o (Fig. 6). At the highest Cu_o, the rate of Cu accumulation in low Cl^-_o was 11-fold higher (significantly different, Kruskal–Wallis test, $P=5\times10^{-6}$) than in normal NaCl conditions (21.6±2.4 in low Cl^-_o compared to 1.88 nmol Cu mg^{-1} cell protein h^{-1} in normal NaCl, means ± S.E.M., N=6; see above). Similar observations were made on application of 0.1 mmol l^{-1} DIDS instead of Cl^-_o removal, except the DIDS effect reached a plateau at Cu_o=200 μmol l^{-1} or more. However, lowering external pH from 7.4 to pH 5.5 produced the greatest increases in Cu accumulation rate compared to Cl^-_o-removal or addition of DIDS (Fig. 6). At low pH, the Cu accumulation rate was 17-fold above the control when Cu_o=800 μmol l^{-1} (reaching 32.6±3.7 nmol Cu mg^{-1} cell protein h^{-1} at low pH, mean ± S.E.M., N=5). Cu accumulation rate at low pH also reached a plateau above 400 μmol l^{-1} Cu_o. The accumulation responses above cannot be explained by increased rapid Cu accumulation by the cells at time zero (not deducted from Fig. 6), which at the highest Cu_o were only 12%, 15% and 27% of the total accumulation for low Cl^-_o, DIDS and low pH treatments, respectively (Table 2).

Effect of Cu_o on cell Na^+ content with normal or low Na^+_o

Additions of Cu_o had no statistically significant Cu-dose-dependent effect on cell Na^+ content when Na^+_o was normal or low (Fig. 7A), nor did a decrease of Na^+_o from 140 to 11 mmol l^{-1}, regardless of Cu_o treatment, alter cell Na^+ content (Fig. 7A). However, a Cu_o-dependent depletion of cell Na^+ was revealed when cells were chilled to 4°C in the presence of normal NaCl (Fig. 7B). In the cold, cells showed about a fivefold increase in cell Na^+ content compared to cells at room temperature (statistically significant at all Cu_o tested; student's t-test, P<0.05). Cell Na^+ content also declined at Cu_o of 400 μmol l^{-1} and or more compared to no added Cu controls at 4°C, indicating a net Na^+ loss from cells at high Cu_o in the cold.

Effect of epithelial Na^+ channel blocking agents on cell Na^+ and K^+ content

Pre-incubation of intestinal cells with ENaC inhibitors followed by Cu_o exposure caused statistically significant depletions of cell Na^+ content compared to drug-free controls in the presence of Cu_o (Fig. 8A), with amiloride and CDPC reducing cell Na^+ content by ninefold or more. This response was slightly different in the absence of added Cu_o (with or without EDTA), so that only amiloride produced a

statistically significant reduction in cell Na^+ content (Fig. 8A). However, Cu_o modulated the effectiveness of ENaC inhibitors at reducing cell Na^+ content, with all drugs being more effective with increasing Cu_o (e.g. with amiloride, cell Na^+ showed an exponential decrease with increasing Cu_o). The biggest reductions in cell Na^+ content were observed at 800 μmol l^{-1} Cu_o, and of the ENaC blocking agents used, the effect of amiloride was enhanced the most by the presence of Cu compared to drug-containing controls without Cu (Fig. 9A).

Pre-incubation of intestinal cells with ENaC inhibitors followed by Cu_o exposure caused statistically significant increases in cell K^+ content compared to controls in normal saline (drug-free) at the same Cu_o (Fig. 8B). No effect of ENaC inhibitors on cell K^+ content was observed in the absence of Cu (Fig. 8B, Kruskal–Wallis test, $P>0.05$), and there was no Cu_o-dependent modulation on cell K^+ content within drug treatments (Fig. 9B).

