Abstract

The paper reviews the evidence for apparent sodium-dependent copper (Cu) uptake across epithelia such as frog skin, fish gills and vertebrate intestine. Potential interactions between Na⁺ and Cu during transfer through epithelial cells is rationalized into the major steps of solute transfer: (i) adsorption on to the apical/mucosal membrane, (ii) import in to the cell (iii) intracellular trafficking, and (iv) export from the cell to the blood. Interactions between Na⁺ and Cu transport are most likely during steps (i) and (ii). These ions have similar mobilities (λ) in solution (λ, Na⁺, 50.1; Cu²⁺, 53.6 cm² Int. ohms⁻¹ equiv⁻¹); consequently, Cu²⁺ may compete equally with Na⁺ for diffusion to membrane surfaces. We present new data on the Na⁺ binding characteristics of the gill surface (gill microenvironment) of rainbow trout. The binding characteristics of Na⁺ and Cu²⁺ to the external surface of trout gills are similar with saturation of ligands at nanomolar concentrations of solutes. At the mucosal/apical membrane of several epithelia (fish gills, frog skin, vertebrate intestine), there is evidence for both a Cu-specific channel (CTR1 homologues) and Cu leak through epithelial Na⁺ channels (ENaC). Cu²⁺ slow the amiloride-sensitive short circuit current (Isc) in frog skin, suggesting Cu²⁺ binding to the amiloride-binding site of ENaC. We present examples of data from the isolated perfused catfish intestine showing that Cu uptake across the whole intestine was reduced by 50% in the presence of 2 mM luminal amiloride, with 75% of the overall inhibition attributed to an amiloride-sensitive region in the middle intestine. Removal of luminal Na⁺ produced more variable results, but also reduced Cu uptake in catfish intestine. These data together support Cu²⁺ modulation of ENaC, but not competitive entry of Cu²⁺ through ENaC. However, in situations where external Na⁺ is only a few millimoles (fish gills, frogs in freshwater), Cu²⁺ leak through ENaC is possible. CTR1 is a likely route of Cu²⁺ entry when external Na⁺ is higher (e.g. intestinal epithelia). Interactions between Na⁺ and Cu ions during intracellular trafficking or export from the cell are unlikely. However, effects of intracellular chloride on the Cu-ATPase or ENaC indicate that Na⁺ might indirectly alter Cu flux. Conversely, Cu ions inhibit basolateral Na⁺K⁺-ATPase and may increase [Na⁺].

Keywords: Copper transport; Sodium transport; Gill microenvironment; Intestine; Amiloride

1. Introduction

Over the last 50 years there has been a wealth of research on the epithelial ion transport of sodium (Na⁺), which is arguably the most characterized solute transport process of all eukaryote cells and epithelia [1–8]. In most cases Na⁺ absorption across epithelia involves the diffusive entry of Na⁺ into the cell from the external medium through ion channels or protein carriers (facilitated diffusion). Export of Na⁺ from the cell into the blood is against the electrochemical gradient, and is ultimately driven by the ubiquitous (usually basolaterally located) Na⁺K⁺-ATPase (the “sodium pump” [9–14]). Under normal circumstances the Na⁺K⁺-ATPase utilizes the energy released from the hydrolysis of ATP to export 3 Na⁺ ions from the cell in exchange for 2 K⁺ ions entering the cell from the blood. The Na⁺ pump is therefore electrogenic and contributes to the resting membrane potential of all animal cells. In more recent times, there has been growing interest in the epithelial ion transport of other metals, including copper (Cu). The epithelial ion transport of Cu is of particular interest because a number of often fatal clinical conditions arise from genetic defects of Cu metabolism. In Menkes Disease a gene (ATP7A) encoding an epithelial cell
isoform of Cu-ATPase (a Cu\(^{+}\) pumping ATPase in the same family of proteins as the Na\(^{+}\) pump) is defective and these patients are unable to absorb dietary Cu across the intestinal epithelium, to regulate Cu in somatic epithelial cells (e.g. vascular endothelium, fibroblasts [15,16]). This results in a fatal Cu deficiency. Conversely, in Wilson’s Disease the absence of an hepatic isoform of the Cu-ATPase (defective ATP7B gene) results in failure of whole body Cu excretion and severe Cu toxicosis [17].

