ORIGINAL PAPER

Mechanisms of dietary Cu uptake in freshwater rainbow trout: evidence for Na-assisted Cu transport and a specific metal carrier in the intestine

Sunita Rao Nadella · Martin Grosell · Chris M. Wood

Abstract Copper (Cu) is both a vital nutrient and a potent toxicant. The objective of this study was to analyze the mechanistic nature of intestinal Cu transport in rainbow trout using radiolabeled Cu (^{64}Cu) and an in vitro gut sac technique. Reduction of mucosal NaCl levels inhibited Cu transport while increase caused stimulation; Na_2SO_4 had an identical effect, implicating Na^+ rather than the anion. These responses were unrelated to solvent drag, osmotic pressure or changes in transepithelial potential. The presence of elevated luminal Ag stimulated Cu and Na^+ uptake. Phenamil caused a partial inhibition of both Cu and Na^+ uptake while hypercapnia stimulated Na^+ and Cu transport. Cu uptake was sensitive to luminal pH and inhibited by a tenfold excess of Fe and Zn. These factors had no effect on Na^+ uptake. On the basis of these results we propose a novel Na^+-assisted mechanism of Cu uptake wherein the Na^+ gradient stimulates an increase in the H^+ concentration of the brushborder creating a suitable microenvironment for the effective transport of Cu via either DMT1 or Ctr1.

Communicated by G. Heldmaier.

S. R. Nadella (✉) · C. M. Wood
Department of Biology, McMaster University,
1280 Main Street West, Hamilton, ON L8S4K1, USA
e-mail: nadellsr@mcmaster.ca

M. Grosell · C. M. Wood
Rosenstiel School of Marine and Atmospheric Science,
University of Miami, Miami, FL 33149, USA

Introduction

In complex organisms including fish copper homeostasis is believed to be maintained by regulating uptake, distribution and excretion (Grosell et al. 1998a, b; Kamunde et al. 2001) However, fish possess two potential routes of uptake from the environment: from the water via the gills and from the diet via the digestive tract. Recent studies investigating interactions between branchial and gastrointestinal uptake of Cu (Kamunde et al. 2001, 2002a, b) provide evidence that under normal levels of Cu in the water and food, the rainbow trout sources over 80% of its Cu from food. However, while the mechanisms of waterborne Cu uptake have been the focus of most current research, mechanisms of dietary Cu uptake in fish have received little attention.

Two possible candidate proteins responsible for the apical absorption of dietary Cu in mammals have emerged: the divalent metal transporter 1 (DMT1, also known as solute carrier family-SLC11A2) and the high affinity Cu transporter 1 (Ctr1). When expressed in *Xenopus* oocytes, DMT1 was shown to be capable of transporting a range of metals including Fe, Zn, Cu, and Mn (Gunshin et al. 1997). Ctr1 has also been shown to have Cu transport activity in transfected cell lines, displaying a substrate preference for Cu^+ (Lee et al. 2002a). Results from studies that examined Ctr1 protein expression are somewhat puzzling—Kuo et al. (2006) found increased Ctr1 expression in duodenum, kidney and brain of copper-deficient mice but not in the liver. In suckling rats increased Ctr1 protein levels were observed in the intestine and liver following Cu supplementation (Bauerly et al. 2004). However, mammalian Ctr1 mRNA expression is not regulated

following dietary Cu restriction (Lee et al. 2000) or in human Caco-2 cells following exposure to high Cu conditions (unpublished data cited by Sharp 2003). Alternatively Sharp (2003) has reported that high Cu levels could modify the expression of DMT1 in Caco-2 cells and suggest that DMT1 and not Ctr1 acts as the major intestinal Cu transporter. Arredondo et al. (2003) have recently demonstrated an association between Cu and Fe transport and suggest that DMT1 preferentially transports monovalent Cu^+ relative to Cu^{2+} in cultured Caco-2 cells.

In addition there is older evidence for a Na^+-related pathway of Cu absorption in mammals. Wapnir and Stiel (1987) demonstrated an association between Cu and Na in the luminal phase of Cu absorption in the jejunal and ileal segments of the perfused rat intestine, with Na removal tending to slow Cu absorption. Further investigation of this association using an in situ perfusion procedure in the rat intestine revealed an inhibition of Cu uptake in the presence of amiloride, an inhibitor of Na^+ channels and some Na^+-linked transporters (Wapnir 1991), providing evidence for Cu entry via a Na^+-linked mechanism.

Several aspects of the mechanisms of waterborne Cu uptake at the gills in fish have now been characterized. Grosell and Wood (2002) have identified both a Na^+-sensitive and a Na^+-insensitive pathway of apical Cu uptake directly using Na^+-competition experiments and pharmacological studies. Kamunde and Wood (2003) and Pyle et al. (2003) have demonstrated in trout that increased dietary Na^+ intake resulted in reduced branchial uptake of Na^+, accompanied by a reduction in branchial Cu uptake. Together these studies provide considerable evidence for a Na^+-associated Cu transport mechanism in the fish gills. The Na^+-insensitive pathway (Grosell and Wood 2002) is believed to involve the high affinity Cu transporter (Ctr) analogous to hCtr1. An orthologue of hCtr1 has recently been cloned in zebrafish embryos (Mackenzie et al. 2004) and while its precise location is yet to be identified, the gill and intestine appear to be prime candidates.

The Menkes protein (MNK; ATP7A), which is a copper-translocating P-type ATPase, is one of the key elements of the copper homeostasis machinery in mammals (Camakaris et al. 1999). It is found in most tissues, except the liver, where a homologous copper ATPase, the Wilson protein (WND; ATP7B) is expressed. Catalytic activity studies have shown that both MNK and WND require reduced copper, Cu^+, in order to translocate it in an ATP-dependent manner, suggesting that Cu^+ is the substrate for human copper ATPases (Vascoboinik et al. 2001). Campbell et al. (1999) and Grosell et al. (2001a) provide evidence for basolateral export of Cu via a Cu ATPase in fish gills.

Despite these recent gill findings, and the knowledge that diet is the dominant route of Cu uptake, transport mechanisms for Cu in the fish gut remain unclear. In the African walking catfish, basolateral transport presumably involves a Cu ATPase and/or a Cu/anion symport (Handy et al. 2000, 2002). Removal of Na^+ from the mucosal solution tended to slow rather than accelerate Cu transport, suggesting that Na^+ channels are not involved, but pointing to the potential presence of a Na^+-linked mechanism similar to that postulated by Wapnir (1991) in the rat. Very recently, Burke and Handy (2005) have developed an isolated trout enterocyte preparation in which uptake is thought to be dominated by apical transport rates. In these dispersed cells, there was no evidence that lowering the bathing Na^+ concentration altered the rate of Cu accumulation at low Cu concentrations, but at very high Cu levels in the bathing solution (800 μM), Na^+ removal did accelerate Cu uptake.

Arising from this somewhat confusing background, our objective was to characterize Cu uptake in the isolated but intact intestine of the rainbow trout. A preceding study (Nadella et al. 2006b) has established the in vitro trout gut sac made from either the mid- or posterior intestine as a robust preparation which exhibits saturable, apparently carrier-mediated Cu-uptake over the normal concentration range of Cu in the chyme, and which maintains stable transport rates for up to 4 h. The anterior intestine is less suitable for this approach because it contains the delicate pyloric caecae. In particular, we focused on the mid- and posterior intestinal segments and employed a mucosal concentration (50 μM) typical of that measured in the chyme in vivo (Nadella et al. 2006a). Possible Na^+-sensitive Cu uptake was assessed by manipulation of Na^+ levels in the gut lumen and use of relevant pharmacological agents that are known inhibitors of Na^+ transport. P_{CO_2} elevation at constant pH was used to investigate the possible contribution of proton supply. The potential of DMT1 and Ctr1 to mediate Cu transport was investigated through examination of pH sensitivity, known to stimulate both transport proteins (Nelson 1999) and the possibility of inhibition of Cu uptake in the presence of tenfold excess of other metals. Transepithelial potential (TEP) was also measured in these experiments to determine whether any of the observed effects on Cu transport were mediated indirectly by changes in the voltage gradient.

Methods

Experimental organisms

Rainbow trout (*Oncorhynchus mykiss* weight 200–300 g) were obtained (McMaster University Animal Utilization Protocol # 02-10-61) from Humber Springs Trout Hatchery (Orangeville, ON, Canada). Fish were acclimated to the laboratory in a 500 L tank holding 35 fish and supplied with aerated, flow-through, dechlorinated Hamilton tap water from Lake Ontario [Na^+ = 0.5 mM, Cl^- = 0.7 mM, Ca^{2+} = 1.0 mM, hardness ~140 ppm as $CaCO_3$, background Cu = <16 nM (<1 µg L^{-1}), pH ~8; temperature = 12 ± 2°C]. During the 2-week acclimation period, fish were fed Martin's commercial dried pellet feed (5-point; Martin Mills Inc., Elmira, ON, Canada, containing 41.0% crude protein, 11.0% crude fat, 3.5% crude fibre, 1% Ca, 0.85% total P, 0.45% Na) daily, at a ration of 2% wet body mass per day. The Cu content of the food was 27 ± 0.01 µg g^{-1} dry wt. Prior to a sampling day, fish were not fed for 48 h to clear the gut of ingesta.