Effect of manipulating anion transport and external pH on cell Na^+ and K^+ content during Cu_o exposure

Exposure to Cu_o had no dose-dependent effect on either cell K^+ or Na^+ content in low Cl^-_o conditions (Fig. 10A, Kruskal–Wallis for Na^+, $P=0.058$; for K^+, $P=0.59$), except for an increase in cell [Na^+] at 50 μmol l^{-1} Cu_o and a decrease in cell [Na^+] at 800 μmol l^{-1}, compared to the control with no added Cu (Fig. 10A). Removal of Cl^-_o caused cell Na^+ content to generally decline compared to controls in normal NaCl, regardless of Cu exposure, but the differences were not statistically significant (Kruskal–Wallis test, $P>0.05$, compare Fig. 10A with normal Na^+ in Fig. 7A).

Treatment with 0.1 mmol l^{-1} DIDS caused a clear Cu_o-dose-dependent decline in both cell [Na^+] and [K^+] (Fig. 10B; Kruskal–Wallis test for Na^+, $P=1.8\times10^{-9}$; for K^+, $P=3.5\times10^{-6}$), with electrolyte levels decreasing at Cu_o=10 μmol l^{-1} or more. Incubation of cells with DIDS in the absence of Cu_o also increased cell Na^+ and K^+ content compared to either the low Cl^-_o or normal NaCl treatments without Cu_o (Kruskal–Wallis test, $P<0.05$), suggesting effects of DIDS on cell electrolytes regardless of Cu_o exposure.

Reducing external pH from 7.4 to 5.5 caused no Cu_o-dependent effect on cell K^+ content (Kruskal–Wallis test, $P=0.166$), but did cause a Cu_o-dependent fall in cell [Na^+] (Fig. 10C; Kruskal–Wallis test, $P=0.0038$). The latter effect occurred at Cu_o=200 μmol l^{-1} or more compared to the control with no added Cu. Acidification of the medium also tended to increase cell Na^+ compared to normal pH in the absence of added Cu_o (compare controls in Fig. 10C with Fig. 7A), but these were not statistically different (Kruskal–Wallis test, $P>0.05$).

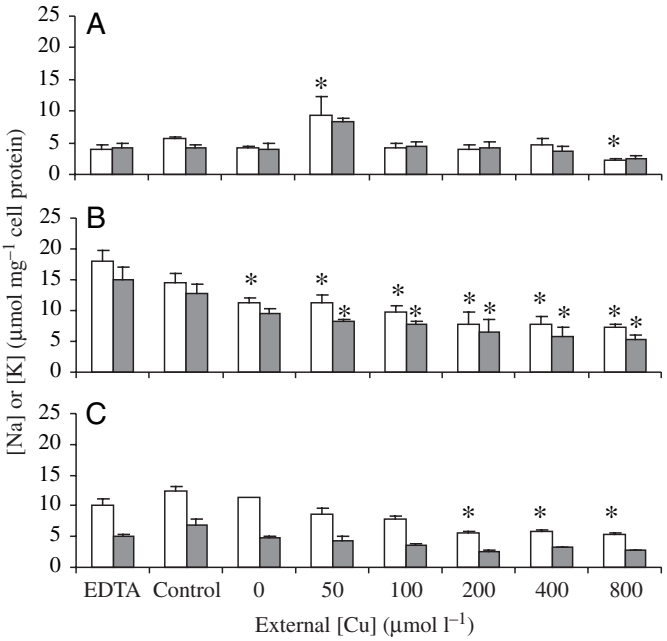

Fig. 10. Na^+ (white bars) and K^+ content (grey bars) content of intestinal cells exposed external [Cu] (Cu_o) ranging from 0 (no added Cu) to 800 μmol l^{-1} for 15 min (A) in low external chloride (Cl^-_o=6.6 mmol l^{-1}, Cl^-_o replaced by sodium gluconate), (B) with 0.1 mmol l^{-1} DIDS in normal saline, and (C) at low external pH (pH 5.5). Values are means ± S.E.M. of N=5–6 separate experiments using fresh cells from different individual fish. *Statistically significant difference from control with no added Cu within treatment and electrolyte (Kruskal–Wallis test, $P<0.05$).