Hans Ussing was probably the first physiologist to recognize that Cu and Na\(^{+}\) ions shared some similarities in solute chemistry. Ussing and Zerahn [1] demonstrated in 1951 that 0.2 mg l\(^{-1}\) of copper sulfate added to the external medium could slow Na\(^{+}\) uptake across the isolated frog skin to the blood side (serosal) solution. Over the last 50 years there have been other reports of Na\(^{+}\) and Cu interactions in the epitheliology literature (Table 1). However, at first glance the evidence seems to be conflicting. Some data suggest that Na\(^{+}\) and Cu ions may share a common pathway for uptake into epithelial cells from the external medium, e.g. through ion channels. Alternatively, Cu absorption across epithelia may occur via Cu-specific pathways that are somehow modulated by the presence of external Na\(^{+}\). This apparent conflict in the literature my arise because of differences in the choice of the epithelia used for the experiments and the experimental conditions. However the existing literature is dispersed and has not been constructed around a rationale defining similarities (or differences) in the epithelial transport of the two metals. It is currently unclear exactly how much of apparently “Na\(^{+}\)-sensitive” Cu transport can be attributed to new Cu-specific pathways that are modulated by Na\(^{+}\), or whether it is simply non-specific Cu flux through existing Na\(^{+}\) uptake routes. Copper probably moves across cell membranes as the Cu\(^{2+}\) or Cu\(^{+}\) ion, and these charged solutes will be influenced by changes in membrane potential. Unfortunately, Cu inhibits the Na\(^{+}/K\(^{+}\) -ATPase which contributes to the resting membrane potential of cells. If membrane potential is altered, then so is the electrochemical gradient for Cu uptake from the medium. Thus, apparent “Na\(^{+}\)-dependent” Cu uptake could also be artifacts of changes in Na\(^{+}/K\(^{+}\) -ATPase activity. In this paper we adopt an approach in the manner of Hans H. Ussing, by starting with the fundamental processes in epithelial ion transport, reviewing the evidence for Na\(^{+}\) and Cu interactions, and building this evidence into the fundamental models for Cu or Na\(^{+}\) transport in order to resolve the controversies outlined above. In this paper we use “Cu\(^{+}\)” to describe Cu, and give valency (Cu\(^{+}\) or Cu\(^{2+}\)) only where a particular oxidation state is known to be relevant.

2. Fundamental steps in solute transfer

The fundamental steps of solute transfer through epithelial cells include: (i) adsorption of the solute on to the surface of the cell membrane, (ii) import of the solute across the cell membrane in to the cell, (iii) intracellular trafficking and/or storage of the solute in membrane bound compartments, and (iv) export of the solute from the cell into the blood (Fig. 1). The initial adsorption of solutes on to the cell surface may involve a series of physico-chemical events, depending on the complexity of the epithelial surface. For example, electrolytes may have to diffuse through layers of relatively unstimred (static or non flowing) water and negotiate pathways through complex mixtures of mucoproteins secreted on to the surface of the epithelia. Import of solutes into the cell from the external medium may involve a variety of carriers (e.g. symporters, antiporters, and channels). Once inside the cell, solutes may be trafficked (direct by intracellular carrier proteins) to particular sub-cellular compartments (e.g. mitochondria, endoplasmic reticulum) where they may be stored, or become available for export from the cell at a later time. Export from the cell into the blood