In vitro: intestinal sacs

Intestinal sacs were used to analyze the mechanism of Cu uptake, which was evaluated at a single concentration of 50 µM (3 µg mL^{-1}) ^{64}Cu as $Cu(NO_3)_2$ which was chosen on the basis of an in vivo sampling study of chyme in trout (Nadella et al. 2006a). Trout were euthanised by an overdose of MS-222 (0.25 g L^{-1}), the intestine was excised, rinsed and placed in ice-cold saline. Intestinal sacs were made from mid- and posterior intestine segments according to protocol described by Nadella et al. (2006b). In brief, the posterior end of each intestinal segment was tightly sealed by double silk ligatures, while at the anterior end a small PE-50 catheter was secured in place, allowing administration of experimental solutions. The resulting intestinal sacs were filled with 1 mL of mucosal saline containing radiolabeled ^{64}Cu (0.04 mCi mL^{-1} as $Cu(NO_3)_2$; Nuclear Research Reactor McMaster University) or ^{22}Na (0.04 µCi mL^{-1} as NaCl; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) in a modified Cortland saline [in mM: NaCl 133, KCl 5, $CaCl_2 \cdot 2H_2O$ 1, $MgSO_4 \cdot 7H_2O$ 1.9, $NaHCO_3$ 1.9, $NaH_2PO_4 \cdot H_2O$ 2.9, glucose 5.5, pH 7.4; Wolf (1963)]. The catheter was heat-sealed and the preparation blotted dry, weighed (to the nearest 0.1 mg) and immersed in a serosal bath containing 12 mL of the modified Cortland saline. During the standard 2 h flux period, the serosal saline was aerated with 99.7% O_2 and 0.3% CO_2 (i.e. P_{CO_2} = 2.25 torr) gas mixture, temperature 13–15°C.

At the end of the flux period, intestinal sacs were drained, reweighed, thoroughly rinsed (with 1 mM EDTA in saline) and the mucosal surface scraped with a glass microscope slide to remove the mucosal enterocytes. The intestinal surface area was determined using the method of Grosell and Jensen (1999). Samples of mucosal saline, intestinal tissue (muscle tissue) remaining after removal of the mucosal epithelium by scraping with a glass slide, and the serosal flux bath were counted for ^{64}Cu or ^{22}Na activity in a Canberra-Packard Minaxi Auto Gamma 5000 Series (Meriden, CT, USA) Auto Gamma counter with an on-board program for decay correction of ^{64}Cu. The concentrations of Cu and Na^+ in mucosal saline were measured by graphite furnace atomic absorption spectroscopy [GFAAS; Varian SpectrAA-220 with graphite tube atomizer (GTA-110), Mulgrave, Australia] and flame atomic absorption spectroscopy (FAAS; Varian SpectrAA-220 FS, Mulgrave, Australia), respectively, for initial specific activity calculations. National Research Council of Canada-certified analytical standards run at the same time were within the specified range.

As explained by Nadella et al. (2006b), radioactivity which had been transported beyond the mucosal epithelium (i.e. the sum of the muscle tissue and the serosal bath radioactivity) was taken as the estimate of transported ^{64}Cu and ^{22}Na. Rates of Cu, Na^+ and fluid transport, per unit intestinal surface area, were calculated exactly as described by Nadella et al. (2006b).

TEP measurements

For selected treatments, transepithelial potential measurements were performed on sac preparations bathed with mucosal saline on the mucosal surface and serosal saline on the serosal surface. TEP was measured using agar/salt bridges (3 M KCl in 4% agar) connected through Ag/AgCl electrodes to a Radiometer PHM 82 standard pH meter (Radiometer; Copenhagen). All TEP values are expressed with mucosal reference at 0 mV, while the sac preparation was exposed to mucosal and serosal salines of appropriate composition. Tip potential was routinely less than 1 mV, and the electrodes were checked for symmetry. The mucosal side was accessed via the cannulation catheter and the serosal side via the outside bathing solution. Triplicate measurements over a 5-min period were averaged.

Experimental series

While every effort was made to maintain uniform physiological conditions for holding the fish, differences

in uptake rates of ~2–3-fold were observed even under control conditions probably resulting from seasonal variations. Hence controls were used simultaneously for each experimental series.

Series 1: effect of Na^+ on Cu transport

To examine the effect of increasing Na^+ concentration on Cu uptake, five treatment groups were employed. NaCl levels in mucosal saline were altered to achieve Na^+ concentrations of 3, 35, 70, 140 and 280 mM while serosal saline remained at 140 mM. Osmolality was measured using a 5100C vapor pressure osmometer (Wescor Inc., Utah, USA). At control levels of 140 mM Na^+, measured osmolality of the modified Cortland saline was 280 mOsm. Thus osmolality of mucosal saline at 3, 35 and 70 mM Na^+ was raised from 37, 80 and 187 mOsm, respectively, to 280 mOsm with 125–260 mM mannitol. This ensured that there was no osmotic gradient. For the 280 mM Na^+ treatment, measured osmolality of the mucosal saline was 494 mOsm so the osmolality of serosal saline was also raised to this value with 231 mM mannitol to eliminate any osmotic gradient. Following a 2 h flux period, intestinal sacs were sampled as per the protocol described.

Series 2: role of solvent drag in Cu absorption

To evaluate whether solvent drag contributed to Cu uptake, osmolality of mucosal saline as compared to serosal saline was increased by 300 mOsm using 330 mM mannitol. This created a gradient for inhibition/reversal of mucosal to serosal fluid transport. Gut sacs were infused with this osmotically elevated mucosal saline spiked with ^{64}Cu and following a 2 h flux period, sampled as described previously.

Series 3: osmotic pressure

Observations of stimulated Cu uptake in the presence of elevated mucosal Na^+ raised the possibility that elevated osmolality, rather than elevated NaCl, could be responsible (even in the absence of a solvent drag effect). To test this possibility, osmolality of the mucosal saline was raised to 494 mOsm using mannitol, to mimic osmolality at 280 mM Na^+ while Na^+ concentration in mucosal saline was maintained at control levels of 140 mM. Osmolality of the serosal saline was raised accordingly to be iso-osmotic with mucosal saline.

Series 4: effect of Cl^- substitution by SO_4^{2-} on Cu transport

To evaluate whether the observed response to NaCl manipulation in Series 3 was related to changes in Cl^- rather than in Na^+ concentration, the Cortland saline was modified to ensure substitution of all Cl^- ions by SO_4^{2-} ions. The following saline was used—(in mM) Na_2SO_4, 133; K_2SO_4, 5; $CaSO_4$, 1; $MgSO_4 \cdot 7H_2O$, 1.9; $NaHCO_3$, 1.9; $NaH_2PO_4 \cdot H_2O$, 2.9; glucose, 5.5; pH 7.4. Three treatment groups were employed by altering Na_2SO_4 concentration in the mucosal saline to obtain 70, 140 and 280 mM Na^+. In all cases salines were osmotically compensated with mannitol as in Series 3 to ensure the same osmolality of mucosal and serosal saline. Cl^- levels were measured in the mucosal solution at the end of the 2 h flux using a chloridometer (Radiometer CMT-10). Measured values were in the range 10 mM which was well below the normal level of 140 mM.

Series 5: effect of phenamil

To test the possible involvement of an apical Na^+ channel or a Na^+/H^+ exchange mechanism in gut Cu and Na^+ uptake, phenamil (RBI Sigma-Aldrich, Canada), an amiloride analogue that is an irreversible inhibitor of such processes (Garvin et al. 1985; Kleyman and Cragoe 1988) was added to the mucosal saline at a concentration of 100 μM, dissolved in 0.1% DMSO (Caledon Lab., ON, Canada). Gut sections of control and treatment groups were incubated in 0.1% DMSO and 100 μM phenamil + 0.1% DMSO (both in mucosal saline), respectively, for 1 h. After this preincubation period, control and phenamil-treated gut sections were thoroughly rinsed in saline to remove traces of DMSO and phenamil. This procedure eliminated any potential problem with phenamil–^{64}Cu complex formation possibly rendering Cu unavailable for uptake. Gut sacs were then infused with appropriate mucosal saline to separately measure Cu and Na^+ uptake.