Discussion

Viability and stability of freshly isolated intestinal cells

The viability of freshly isolated intestinal cells used in our experiments was high, with a mean viability of 94% from 54 separate cell isolations, and cells remained viable for several hours with or without added Cu (Fig. 1). Thus cells can be kept in suspension for much longer than the 15 min required for experiments with Cu here. Freshly isolated cells also showed dose-dependent increases in cell Cu content (Fig. 2A) without loss of major electrolytes over a 30 min period (Table 1). Measurements of Na^+ and K^+ content in isolated gut mucosa of freshwater fish are rare (Albus et al., 1979; Handy et al., 2000, 2002), and our values for isolated cells (Table 2) are broadly comparable to these reports, assuming a cell protein content of 100 mg g^{-1} mucosal tissue in the present study. The absence of LDH leak into the medium (Table 1) showed that cell membrane permeability was not compromised by exposure to micromolar concentrations of Cu in physiological saline over the time course of the experiments. Interestingly, freshly suspended Caco-2 cells (pre-confluent cells) also do not leak LDH in the presence of Cu, unlike mature confluent Caco-2 cultures (Zodl et al., 2003). In this study, trout intestinal cells were metabolically active because isolated cells showed responsiveness to ENaC inhibitors (Fig. 8), and the elevation of cell Na^+ content in ice-cold conditions (Fig. 7) is diagnostic of metabolic depression of Na^+ pump activity (Skou, 1983). Together, these observations suggest the freshly isolated cells are both viable and physiologically intact for studies on Cu accumulation.

Copper accumulation with normal Na^+_o

Copper accumulation by isolated intestinal cells in normal physiological saline was Cu_o-dose dependent, with saturable and non-saturable components (Fig. 4), and we argue that this is indicative of at least two Cu accumulation processes including a slower carrier-mediated Cu accumulation (saturable), and a faster (non-saturable) diffusive process. Saturable Cu accumulation has been demonstrated in cultured human intestinal cells (Caco-2 cells; Arredondo et al., 2000), mouse embryo cells (Lee et al., 2002b), jejunum segments of rat intestine (Linder, 1991), across perfused catfish intestine (Handy et al., 2000); and is suggested in trout gut *in vivo* (Clearwater et al., 2000). The Cu accumulation response here showed a curvilinear rise (Fig. 4A), and when the rapid Cu accumulation component (Fig. 3) was deducted, a saturable accumulation profile was revealed (Fig. 4B). This is very similar to rat intestine, where deduction of a linear component (assumed to be diffusion by Linder, 1991), revealed a saturable Cu-uptake curve (Linder, 1991). The saturable component in this study (Fig. 4B) probably involves a carrier-mediated process (as in mammals; Linder, 1991; Arredondo et al., 2000) and cannot be explained by net Cu accumulation being a passive equilibrium between diffuse influx and diffusive efflux. Electrochemical theory predicts that passive diffusive efflux of Cu is impossible in our experimental conditions (e.g. if intracellular free [Cu^{2+}] is 10 nmol l^{-1} or less, when Cu_o=10 μmol l^{-1}, the equilibrium potential = +90 V or more, membrane potential –70 mV). This is further supported by the fact that exposure of intestines to micromolar concentrations of Cu has no effect on transepithelial potential (Handy et al., 2000), and trout intestinal mucosa shows no decrease in newly acquired Cu content 4 h after exposure (Clearwater et al., 2000). Arredondo et al. (2000) argues the rapid component is fast Cu uptake into the cells (see below) and the slow saturable Cu accumulation component involves Cu storage by active uptake from the cytoplasm into the Golgi network and/or cytosolic Cu buffering. The observations on trout in the present work are also consistent with this idea.

There may also be some species differences in Cu accumulation rates between fish and mammalian intestinal cells. Cu accumulation rates measured in the present work were around 0.05–3.4 nmol mg^{-1} cell protein h^{-1} (Fig. 4A), which is higher than those measured in adherent Caco-2 cells (20–90 pmol mg^{-1} cell protein h^{-1}, Arredondo et al., 2000) or isolated segments of rat intestine (72 pmol mg^{-1} cell protein h^{-1}, Linder 1991), but are similar to rates measured in isolated fish intestine (assuming 100 mg protein g^{-1} gut tissue, 8–27 nmol mg^{-1} cell protein h^{-1}; Handy et al., 2000). The apparent K_m was also higher in the present study (216 μmol l^{-1} Cu_o; Fig. 4B) compared to Caco-2 cells (0.3 μmol l^{-1}; Arredondo et al., 2000) or rat intestine (5 μmol l^{-1}; Linder 1991). These differences may partly reflect species differences in Cu homeostasis, where trout regulate plasma [Cu] to levels about tenfold higher than rats (compare Clearwater et al., 2000; Linder, 1991).