Series 6: influence of P_{CO_2}

In these experiments, the serosal saline was gassed with 0.3% CO_2 (P_{CO_2} = 2.25 torr) in O_2 (control), 1% CO_2 (7.5 torr) in O_2 and 3% CO_2 (22.5 torr) in O_2 delivered from a Wösthoff gas mixing pump (Bochum, Germany). The goal was to use elevated P_{CO_2} to create intracellular acidosis and thereby increase proton supply to drive any proton pump or Na^+/H^+ exchange

system which might be present, and to assess its possible role in Cu and Na^+ uptake. Mucosal and serosal saline were pre-equilibrated with these gases prior to the 2 h flux period and appropriate gassing of serosal saline was continued throughout the experiment. The pH of both salines was kept at 7.4 using the Henderson–Hasselbach equation

$$pH = pK + \log [HCO_3]/[(\alpha CO_2)(P_{CO_2})]$$

(with constants from Boutilier et al. 1984) to calculate the necessary $NaHCO_3$ concentration required to maintain this pH. Na levels in the saline were accordingly adjusted by reducing NaCl to accommodate the additional $NaHCO_3$, and the pH was checked to ensure that it remained at 7.40 ± 0.05.

Series 7: effect of Ag^+ on Cu and Na^+ transport

500 μM $AgNO_3$ (Sigma-Aldrich), tenfold excess in relation to Cu, was added to mucosal saline. Ag is known to enter via Na^+ channels in the trout gill (Bury and Wood 1999) but to augment Na^+ transport in other systems (Klyce and Marshall 1982; Rangachari and Matthews 1985). Cl^- salts in the saline were substituted with SO_4^{2-} salts as in Series 4 to avoid precipitation of Ag^+ as AgCl. The stock solution of $Ag(NO_3)_2$ was prepared fresh and stored in the dark.

Series 8: effect of luminal pH on Cu and Na^+ transport

To assess the effect of pH on Cu uptake, the pH of mucosal saline was adjusted to 6.0, 7.4 and 8.0 using 1 N H_2SO_4 and 2 N KOH, respectively. The saline was buffered with addition of 10 mM MES (Sigma-Aldrich). MES belongs to a range of ''better buffers'', tertiary amines which do not complex metals (Kandegedara and Rorabacher 1999). The pH of serosal saline was maintained at 7.4. The geochemical equilibrium modeling program MINEQL+ (Version 4.01; Environmental Research Software) was used to determine the effect of pH on Cu^{2+} and Na^+ speciation.

Series 9: effect of metal cation competition on Cu and Na^+ transport

500 μM $ZnSO_4$ or $Fe(NO_3)_3$, tenfold excess in relation to Cu, was added to mucosal saline. Zn and Fe are known to be transported by DMT1 (Gunshin et al. 1997). As in Series 4 and 7, Cl^- salts in the saline were substituted with SO_4^{2-} salts to avoid precipitation of the cations.

Series 10: combined effect of phenamil and $Fe(NO_3)_3$ on Cu uptake

To determine whether the observed inhibition of Cu uptake with phenamil and Fe involved two separate mechanisms or a common pathway, intestinal sacs of control and treatment groups were incubated in 0.1% DMSO and 100 μM phenamil + 0.1% DMSO (both in mucosal saline), respectively, for 1 h. Cu uptake was then measured in these sacs in the absence and presence of a tenfold excess of $Fe_2(NO_3)_3$.

Statistical analyses

Statistically significant differences between control and treated groups were evaluated by unpaired Student's t tests (two-tailed). A one-way analysis of variance (ANOVA) was used to compare multiple treatment groups. Differences between specific means were then compared with a least significant difference (LSD) test (SPSS10 for Windows). Data are reported as mean ± SEM and differences were considered significant at $P < 0.05$.

Results

For all series, experiments were performed using mid- and posterior intestinal segments. The two segments showed similar trends for most treatments, hence data are reported only for the mid-intestine and differences when observed are noted for the posterior intestine in the text of results.

Series 1: effect of Na^+ on Cu transport

Mucosal NaCl concentrations were manipulated over the range 3–280 mM revealing a clear stimulatory effect on Cu transport. Cu absorption increased significantly from 0.007 nmol cm^{-2} h^{-1} at 3–35 mM Na^+ to 0.029 nmol cm^{-2} h^{-1} at 140 mM Na^+ and 0.075 nmol cm^{-2} h^{-1} at 280 mM Na^+ in the mid-intestine (Fig. 1a). Cu transport in the posterior intestine was more or less uniform with control values at the lower Na^+ concentrations (3–35 mM) but increased more than twofold at higher Na levels (280 mM—data not shown). Serosal transepithelial potential (TEP) measured against a mucosal reference set at 0 mV was –6 mV at 3 mM Na^+ in the mid-intestine (Fig. 1b). Increasing Na^+ concentration resulted in a progressively less negative TEP reaching +1.2 mV at 280 mM Na^+.
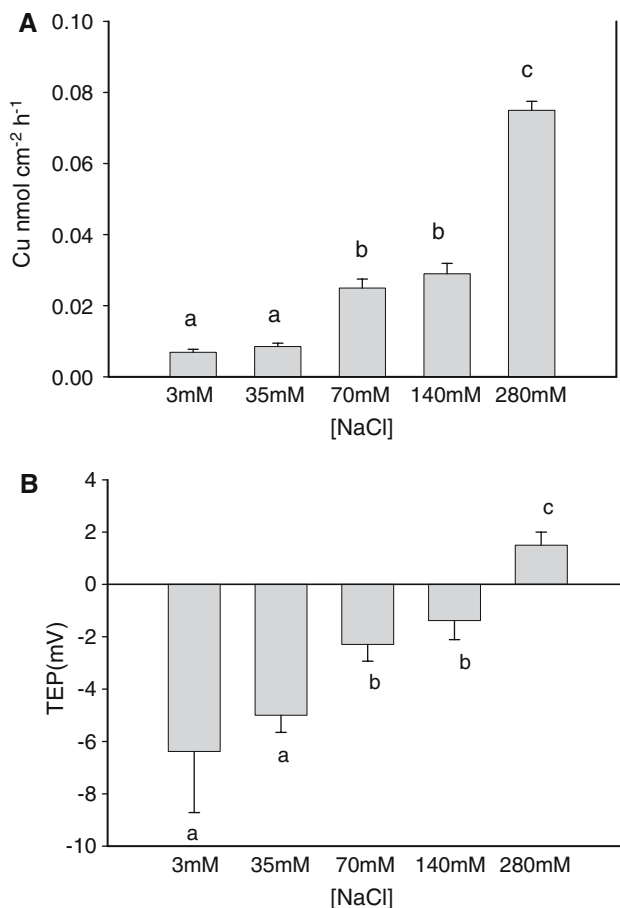
A

B

Fig. 1 Effect of increasing luminal Na^{+} (as NaCl) on **a** Cu transport rate (50 μmol ^{64}Cu) and **b** transepithelial potential relative to a mucosal reference at zero in the isolated mid-intestine segment from rainbow trout. *Plotted values* represent mean ± SEM (*n* = 5 per treatment). Statistical significance was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with different letters are significantly different (*P* < 0.05)

Series 2: effect of solvent drag on Cu transport

Cu transport occurred simultaneously with considerable fluid transport. Net water flux was elevated 2–5-fold with increasing mucosal Na^{+} concentration in Series 1 (Fig. 2). Therefore this series evaluated whether solvent drag was responsible for the increased Cu transport. However, reversing fluid transport by manipulation of the osmotic gradient had no effect on Cu transport (Fig. 3), indicating that solvent drag was not involved.

Series 3: influence of osmotic pressure

Increasing the osmolality of the mucosal fluid to 495 mOsm with mannitol (serosal fluid set to the same level) to mimic the 280 mM Na^{+} level had no signifi-
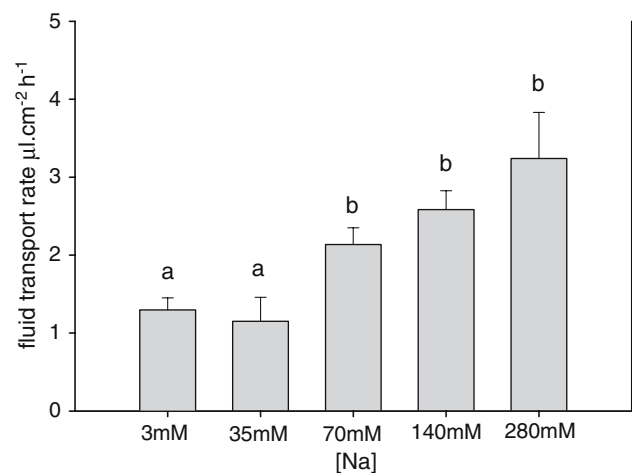

Fig. 2 Effect of increasing luminal Na^{+} (as NaCl) in the presence of 50 μmol ^{64}Cu on mucosal-to-serosal fluid transport rate in the isolated mid-intestine segment from rainbow trout. *Plotted values* represent mean ± SEM (*n* = 5 per treatment). Statistical significance was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with different letters are significantly different (*P* < 0.05)

cant effect on Cu transport with uptake rates of 0.024 nmol cm^{-2} h^{-1} ± 0.0034 and 0.021 nmol cm^{-2} h^{-1} ± 0.0012 in control and experimental groups, respectively, eliminating the possible role of elevated osmotic pressure in stimulating Cu uptake.

Series 4: possible Cl^{−} dependency of Cu transport

Replacing all Cl^{−} with SO_4^{2-} confirmed the Na^{+}-dependent pattern of Cu transport seen with NaCl
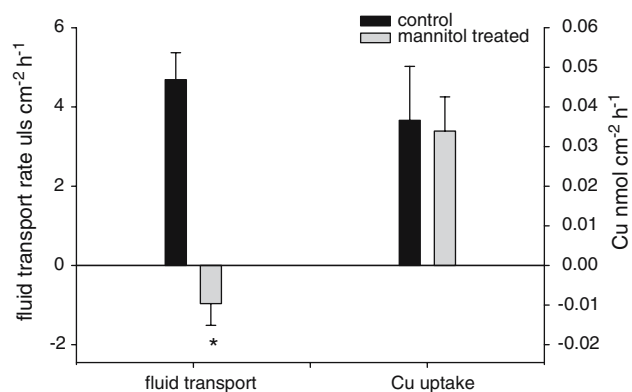

Fig. 3 An analysis of the possible relationship between fluid transport rate and Cu transport rate (50 μmol ^{64}Cu) in the isolated mid-intestine segment from rainbow trout. Cu uptake rate measured in the presence and absence of net mucosal-to-serosal transport of fluid. Values represent mean ± SEM (*n* = 5 per treatment). Statistical significance was tested using unpaired *t* test (two-tailed). Means labeled with *asterisks* are significantly different (*P* < 0.05)

manipulation in Series 1 (Fig. 1a). A twofold increase in Cu uptake rate with increasing Na^+ concentration from 70 to 280 mM (as Na_2SO_4) was observed (Fig. 4a) similar to that seen with Na^+ present as NaCl (Fig. 1) suggesting Cu transport to be sensitive to Na^+ and insensitive to Cl^-. TEP exhibited an increasing serosal side positive trend with the increase in Na^+ concentration, as was the case for the NaCl experiments (Fig. 4b).

Series 5: effect of phenamil on Cu and Na^+ transport

Mid-intestine segments exposed to 100 μM phenamil exhibited an approximate 35% decrease in Cu and Na^+ transport (Fig. 5) compared to drug-free, DMSO solvent control. Cu and Na^+ transport were inhibited significantly in the posterior intestine as well but only by 20% (data not shown). A simultaneous inhibition of fluid transport rates was also observed in these prep-
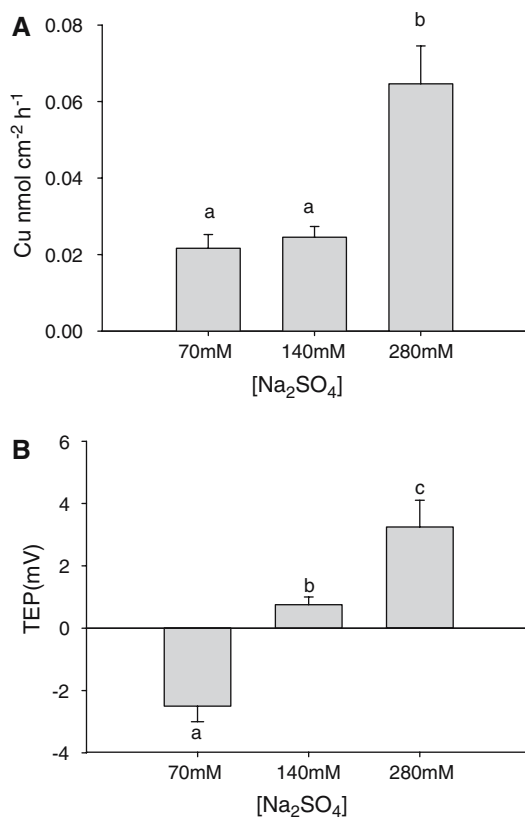

Fig. 5 Effect of pre-incubating isolated mid-intestine segment from rainbow trout in 100 μM phenamil on Cu (50 μmol ^{64}Cu) and ^{22}Na (140 mM) transport rate. Values are mean ± SEM ($n = 5$ per treatment). Statistical significance was tested using unpaired t test (two-tailed). Means labeled with *asterisks* are significantly different ($P < 0.05$)

arations(data not shown). Pretreatment with 100 μM phenamil, however, did not significantly influence TEP which remained unchanged at 1 and 0 mV, respectively (data not shown).

Series 6: influence of elevated CO_2 on Cu and Na^+ transport

Inducing hypercapnia by changing the gassing of the saline from 0.3% CO_2 to 1 and 3% CO_2 (at constant pH 7.4) caused a general trend of elevated Cu transport which was significant (Fig. 6) at the highest CO_2 concentration (threefold increase). Na^+ transport was also elevated by about 50%, which was significant at 1% CO_2, with no further rise at 3% CO_2 (Fig. 6).

Fig. 4 Effect of increasing luminal Na^+ as (Na_2SO_4) on **a** Cu (50 μmol ^{64}Cu) transport rate and **b** transepithelial potential in the isolated mid-intestine segment from rainbow trout. *Plotted values* represent mean ± SEM ($n = 5$ per treatment). Statistical significance was tested by ANOVA followed by least significant difference (LSD) test. Means labeled with *different letters* are significantly different ($P < 0.05$)
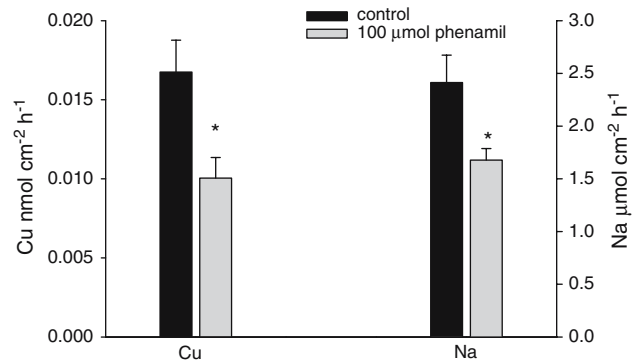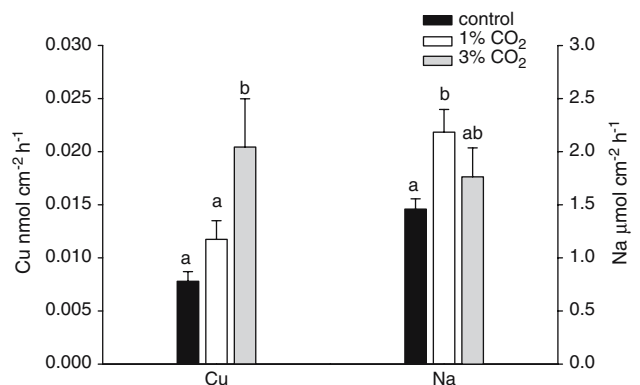

Fig. 6 Cu (50 μmol ^{64}Cu) and ^{22}Na (140 mM) uptake rates in the isolated mid-intestine segment from rainbow trout bathed in solutions gassed with 0.3, 1 and 3.0% CO_2 in O_2. Values are mean ± SEM ($n = 5$ per treatment). Statistical significance was tested using one-way ANOVA followed by least significant difference (LSD) test. Means labeled with *different letters* are significantly different ($P < 0.05$)

Hypercapnia did not induce any significant change in TEP which remained stable at 1 mV (data not shown).

Series 7: effect of Ag^+ on Cu and Na^+ transport

The presence of 500 μM $AgNO_3$ in the mucosal saline significantly increased transepithelial transport of Cu threefold in the mid-intestine. Similarly, Na^+ transport rate increased significantly by about 50% in the mid-intestine (Fig. 7a). While Cu transport increased likewise in the posterior intestine, increase in Na transport was not observed in this segment (data not shown). Transepithelial potential increased significantly from +0.75 mV in control mid-intestine preparations to approximately +2 mV in Ag^+-treated groups, displaying a strong serosal side positive trend (Fig. 7b).