Is the rapid Cu accumulation component fast uptake into the cells or surface adsorption of Cu?

Some of the 'time zero' control experiments in this study (Fig. 3, Table 2) revealed a rapid component of Cu accumulation that occurred in less than 1 min. This component was probably very rapid Cu uptake into the cells, rather than just surface binding (adsorption) of Cu on to the outside of the cells (Handy et al., 2002). The characteristics of the rapid component include sensitivity to ENaC inhibitors, DIDS and ice-cold chilling (Table 2), and are best explained by rapid changes in the rate of Cu transport rather than adsorption chemistry. Cells were also washed several times in 0.1 μmol l^{-1} EDTA, and this would chelate/wash off some of the surface-bound Cu^{2+}. Furthermore, the Cu content of cells in Cu-free conditions (no added Cu + 1 μmol l^{-1} EDTA) were the same as in conditions of no added Cu (saline without EDTA) in all experiments (Figs 4–6), suggesting that surface-bound Cu was a small component relative to the total background Cu content of the cells (albeit after washing the cells in EDTA). Although we cannot completely exclude some instantaneous surface binding of Cu^{2+} to the cells in our experiments, it is probably fortuitous that the rapid Cu accumulation component in normal saline (12% of the total, Table 2) is similar to that for apparent Cu binding to trout gills at the tissue Cu levels found here (MacRae et al., 1999).

Does mucosal Cu entry control Cu accumulation by intestinal cells?

In our experimental conditions, passive diffusion of Cu from the cells into the external medium is thermodynamically impossible, and intestinal cells are not known to secrete Cu across the mucosal membrane into the gut lumen by active transport (Linder, 1991; Arredondo et al., 2000). Therefore, Cu accumulation by intestinal cells must be controlled by passive Cu influx, and active Cu efflux across the serosal membrane (Arredondo et al., 2000). In cell suspensions there is a thermodynamic possibility of passive Cu influx across the serosal membrane into the cells. However this is unlikely, because the putative ion channels involved in passive Cu entry into the cells (e.g. ENaC, Ctr1, see below) are mainly localised on the mucosal membrane (Staub et al., 1992; Bauerly et al., 2004). Thus there is no obvious pathway for passive Cu leak into intestinal cells across the serosal membrane. Indeed, the absence of such a pathway partly contributes to Cu overload diseases because excess Cu cannot be excreted *via* the intestine to the gut lumen (e.g. Wilson's disease; Menkes, 1999). Copper accumulation in our cells is therefore the sum of Cu influx across the mucosal membrane and active efflux across the serosal membrane.

We believe that Cu accumulation by isolated trout cells is mainly controlled by influx across the mucosal membrane into the cell, as in perfused catfish intestine (Handy et al., 2000), and in mammalian gut cells (Arredondo et al., 2000). If active Cu efflux controlled cell Cu accumulation, then lowering metabolism by chilling should slow active efflux and cause a

large increase in cell Cu content. This was not observed (Fig. 5). Chilling cells in an iced water bath (Fig. 5) had no effect on cell Cu accumulation, except for a small increase in Cu accumulation at the highest Cu_o compared to cells at room temperature. Furthermore Cu-loaded cells that are placed in normal saline should show rapid Cu depletion if efflux controls cell Cu content, but this was not observed over the 15 min period in the present work (Fig. 2B), or in mammalian intestinal cells (Zerounian et al., 2003). Zerounian and Linder (2002) also argue that Cu is tightly bound to intracellular chaperones and cannot be simply dialysed from ligands to facilitate passive efflux. Instead, we suggest that passive Cu entry into the cell down the electrochemical gradient is more important in controlling cell Cu content. Arredondo et al. (2000) made similar observations in Caco-2 cells using ^{64}Cu, where 70% or more of the cell Cu content was controlled by flux across the mucosal membrane in cells equilibrated with 1 mmol l^{-1} Cu or less. Copper accumulation by mouse embryo cells also continued in ice-cold conditions (passive influx conditions), as measured by net influx of ^{64}Cu (Lee et al., 2002b).