Series 8: effect of luminal pH on Cu and Na^+ transport

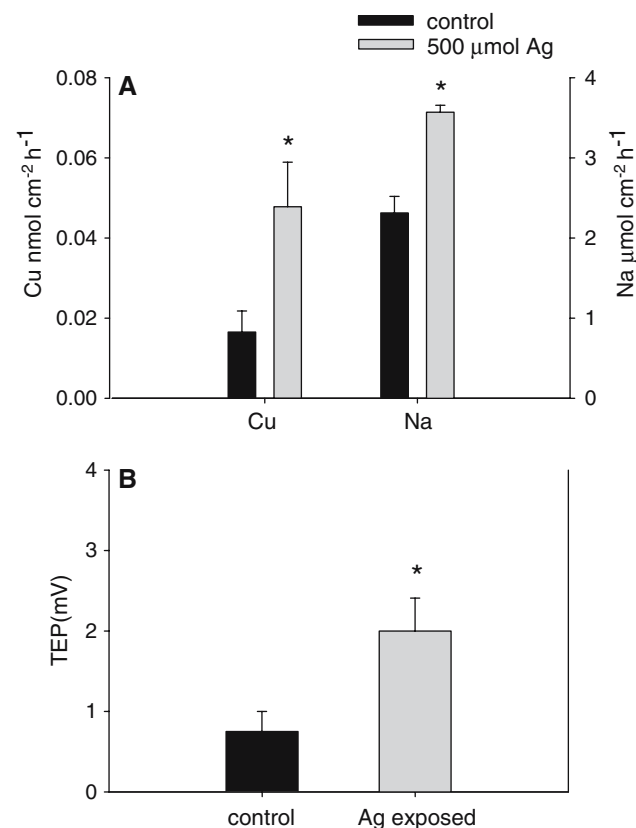Cu transport in rainbow trout intestine appeared to be sensitive to variation in luminal pH. There was no significant difference in Cu transport when the pH of mucosal saline was lowered from 7.4 to 6.0. However, Cu uptake declined by approximately 50% in the mid-intestine when the pH of mucosal saline was raised to 8.0 (Fig. 8). Similar trends of significantly lower rates of Cu uptake were observed in the posterior intestine at pH 8.0. The geochemical equilibrium modeling program MINEQL+ (Version 4.01; Environmental Research Software) was used to determine the effect of pH on Cu^{2+} speciation. About 60% of the total concentration of Cu was present as Cu^{2+} at pH 7.4, increasing to 90% at pH 6.0. However, a steady decrease in % total concentration of Cu^{2+} was evident as pH increased particularly beyond 7.4, and only 18% of the total concentration of Cu remained as Cu^{2+} at pH 8.0. The decline in Cu^{2+} correlated with a steep increase in the % total concentration of $CuCO_3$ and modest increases in the % total concentration of CuOH and $Cu(OH)_2$.

Na^+ speciation on the other hand was unaffected with changes in pH; Na^+ accounted for >99.5% of total Na at all pHs. Furthermore, the uptake of Na^+ was not significantly different between segments maintained at a mucosal pH of 6.0, 7.4 or 8.0. Variation of pH did not influence TEP significantly which remained unchanged at –1 and –0.5 mV in the mid- and posterior intestine, respectively (data not shown).

Series 9: effect of cation competition on Cu and Na^+ transport

The presence of a tenfold excess of Zn (500 μM $ZnSO_4$) in the mucosal saline significantly decreased transepithelial transport of Cu by 75% in the
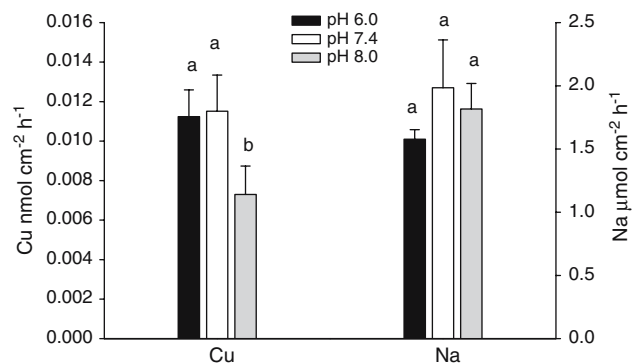

Fig. 7 Influence of luminal $AgNO_3$ (500 μM) on **a** Cu(50 μmol ^{64}Cu) and ^{22}Na (140 mM) transport rate and **b** TEP in the isolated mid-intestine segment from rainbow trout. Values are mean ± SEM ($n = 5$ per treatment). Statistical significance was tested using unpaired t test (two-tailed). Means labeled with *asterisks* are significantly different ($P < 0.05$)

Fig. 8 Influence of luminal pH buffered with 10 mM MES on Cu (50 μmol ^{64}Cu) and ^{22}Na (140 mM) transport rate in the isolated mid-intestine segment from rainbow trout. Values are mean ± SEM ($n = 5$ per treatment). Statistical significance was tested using one-way ANOVA followed by least significant difference (LSD) test. Means labeled with *different letters* are significantly different ($P < 0.05$)

mid-intestine (Fig. 9a). Similarly, Cu uptake rate significantly declined by 80% in the mid-intestine when exposed to 500 μM Fe(NO$_3$)$_3$ (Fig. 9b). In the posterior intestine Cu uptake rates declined by 40% in the presence of Zn but only a modest inhibition of 20% (non-significant) was observed in the presence of Fe (data not shown). There was no effect of either cation on Na transport rates, and no significant changes in TEP were observed upon exposure to either Zn or Fe (data not shown).

Series 10: combined effect of phenamil and Fe treatment on Cu uptake

While Cu uptake was inhibited independently in the presence of Fe (Fig. 9b) and phenamil (Fig. 5) no additive inhibitory effect of phenamil treatment was observed on Cu uptake when the two treatments were combined (Fig. 10).
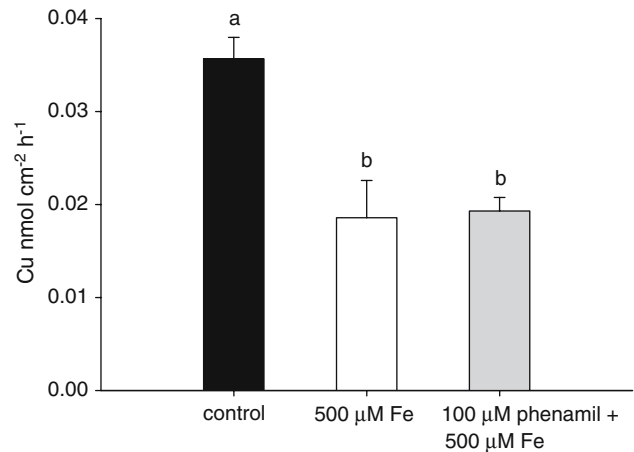

Fig. 10 Influence of 500 μM Fe(NO$_3$)$_3$ on Cu uptake in phenamil (100 μM) treated mid-intestine segments from rainbow trout. Values are mean ± SEM ($n = 5$ per treatment). Statistical significance was tested using one-way ANOVA followed by least significant difference (LSD) test. Means labeled with *different letters* are significantly different ($P < 0.05$)

Discussion

Based on observations of a greater transport of Cu in the presence of Na$^+$, Ag$^+$, high P_{CO_2} and inhibition by phenamil, our data provide evidence for an apparent Na$^+$-assisted pathway of intestinal Cu uptake in rainbow trout. Alternately, sensitivity of Cu uptake to pH and competition with other divalent cations suggests a specific metal transporter with features similar to a recently characterized Cu transporter from Ctr1-deficient embryonic cells in mammals (Lee et al. 2002a, b). Consequently we discuss if these two mechanisms are linked, or whether separate mechanisms of Cu transport exist in the trout gut, one which is Na$^+$-assisted and the other a metal-specific pathway independent of the Na$^+$-gradient.

Na$^+$-dependent Cu uptake

A notable finding of this study was the observation of a Na$^+$ concentration-dependent increase in Cu transport (Fig. 1) which provides compelling evidence that Na$^+$ may be involved in the uptake of Cu in trout intestine. Na$^+$-sensitive Cu transport has been earlier described in rainbow trout gills (Grosell and Wood 2002). However, the mechanism at the gills where branchial Cu uptake was reduced with increasing ambient Na$^+$ concentration is diametrically different from our observation of stimulated Cu uptake across the intestinal epithelium.

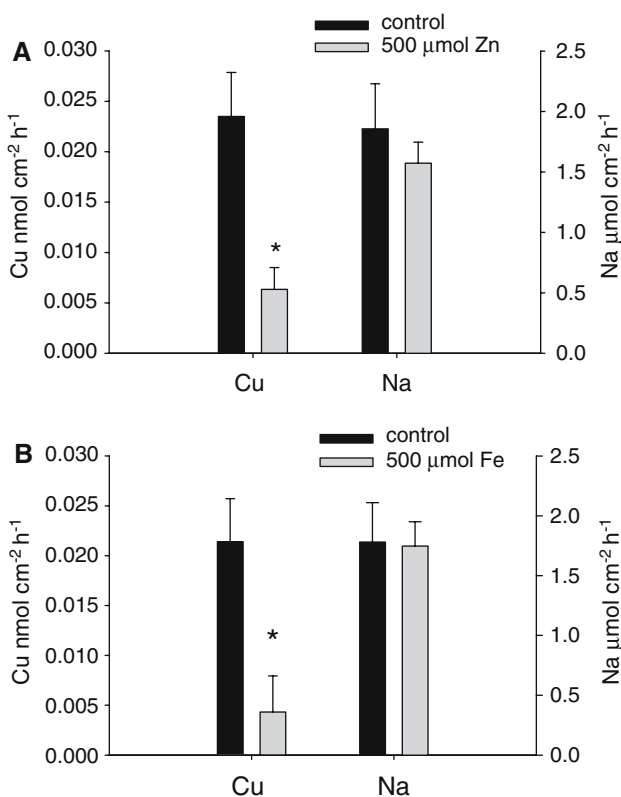Under normal physiological Na$^+$ levels and symmetrical conditions, the measured TEP was about

Fig. 9 Influence of luminal **a** (500 μM) ZnSO$_4$ and **b** (500 μM) Fe(NO$_3$)$_3$ on Cu (50 μmol ^{64}Cu) and ^{22}Na(140 mM) transport rate in the isolated mid-intestine segments from rainbow trout. Values are mean ± SEM ($n = 5$ per treatment). Statistical significance was tested using unpaired t test (two-tailed). Means labeled with *asterisks* are significantly different ($P < 0.05$)

–1.5 mV, which would slightly assist Cu uptake. Serosal transepithelial potential (TEP) was progressively less negative with increasing Na^+ levels (Fig. 1b), suggesting that stimulation of Cu uptake was not related to changes in TEP as the trend towards a serosa-positive electrical gradient with increasing luminal Na^+ would have provided progressively less assistance for the transport of positively charged Cu ions.