Is Na^+_o-sensitive Cu accumulation through epithelial Na^+ channels?

The main candidate pathways for Cu entry into intestinal cells across the mucosal (apical) membrane are incidental Cu entry through ENaCs or Cu influx through the Cu-specific pathway encoded by Ctr1 (for a review, see Handy et al., 2002). The available evidence suggests that diffusive Cu entry through ENaCs is unlikely at the Na^+_o concentrations found in the intestine (+100 mmol l^{-1} NaCl, Handy et al., 2002) or frog skin (short circuit conditions; Flonta et al., 1998), although Cu entry into epithelial cells through ENaCs is more likely at much lower Na^+_o (around 1 mmol l^{-1}, e.g. freshwater fish gills, Grosell and Wood, 2002; frogs in freshwater, Handy et al., 2002). In the present study our data support the notion of Cu accumulation by a pathway(s) that is characteristic of Ctr1, with some Cu entry through ENaCs only when Cu_o is high (800 μmol l^{-1}) in combination with low Na^+_o.

Cu uptake in competition with Na^+ through ENaCs requires increased Cu accumulation by the cell on removal of Na^+_o, and at least slowed Cu accumulation in the presence of ENaC inhibitors. This is not the case in isolated intestinal cells (Figs 4 and 5). Lowering Na^+_o to 11 mmol l^{-1} generally did not increase Cu accumulation rates (Fig. 4A), and even after deduction of the rapid Cu-component (Fig. 3), a non-saturable Cu_o-dependent and mainly Na^+_o-insensitive curvilinear (diffusive) component remained. The latter Na^+_o-insensitive component to accumulation was not significantly different from Cu accumulation rates in normal NaCl over most of the Cu_o range (Fig. 4B). Removal of Na^+_o also slows Cu uptake in intact intestine (fish, Handy et al., 2002; rats, Wapnir and Stiel, 1987), an observation that also excludes Cu uptake through ENaCs. However, reducing Na^+_o does stimulate Cu accumulation when Cu_o is also high (800 μmol l^{-1} Cu_o; Fig. 4A), implying some Cu entry through EnaCs, but only when Cu_o is above normal (nutritional range equates to 200–400 μmol l^{-1} Cu_o, Watanabe et al., 1997; Clearwater et al., 2002) and in combination with abnormally low Na^+_o (non-physiological).

The rapid Cu accumulation component was Na^+_o-dependent (Fig. 3), and this is consistent with the similar charge densities and ionic mobilities of hydrated Cu^{2+} and Na^+ ions that ensure close competition of these ions for binding on cell membranes (Handy et al., 2002). The possibility that the instantaneous component also includes some rapid Cu uptake through ENaCs is unlikely, since Flonta et al. (1988) showed that Cu binds externally on the channel rather than going through it. However, we cannot exclude rapid Cu uptake through Cu specific channels (Ctr1, see below).

Does Cu accumulation fit the characteristics of Ctr1?

Our data show that Cu accumulation by trout cells over most of the Cu_o range used here has several of the key characteristics of Ctr1; including Na_o^+-independence and stimulation by low pH. This supports the notion of Cu uptake through Ctr1 rather than ENaC when Cu_o is 400 μmol l^{-1} or less. In mammals, Cu flux through the pathway encoded by Ctr1 is not Na^+_o-dependent (Lee et al., 2001; Lee et al., 2002a). Ctr1 is not affected by the removal of Na^+_o or elevation of intracellular Na^+ *via* ouabain addition (human Ctr1 in transfected Hek293 cells, Lee et al., 2002a). In the present study, Cu accumulation is also independent of Na^+_o over most of the Cu_o range used (except at 800 μmol l^{-1} Cu_o, Fig. 4B). The low Na^+_o used here (11 mmol l^{-1}, not complete Na^+_o-removal) did not alter cell Na^+ content (Fig. 7A). Even the ice treatment, which caused large increases in cell Na^+ content (Fig. 7B), did not alter Cu accumulation rates, except for a small increase in Cu accumulation rate at 800 μmol l^{-1} Cu_o (Fig. 5).

Cu flux through Ctr1 is increased by the depolarising effects of high K^+_o, and is strongly increased by acidification of the external medium (Lee et al., 2002a). Trout intestinal cells show both of these properties. The incidental elevation of cell K^+ associated with ENaC inhibitors in the presence of Cu_o (Fig. 8B) would aid depolarisation and Cu entry through Ctr1. Importantly, acidification of the external medium to pH 5.5 enhanced Cu accumulation up to 17-fold over that at normal pH (Fig. 6). This effect occurred without significant changes in cell Na^+ or K^+ below Cu_o=200 μmol l^{-1} (Fig. 10C), and is characteristic of Ctr1 (Lee et al., 2002a).

At Cu_o >200 μmol l^{-1}, there is a big increase in pH-dependent Cu accumulation (Fig. 6) that coincides with a reduction in cell Na^+ content (Fig. 10C). A fall in cell Na^+ content in conditions of low pH is not evidence against Ctr1, but reveals some combined effects of low pH and Cu exposure on Na^+ transporters. The decrease in cell Na^+ content at low external pH in the presence of high Cu_o can be explained by: (i) reduced competitive binding of external Na^+ to the cell in the presence of external Cu^{2+} with H^+ (Handy and Eddy, 1991; Handy et al., 2002), and (ii) reversal of the Na^+/H^+ exchanger during external acidification (Ellis and Macleod, 1985), which is stimulated by Cu_o (Kramhøft et al., 1988). Cu-dependent

inhibition of Na^+K^+-ATPase (Li et al., 1996) would normally raise intracellular [Na^+], but may not apply to uncoupled Na^+K^+-ATPase in acid conditions (Skou, 1983), suggesting the Na^+/H^+ exchanger is more critical for controlling cell Na^+ content in conditions of high Cu_o and low external pH.

Alternative hypotheses to pH_o-dependent Cu accumulation *via* Ctr1, such as Cu uptake on other pH-sensitive divalent ion transporters, or increased Cu entry into the cell as an artefact of Cu speciation at low pH, are excluded. At the low pH used here, free Cu^{2+} in the medium will only increase by 35% (Sylva, 1976), and this is insufficient to explain the 17-fold increase in Cu accumulation observed (Fig. 6). Divalent metal ion transporter 1 (DMT1) is stimulated by low external pH and will transport Cu in oocytes expressing DMT1 (Gunshin et al., 1997). However, iron-deficient trout do not suffer Cu-overload and show less than a twofold increase in intestinal Cu content during 8 weeks of iron deficiency (Carriquiriborde et al., 2004). This suggests that Cu leak through DMT1 is not a major factor in Cu accumulation by trout intestinal cells. Lee et al. (2002b) present a similar argument for mouse embryo cells, and DMT1-deleted mutant mice do not show Cu deficiency (Conrad et al., 2000).

Why do ENaC inhibitors increase Cu accumulation in isolated cells?

Our data (Fig. 4A) suggest that Cu accumulation is mainly Na^+_o-insensitive at the millimolar Na^+_o range used here, and is consistent with previous reports on other epithelia at millimolar Na^+_o (Handy et al., 2002). This also suggests that ENaC inhibitors are unlikely to slow Cu accumulation, and this notion is consistent with observations here on isolated cells, and previous reports on gill and intestine. For example in trout gills, when Na^+_o is below 200 μmol l^{-1}, Cu uptake is inhibited by apical additions of 100 μmol l^{-1} phenamil (Grosell and Wood, 2002), an ENaC inhibitor (Garvin et al., 1985; Reid et al., 2003), or 2 μmol l^{-1} bafilomycin, and can be explained by Cu entry through a Na^+ channel coupled to H^+-ATPase (Grosell and Wood, 2002). However, Grosell and Wood (2002) also identified a Na^+-insensitive Cu uptake pathway (possibly Ctr1), which dominates when Na^+_o is >200 μmol l^{-1}. In gut tissue where Na^+_o is much higher than in the gill, we also see a Na^+_o-insensitive component (59% of Cu accumulation rate when Cu_o=400 μmol l^{-1}; Fig. 4B), which is similar to that in catfish intestine (59%; Handy et al., 2002). The Na^+_o-insensitive component also dominates Cu uptake in rat intestine (85%; Wapnir, 1991). It would therefore seem unlikely that ENaC inhibitors would decrease Cu uptake very much in intestine (<20% in rat intestine, Wapnir, 1991; <50% in catfish intestine; Handy et al., 2002).