Increasing the osmolality of the luminal fluid to mimic the highest Na^+ concentration had no significant effect on Cu transport eliminating osmotic pressure as an explanation. Furthermore, the contribution of solvent drag and the nature of the accompanying anion are unimportant (see below). Thus the interaction of Na^+ with Cu transport appears to be direct rather than indirect. In accord with the present results, Na^+-dependent stimulation of Cu uptake has also been demonstrated in the jejunum and ileum of rat (Wapnir and Stiel 1987), though its exact mechanism remains unclear. In trout the intestinal Na^+–Cu interaction appears to be important in vivo as well as in vitro. Very recently Kjoss et al. (2005) found that juvenile rainbow trout that received elevated dietary Na^+ along with high dietary Cu showed the greatest Cu retention, a result which was considered to reflect a positive interaction between Cu and Na^+ transport in the gastrointestinal tract. In accord with the above observations, Na^+ removal from the lumen of the intestine in African walking catfish tended to slow Cu transport (Handy et al. 2000, 2002). In contrast increased Cu accumulation in isolated intestinal cells from rainbow trout was observed when Na^+ was lowered from 140 to 11 mM (Burke and Handy 2005). However, this response was observed only at a high Cu concentration of 800 μM where there may be a different route of Cu transport. At lower concentrations, down to the 50 μM Cu used in the present study, Burke and Handy (2005) observed no interaction of Na^+ on Cu transport. While the above study measured accumulation in isolated enterocytes (and not net transport as in the current study), it is also possible that the differential response to similar treatments are a reflection on the properties of the preparations especially with regard to polarity which may be lost in isolated enterocytes.

Enhanced Cu transport paralleled a simultaneous and dramatic increase in fluid transport (Fig. 2), raising the possibility that the former was a solvent drag effect. However, since the increase in Cu transport rate was essentially unaffected even under conditions of osmotically manipulated reversal in net water flux (Fig. 3), solvent drag was not associated with the Na^+-stimulated transport of Cu. The effect of solvent drag on Cu uptake was also overruled in the intestine of the

African walking catfish as absolute rates of Cu transfer into the serosal perfusate were higher than those for water, and Cu uptake to the serosal solution occurred when the net water flux was in the opposite direction (Handy et al. 2000). Wapnir and Stiel (1987) have shown that Na^+-associated Cu absorption in the rat intestine was not related to water fluxes as maximum Cu absorption corresponded with minimum net water absorption.

When Cl^- was replaced with SO_4^{2-}, an identical response of Na^+-dependent stimulation in Cu uptake was observed at the highest Na^+ concentration (Fig. 4) which was independent from changes in TEP, suggesting Cu transport to be insensitive to Cl^- and that the nature of the accompanying anion is unimportant. These results are contrary to observations of Burke and Handy (2005) in isolated rainbow trout enterocytes and Handy et al. (2000) in the African walking catfish. The latter study reported a tenfold decrease in the rate of Cu uptake when Cl^- was removed simultaneously from both mucosal and serosal solutions. The authors related this decrease in Cu uptake to an inhibition of Cu/anion symport mechanism for export on the basolateral membrane resulting from the blockade of DIDS-sensitive basolateral Cl^- transporters.

The partial inhibition of both Cu and Na^+ uptake with 100 μM phenamil, an amiloride analogue (Fig. 5) implicates an apical Na^+ channel or Na^+/H^+ exchanger in the process. Similar evidence linking the transport of Cu to a Na^+-coupled mechanism is available from the rat intestine on the basis of a considerable decrease in Cu absorption with 1 mM amiloride (Wapnir 1991). This observation, together with earlier findings that the presence of luminal Na^+ stimulated Cu transport (Wapnir and Stiel 1987) led to the proposal of Na^+-associated Cu transport (Wapnir 1991).

The results from the present study with trout intestinal sacs correspond with a reported inhibition of Cu uptake in rainbow trout gills exposed to 100 μM phenamil (Grosell and Wood 2002). However, Grosell and Wood (2002) reported a discrepancy between the inhibitory effect of phenamil on Na^+ and Cu uptake, phenamil inhibiting Na^+ uptake more effectively than Cu uptake suggesting multiple Cu uptake pathways. In the trout intestine, however, phenamil inhibited Cu uptake to an equal or greater extent. When taken together with our finding of stimulated Cu uptake with increasing Na^+ levels in the trout intestine (in contrast to inhibition of Cu uptake by increasing Na^+ levels at the trout gills), this clearly indicates that Na^+-dependent Cu uptake in the trout intestine occurs via a mechanism different from that found in the gills.

Additional evidence linking Na^+ and Cu transport in the trout intestine was the significant increases in both Na^+ and Cu transport observed under hypercapnia, a treatment intended to induce intracellular acidosis (Fig. 6). Previous studies in isolated frog skin and urinary bladder of the water turtle (Ehrenfeld and Garcia-Romeu 1977; Schwartz and Steinmetz 1977; Harvey 1992) indicate that H^+ excretion is dependent on availability of CO_2 and the transport of Na^+. Protons are generated from the catalyzed hydration of CO_2, and Na^+ provides the electrically balancing positive charge via entry through apical Na^+ channels which are electrically coupled to the proton pumps, or via a Na^+/H^+ antiport. Substantial evidence exists for the presence of amiloride-sensitive NHE isoforms associated with a Na^+/H^+ antiport on both the apical and basolateral membranes of the mammalian gastrointestinal tract (Yun et al. 1995; Hoogerwerf et al. 1996). It is possible that such a mechanism exists in the trout gut as the normally high external $[Na^+]$ in the gut lumen, arising from a largely carnivorous diet, would thermodynamically favor Na^+/H^+ exchange and explain the increased uptake of Na^+ under induced hypercapnia. By this scenario, high P_{CO_2} would drive Na^+ uptake by the NHE (or H^+ ATPase/Na^+ channel) and at the same time acidify the boundary layer on the mucosal surface. It remains to be determined whether Cu is transported at a higher rate along with Na^+ by a common mechanism, or because the elevated H^+ extrusion creates a suitable microenvironment facilitating Cu transport.

There was also a marked effect of Ag^+ in stimulating both Cu and Na^+ transport (Fig. 7a), again suggesting linkage. The TEP became significantly more serosa-positive (Fig. 7b) indicating that the stimulated transport was again unrelated to the change in voltage. This suggests that Ag^+ activates some sort of Na^+-coupled Cu transport mechanism in the trout intestine. In support of this suggestion are several reports wherein Ag^+ appears to increase the apical conductance to Na^+ in a variety of epithelia such as the rat ileum (Clarkson and Toole 1964), toad skin (Gerencser et al. 1977), toad bladder (Walser 1970) and frog skin (Curran 1972; Li and DeSouza 1977) with the increased entry of Na^+ contributing to the current generated. In contrast Ag^+ is known to inhibit Na^+ uptake at the gills in rainbow trout (Morgan et al. 1997) and vice versa (Bury and Wood 1999), but this again reinforces the differences between the Na^+–Cu interactions at the two epithelia.

Na-independent Cu uptake

Two of the possible candidate proteins responsible for the absorption of dietary Cu in mammals are recognized to be pH-sensitive. Ctr1 is stimulated by low extracellular pH (Lee et al. 2000) and the transport of metal ions by all the members of the NRAMP family, which includes DMT1, is driven by protons (Gunshin et al. 1997; Nelson 1999).

No effect on Cu transport was seen when pH was lowered to 6.0, but a marked decrease in Cu uptake was observed when pH was raised to 8.0 (Fig. 8). These effects were specific to Cu and not Na^+ transport suggesting the presence of a non Na^+-dependent pathway for Cu uptake that demonstrates characteristics of DMT1 or Ctr1 mediated transport. However, the pH effect upon intestinal Cu uptake in rainbow trout could also be explained by altered Cu speciation, related to the decline in % total concentration of Cu^{2+} as it is replaced mainly by $CuCO_3$ at high pH. At pH 8, Cu uptake rate declined by 50%, whereas MINEQL speciation analysis indicated that Cu^{2+} concentration declined by ~70%. This result might suggest that Cu transport is dependent on the presence of Cu^{2+} as the primary substrate and thereby allude to a role for DMT1 in Cu transport according to the traditional view that DMT1 preferentially transports divalent metals (Gunshin et al. 1997). However, the experiments of Gunshin et al. (1997) were performed in the presence of 100 μM ascorbic acid, a reducing agent, so it is possible that DMT1 transports Cu^+. Indeed Arredondo et al. (2003) recently provided evidence that DMT1 may preferentially transport Cu^+ over divalent Cu^{2+} in cultured human intestinal Caco-2 cells. Further complicating interpretation is the fact that treatment with the reducing agent ascorbate (100 μM–2.5 mM) had no effect on Cu uptake on our trout gut system (Nadella et al. 2006b) indicating either that the valence of Cu present is not critical or that sufficient quantities of endogenous reductase are present on the intestinal epithelium of trout. The presence of endogenous plasma membrane reductases capable of reducing Cu has been reported in mammalian brush border membranes (Knopfel and Solioz 2002). If this were the case, the plasma membrane reductase would reduce Cu^{2+} to monovalent Cu^+ which would be taken up more readily by Ctr1 (Sharp 2003). However, Ctr1 is inhibited rather than stimulated by Ag^+ (Lee et al. 2002a) in contrast to our data, a detail that moderates its likely contribution in Cu uptake in the trout intestine. Given that DMT1 accepts Cu^+ as substrate, an endogenous reductase could also facilitate Cu uptake via this H^+-metal symport.

At the gene level DMT1 has been identified in fish (Donovan et al. 2002; Dorschner and Phillips 1999) and it has been implicated in intestinal Fe uptake in flounder (Bury et al. 2001) and the branchial uptake of

Fe in the zebrafish (Bury and Grosell 2003). The present study revealed significant inhibition of Cu uptake in the presence of tenfold greater Fe [500 μM Fe(NO₃)₃] or Zn (500 μM ZnSO₄) in the intestine of the rainbow trout (Fig. 9). Notably Glover and Hogstrand (2003) also reported significant inhibition of intestinal Zn absorption by equimolar Cu levels in rainbow trout in vivo. In the present study Na⁺ uptake rate remained unchanged in the presence of either metal cation, indicating a specific effect on Cu transport. DMT1 accounts for at least 50% of Cu transport in human intestinal Caco-2 cells (Arredondo et al. 2003; Tenant et al. 2002), where there is a similar competition between Cu and Fe. When cultured in high Cu medium these cells exhibited decreased expression of DMT1 protein and mRNA, similar to the down-regulation of intestinal Cu uptake observed in vivo (Turnlund et al. 1989) following exposure to high dietary Cu levels. Very recently Knöpfel et al. (2005) observed that DMT1 was involved in Cu uptake in the rat small intestine BBM vesicles.

In mammalian systems, there is also extensive evidence of inhibition of Cu absorption when dietary or intra-luminal Zn: Cu ratios are elevated (Oestreicher and Cousins 1984; Evans et al. 1970; Van Campen 1969). Taken together with these mammalian observations, the present evidence of Cu–Fe and Cu–Zn interaction in the trout intestine and the identification of DMT1 in the zebrafish argue that Cu uptake in the trout intestine occurs at least in part by DMT1.

A proposed model for intestinal copper transport in the rainbow trout

Overall our data clearly implicate a Na⁺-dependent mechanism in mediating Cu transport across the trout intestine, but there is a dichotomy in the results. On the one hand, phenamil partially inhibits both Na⁺ and Cu transport and hypercapnia stimulates both Na⁺ and Cu transport, results which are best explained by both Cu and Na⁺ entering through an apical Na⁺-channel as in the trout gill (Grosell and Wood 2002). On the other hand, elevated luminal Na⁺ stimulates rather than competitively inhibits Cu transport and the presence of Ag⁺ stimulates both Na⁺ and Cu transport, results which are best explained by a Na⁺–Cu co-transport system. However, we suggest that these apparently dichotomous trends can be explained by a common mechanism. In support of this proposal is evidence that phenamil treated gut sections did not show an added inhibition of Cu uptake in the presence of a tenfold excess of Fe (Fig. 10). Based on this evidence and the fact that treatments which accelerate Na⁺ transport
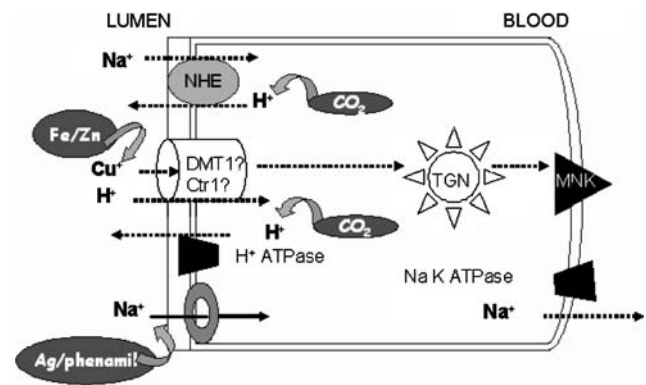

Fig. 11 Schematic diagram of a conceptual model for transcellular Cu uptake pathway in the trout intestine. Cu from the diet is possibly transported by DMT1 (or Ctr1), the process being facilitated by the proton gradient created from the transport of Na via an NHE-like transporter or H⁺ ATPase/Na⁺ channel system. Absorbed Cu is directed to the transgolgi network (*TGN*) and exported across the basolateral membrane into the extracellular fluid (*ECF*) via a Menkes-like ATPase (*MNK*). The potential sites of interaction of Ag and phenamil, of CO₂, and of Fe and Zn on Cu uptake are indicated

also accelerate Cu uptake, while treatments which depress Na⁺ transport inhibit Cu uptake, we propose a novel Na⁺-assisted mechanism of Cu uptake (Fig. 11).

By this scenario, the Na⁺ gradient stimulates Na⁺/H⁺ exchange via an NHE type transporter or a Na⁺ channel/H⁺ATPase system at the brush border. The resulting increase in H⁺ concentration in the brush border microenvironment would thereby create a suitable H⁺ gradient for the effective transport of Cu via either DMT1 and/or Ctr1. Increased H⁺ extrusion associated with high P_{CO_2} would also have this effect. This proposed mechanism serves to link the observed stimulation of Cu transport with increasing Na⁺ transport to the inhibition of Cu transport in the presence of phenamil. Since neither DMT1 nor Ctr1 are ATP-dependent, this step would be responsible for the low Q_{10} values associated with apical uptake, while the higher Q_{10} values for overall transport (Nadella et al. 2006b) would be explained by Cu extrusion via a Menkes type Cu ATPase at the basolateral membrane. Based on pH sensitivity and inhibition of Cu uptake with Fe and Zn, DMT1 appears as the most likely candidate involved in apical Cu transport in the trout intestine, although a role for Ctr1 cannot be excluded.

References

Arredondo M, Munoz P, Mura CV, Nunez MT (2003) DMT1 a physiologically relevant apical Cu^{1+} transporter of intestinal cells. Am J Physiol 284:C1525–C1530

Boutilier RG, Heming TA, Iwama GK (1984) Physicochemical parameters for use in fish respiratory physiology: constants in fish physiology. In: Randall DJ, Hoar WS (eds) Fish physiology, vol 10A. Academic, NY, pp 403–431

Burke J, Handy RD (2005) Sodium-sensitive and -insensitive copper accumulation by isolated intestinal cells of rainbow trout Oncorhynchus mykiss. J Exp Biol 208:391–407

Bury NR, Grosell M (2003) Waterborne iron acquisition by a fresh water teleost fish, zebrafish Danio rerio. J Exp Biol 206:1529–1535

Bury NR, Wood CM (1999) Mechanism of branchial apical silver uptake by rainbow trout is via the proton coupled Na^+ channel. Am J Physiol 277:R1385–R1391

Bury NR, Grosell M, Wood CM, Hogstrand C, Wilson RW, Rankin JC, Busk M, Lecklin T, Jensen FB (2001) Intestinal iron uptake in the European flounder (Platichthys flesus). J Exp Biol 204:3779–3787

Camakaris J, Vaskoboinik I, Mercer JF (1999) Molecular mechanisms of copper homeostasis. Biochem Biophys Res Commun 261:225–232

Campbell HA, Handy RD, Nimmo M (1999) Copper uptake kinetics across the gills of rainbow trout (Oncorhynchus mykiss) measured using an improved isolated perfused head technique. Aquat Toxicol 46:177–190

Clarkson TW, Toole SR (1964) Measurement of short-circuit current and ion-transport across the ileum. Am J Physiol 206:658–668

Condomina J, Zoronza ST, Granero L, Polache A (2002) Kinetics of Zn transport in vitro in rat small intestine and colon: interaction with copper. Eur J Pharm Sci 16:289–295

Curran PF (1972) Effect of silver ion on permeability properties of frog skin. Biochim Biophys Acta 288:90–97