The known pharmacology of ENaC inhibitors in the presence of Cu_o also excludes Cu entry through ENaCs, whilst still allowing cell Na^+ depletion due to drug-dependent blockade of Na^+ uptake. Our data are consistent with this pharmacology, but also suggest that ENaC inhibitors may create the conditions (depolarisation, altered pH gradient across the cell membrane) that stimulate Cu uptake *via* a Ctr1-like pathway. In the present study, there was a large increase in Cu accumulation rate following incubation with ENaC inhibitors (Fig. 5), which was not consistent with blockade of Cu entry in to the cell through ENaCs by ENaC inhibitors. Flonta et al. (1998) demonstrated atypical pharmacology of amiloride in the presence of Cu_o, which resulted in 20% closure of ENaC. If the channel is closed, then it cannot be the pathway for Cu entry into the cell in the presence of inhibitors. The ENaC inhibitors used here were active because Cu_o-dependent reductions in cell Na^+ content were observed (Fig. 8). Furthermore, the additional amiloride effect on cell Na^+ content in the presence of Cu_o (Fig. 9) was consistent with the voltage–current recording made by Flonta et al. (1998) on the frog skin ENaC, which showed that the Na^+ current was inhibited more by amiloride in the presence of Cu_o.

In this study, the effect of ENaC inhibitors (Fig. 5) are more easily explained by indirect changes of intracellular pH and/or membrane potential that would stimulate Ctr1-like Cu accumulation (Lee et al., 2002a). Amiloride, in addition to blocking ENaCs, also inhibits the Na^+/H^+ exchanger (K_i=340 μmol l^{-1} amiloride in colonic vesicles; Dudeja et al., 1994). This would partly explain the fall in cell Na^+ content in amiloride treatment during Cu_o exposure (Fig. 8), but would also cause intracellular acidification (the acidifying effects of Cu and amiloride are additive; Kramhøft et al., 1988). A fall of intracellular pH would stimulate Cu flux through Ctr1 (Lee et al., 2002a), resulting in the observed rise in cell Cu accumulation rate (Fig. 5).

In trout gills, phenamil probably blocks ENaCs that are coupled to apical H^+-ATPase (Bury and Wood, 1999; Reid et al., 2003). However, in the intestine this is unlikely as phenamil-sensitive ENaCs may not be coupled to H^+-ATPase (no blockade of apparent Na^+/H^+ activity, Dudeja et al., 1994; Abdulnour-Nakhoul et al., 1999). Alternatively, phenamil inhibits inward rectifying K^+ channels (Guia et al., 1996) and depolarises the mucosal membrane of intestinal cells (Sellin et al., 1993). These effects would cause Cu influx through a Ctr1-like pathway (Lee et al., 2002a) and cell Cu accumulation (Fig. 5), without depleting cell Na^+ (Fig. 8A). Closure of K^+ channels would also contribute to the observed rise in cell K^+ content in the presence of ENaC inhibitors combined with high Cu_o (Fig. 8B).

CDPC is electroneutral at physiological pH and has a different binding site on ENaCs to amiloride, and therefore unlike amiloride, does not compete directly with Cu for binding (Fig. 9; Flonta et al., 1998) to the extracellular loop of the alpha subunit of ENaC (Li et al., 1995). CDPC therefore behaves more like phenamil in the presence of Cu_o and does not produce Cu-dependent depletion of cell Na^+ (Fig. 9). CDPC reduces short circuit current (I_{sc}) in Ussing preparations (frog skin, Flonta et al., 1998; A6 cells, Atia et al., 2002) and makes the transepithelial potential of catfish intestine slightly more positive (R. D. Handy and H. Baines, unpublished observations), so might also depolarise a Ctr1-like pathway.