Donovan A, Brownlie A, Dorschner MO, Zhou Y, Pratt SJ, Paw BH, Phillips RB, Thisse C, Thisse B, Zon LI (2002) The zebrafish mutant gene chardonnay (cdy) encodes divalent metal transporter 1 (DMT1). Blood 100:4655–4659

Dorschner MO, Phillips RB (1999) Comparative analysis of two Nramp loci from rainbow trout. DNA Cell Biol 18:573–583

Ehrenfeld J, Garcia-Romeu F (1977) Active hydrogen excretion and sodium absorption through isolated frog skin. Am J Physiol 233:F46–F54

Evans GW, Majors PF, Cornatzer WE (1970) Mechanism for cadmium and zinc antagonism of copper metabolism. Biochem Biophys Res Commun 40:1142–1148

Garvin JL, Simon SA, Cragoe EJ, Mandel LJ (1985) Phenamil: an irreversible inhibitor of sodium channels in the toad urinary bladder. J Membr Biol 87:45–54

Gerencser GA, Corvette KM, Loo SY, Hong SK (1977) Effect of silver chloride on the short-circuit current across the isolated toad skin. Life Sci 20:1883–1890

Glover CN, Hogstrand C (2003) Effects of dissolved metals and other hydrominerals on in vivo intestinal zinc uptake in fresh water rainbow trout. Aquat Toxicol 62:281–293

Grosell M, Jensen FB (1999) NO_2^- uptake and HCO_3^- excretion in the intestine of the European flounder (Platichthys flesus). J Exp Biol 202:2103–2110

Grosell M, Wood CM (2002) Copper uptake across rainbow trout gills: mechanisms of apical entry. J Exp Biol 205:1179–1188

Grosell M, Hansen HJM, Rosenkilde P (1998a) Cu uptake, metabolism and elimination in fed and starved European eels (Anguilla anguilla) during adaptation to water-borne copper exposure. Comp Biochem Physiol 120C:295–305

Grosell MH, Hogstrand C, Wood CM (1998b) Renal Cu and Na excretion and hepatic Cu metabolism in both Cu-acclimated and non-acclimated rainbow trout (Oncorhynchus mykiss). Aquat Toxicol 40:275–291

Grosell M, Kamunde C, Wood CM and Walsh PJ (2001) Copper transport across fish gills. Society for Experimental Biology, Annual Meeting, University of Kent, Canterbury, England, 2–6 April, p 89

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA (1997) Cloning and characterization of a proton-coupled mammalian metal ion transporter. Nature 388:482–488

Handy RD, Musonda MM, Philips C, Fella SJ (2000) Mechanisms of gastrointestinal copper absorption in the African walking catfish: copper dose-effects and a novel anion-dependent pathway in the intestine. J Exp Biol 203:2365–2377

Handy RD, Eddy FB, Baines H (2002) Sodium-dependent copper uptake across epithelia: a review of rationale with experimental evidence from gill and intestine. Biochim Biophys Acta 1566:104–115

Harvey BJ (1992) Energization of sodium absorption by the H(+)-ATPase pump in mitochondria-rich cells of frog skin. J Exp Biol 172:289–309

Hoogerwerf WA, Tsao SC, Devuyst O, Levine SA, Yun CH, Yip JW, Cohen ME, Wilson PD, Lazenby AJ, Tse CM, Donowitz M (1996) NHE2 and NHE3 are human and rabbit intestinal brush-border proteins. Am J Physiol 270:G29–G41

Kamunde CN, Wood CM (2003) The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout, Oncorhynchus mykiss. Aquat Toxicol 62:235–254

Kamunde CN, Grosell M, Lott JNA, Wood CM (2001) Copper metabolism and gut morphology in rainbow trout (Oncorhynchus mykiss) during chronic sublethal dietary copper exposure. Can J Fish Aquat Sci 58:293–305

Kamunde CN, Clayton C, Wood CM (2002a) Waterborne versus dietary copper uptake in trout and the effects of waterborne copper acclimation. Am J Physiol 383:R69–R78

Kamunde CN, Grosell M, Higgs D, Wood CM (2002b) Copper metabolism in actively growing rainbow trout (Oncorhynchus mykiss): interactions between dietary and waterborne copper uptake. J Exp Biol 205:279–290

Kandegedara A, Rorabacher DB (1999) Noncomplexing tertiary amines as "Better" buffers covering the range of pH 3–11. Temperature dependence of their acid dissociation constants. Anal Chem 71:3140–3144

Kjoss VA, Kamunde CN, Niyogi S, Grosell M, Wood CM (2005) Dietary Na does not reduce dietary Cu uptake by juvenile rainbow trout. J Fish Biol 66:468–484

Kleyman TR, Cragoe EJ Jr (1988) Amiloride and its analogs as tools in the study of ion transport. J Membr Biol 105:1–21

Klyce SD, Marshall WS (1982) Effects of Ag^+ on ion transport by the corneal epithelium of the rabbit. J Membr Biol 66:133–144

Knopfel M, Solioz M (2002) Characterization of a cytochrome b (558) ferric/cupric reductase from rabbit duodenal brush border membranes. Biochem Biophys Res Comm 291:220–225

Knopfel M, Smith C, Solioz M (2005) ATP-driven copper transport across the intestinal brush border membrane. Biochem Biophys Res Comm 330:645–652

Lee J, Prohaska JR Dagenais SL, Glover TW, Thiele DJ (2000) Isolation of a murine copper transporter gene, tissue specific

expression and functional complementation of a yeast copper transport mutant. Gene 254:87–96

Lee J, Pena MMO, Nose Y, Thiele DJ (2002a) Biochemical characterization of the human copper transporter Ctr1. J Biol Chem 277:4380–4387

Lee J, Petris MJ, Thiele DJ (2002b) Characterization of mouse embryonic cells deficient in the Ctr1 high affinity copper transporter. J Biol Chem 277:40253–40259

Li JH, DeSouza RC (1977) Effects of Ag+ on frog skin: interaction with oxytocin, amiloride and ouabain. Experientia 33(4):433–436

Mackenzie NC, Brito M, Reyes AE, Allende ML (2004) Cloning, expression pattern and essentiality of the high-affinity copper transporter1 (ctr1) gene in zebrafish. Gene 328:13–20

Morgan IJ, Henry RP, Wood CM (1997) The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na+ and Cl− transport. Aquat Toxicol 38:145–163

Nadella SR, Bucking C, Grosell M, Wood CM (2006a) Gastrointestinal assimilation of Cu during digestion of a single meal in the freshwater rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol-C 143:394–401

Nadella SR, Grosell M, Wood CM (2006b) Physical characterization of high-affinity gastrointestinal Cu transport in vitro in freshwater rainbow trout *Oncorhynchus mykiss*. J Comp Phys B. E-Pub. doi:10.1007/s00360-006-0101-z

Nelson N (1999) Metal-ion transporters and homeostasis. EMBO J 18:4361–4371

Oestreicher P, Cousins RJ (1984) Copper and zinc absorption in the rat: Mechanism of mutual antagonism. J Nutr 115:159–166

Pyle GG, Kamunde CN, McDonald DG, Wood CM (2003) Dietary sodium inhibits aqueous copper uptake in rainbow trout, *Oncorhynchus mykiss*. J Exp Biol 206:609–618

Rangachari PK, Matthews J (1985) Effect of Ag+ on isolated bullfrog gastric mucosa. Am J Physiol 248:G443–G449

Schwartz JH, Steinmetz PR (1977) Metabolic energy and P_{CO_2} as determinants of H+ secretion by turtle urinary bladder. Am J Physiol 233:F145–F149

Sharp PA (2003) Ctr1 and its role in body copper homeostasis. Int J Biochem Cell Biol 35:288–291

Tennant J, Stansfield M, Yamaji S, Srai SK, Sharp PA (2002) Effects of copper on the expression of metal transporters in human intestinal Caco-2 cells. FEBS Lett 527:239–244

Turnlund JR, Keyes WR, Anderson HL, Acord LL (1989) Copper absorption and retention in young men at three levels of dietary copper by use of the stable isotope 65Cu. Am J Clin Nutr 49:870–878

Van Campen DR (1969) Copper interference with the intestinal absorption of zinc-65 by rat. J Nutr 97:104–108

Vascoboinik I, Mar J, Strausak D, Camakaris J (2001) The regulation of catalytic activity of the menkes copper-translocating p-type ATPase. J Biol Chem 276:28620–28627

Walser M (1970) Calcium transport in toad bladder: permeability to calcium ions. Am J Physiol 218:582–589

Wapnir RA (1991) Copper–sodium linkage during intestinal absorption: inhibition by amiloride. Proc Soc Exp Biol Med 196:410–414

Wapnir RA, Stiel L (1987) Intestinal absorption of copper: effect of sodium. Proc Soc Exp Biol Med 185:277–282

Wolf K (1963) Physiological salines for freshwater teleosts. Prog Fish Cult 25:135–140

Yun CH, Tse CM, Nath SK, Levine SA, Brant SR, Donowitz M (1995) Mammalian Na+/H+ exchanger gene family: structure and function studies. Am J Physiol 269:G1–G11