How does Low Cl^-_o or DIDS treatment stimulate Cu accumulation?

In this study, reductions in Cl^-_o or additions of 0.1 mmol l^{-1} DIDS caused increases in Cu accumulation rates (Fig. 6). These effects can be partly explained by inhibition of Cu efflux across the serosal membrane, and also by the likely acidifying effects of DIDS in the presence of Cu_o that would stimulate Ctr1-like activity (Cu influx) on the mucosal membrane. The combination of reduced efflux and increased influx would thus contribute to increased intracellular Cu accumulation. In fish intestine, Cu export from intestinal cells to the blood across the serosal membrane involves a Cu–Cl^- symport and vesicular Cu trafficking of Cu-loaded vesicles (Handy et al., 2000). Both these processes are sensitive to Cl^-. Lowering Cl^-_o would deplete intracellular Cl^- and therefore inhibit Cu export on the Cu–Cl^- symporter (Handy et al., 2000). Failure of Cu-loading into vesicles by Cu-ATPase at the Golgi complex is also possible in low Cl^-_o conditions (Cl^- is an allosteric effector of vesicular Cu loading in yeast; Davis-Kaplan et al., 1998). In our experiments, the reduction of Cl^-_o in the presence of Cu_o did not deplete cell Na^+ over most of the Cu_o range used (Fig. 10A), and so the effect of low Cl^-_o on Cu accumulation rate is not an artefact of changes in Na^+ balance. DIDS is also known to inhibit anion-dependent divalent metal transport (Mg, Bijvelds et al., 1996; Cd, Endo et al., 1998), and serosal application of DIDS causes tissue Cu accumulation in the perfused catfish intestine (Handy et al., 2000). In the present study DIDS also caused Cu accumulation (Fig. 6), and this observation can be partly explained by blockade of the Cu–Cl^- symport.

DIDS also blocks a variety of other anion-transporters including Cl^- channels (Bicho et al., 1999), Cl^-/HCO_3^- exchanger (Ando, 1990) and Na^+/HCO_3^- cotransporter, but not the $Na^+K^+2Cl^-$ cotransporter family (Ramirez et al., 2000). In particular, the effect of DIDS on the Na^+/HCO_3^- cotransporter is likely to cause intracellular acidification, and indirectly promote Ctr1-like activity. DIDS blocks the electrogenic Na^+/HCO_3^- cotransporter, which is ubiquitously expressed in vertebrate intestine (Romero et al., 1997), and homologues are expressed in trout epithelia (Perry et al., 2003). In rodents, the electrogenic Na^+/HCO_3^- cotransporter is basolaterally located and protects the gut cell from acid load by importing $1Na^+$ and $2HCO_3^-$ (Praetorius et al., 2001; Virkki et al., 2002). Blockade of basolateral Na^+/HCO_3^- would cause cell Na^+ content to fall (Fig. 10), and Cu_o-dependent acidification of the cell, resulting in the observed DIDS-dependent Cu accumulation (Fig. 6). DIDS block of apical Cl^-/HCO_3^- to cause recovery of intracellular pH can be excluded in our experiments because the anion exchanger is only active during base secretion (Grosell and Jensen, 1999). The depolarising effects of low Cl^-_o or DIDS on the cell membrane (Bicho et al., 1999) might also directly contribute to stimulation of Ctr1-like activity, and therefore Cu accumulation. However, this is a less likely mechanism in the presence of both Cu_o and DIDS because cell $[K^+]$ did not rise in these conditions (Fig. 10B).

In conclusion, isolated intestinal cells from rainbow trout show saturable Cu accumulation that is mainly controlled by passive Cu entry to the cell, as in mouse embryo and Caco-2 cells. The Cu accumulation is mostly (two thirds) Na^+-insensitive and is not easily explained by Cu uptake through ENaC. Cu accumulation is more characteristic of a pH and K^+-sensitive Ctr1-like pathway, where the known indirect effects of ENaC inhibitors and external acidification are also consistent with Ctr1 activation. Increased Na^+-sensitive Cu accumulation can occur at high (non-nutritional) Cu_o levels.

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