<table>
<thead>
<tr>
<th>Epithelium/cell type</th>
<th>Observations</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frog skin</td>
<td>Aqueous additions of Cu inhibit Na uptake</td>
<td>[1]</td>
</tr>
<tr>
<td>Frog skin</td>
<td>Cu(^{2+}) alters (I_{m}) Na(^{-})-dependent (increased (K_{m}) and (V_{\text{max}})), amiloride-sensitive, Cu(^{2+}) binding to external surface of Na(^{+}) channels.</td>
<td>[20]</td>
</tr>
<tr>
<td>Frog skin</td>
<td>Dose-dependent increase in (I_{m}) maximum effect with 50 (\mu)M Cu(^{2+}), abolished by 30 (\mu)M amiloride; implies Cu(^{2+}) competes for amiloride binding site.</td>
<td>[21]</td>
</tr>
<tr>
<td>Perfused rat intestine</td>
<td>Copper uptake rates in isotonic NaCl; jejunum 57.5, and ileum 64.4 pM min(^{-1}) cm; Na(^{-}) substitution (NMG) 15 and 18 pM min(^{-1}) cm, respectively. Cu kinetics; (V_{\text{max}}=47.5) pM min(^{-1}) and (K_{m}=21) (\mu)M. Anaesthesia present.</td>
<td>[22]</td>
</tr>
<tr>
<td>Perfused rat intestine</td>
<td>Removal of NaCl from lumen slows the disappearance of Cu from the mucosal solution; NaCl-dependent component accounts for 15% of overall Cu flux.</td>
<td>[23]</td>
</tr>
<tr>
<td>Perfused catfish intestine</td>
<td>Indirect effects on Na uptake via Cl(^{-}) removal; both tissue Na(^{-}) and Cu levels reduced. No circumstantial evidence of Cu entry on Na(^{-})/H(^{+}) exchanger.</td>
<td>[24]</td>
</tr>
<tr>
<td>Rainbow trout gills</td>
<td>In freshwater, 55 (\mu)g l(^{-1}) Cu inhibits net Na(^{-}) uptake, 55% and 49% reduction in (V_{\text{max}}) and (K_{m}), respectively. Injury is reversible, (V_{\text{max}}) partial recovery over 28 days of exposure.</td>
<td>[25]</td>
</tr>
<tr>
<td>Rainbow trout gills</td>
<td>In freshwater, inhibition of Na(^{-}) influx, net efflux of Na(^{-}) in the presence of 6.5 (\mu)M Cu at pH 7.6</td>
<td>[26]</td>
</tr>
<tr>
<td>Rabbit kidney homogenates</td>
<td>Noncompetitive inhibition with respect to Na(^{-}), K(^{+}), and ATP binding; IC(_{50}) for Cu inhibition of Na(^{+}/K(^{+}) -ATPase is 0.1 (\mu)M. Cu(^{2+}) interference with thiol groups on subunits and Mg(^{2+}) binding.</td>
<td>[27]</td>
</tr>
</tbody>
</table>
may involve a variety of carriers, especially primary transporters that use energy stores (e.g. hydrolysis of ATP) to move solutes against the electrochemical gradient into the blood. The cytosolic protein carriers involved in intracellular trafficking of solutes may also have to bind to, or at least stop adjacent to, these ion transporters to ensure a continuous supply of solute at the basolateral membrane for export from the cell. The overall process of solute absorption across epithelia is therefore complex and involving many steps. For the convenience of study, it is often useful to break these steps down into small components or ion binding events. These steps may be described in more detail from a thermodynamic perspective. For example, Fig. 1 shows thermodynamic steps $K_1$–$K_6$, representing the equilibrium constants (log $K$ is inversely related to affinity) for all the steps in solute transfer. See text for details.

3. Literature on interactions between Na$^+$ and Cu transport

Schematic diagrams of Na$^+$ and Cu transport are shown in Fig. 2. However, only a few papers have explored interactions between Na$^+$ and Cu transport since the initial observation by Ussing and Zerahn [1] showed that CuSO$_4$ slowed Na$^+$ uptake across isolated frog skin (Table 1). A series of papers on frog skin [18–20] culminated in the demonstration that micromolar concentrations of external Cu (Cu$^{2+}$) caused a dose-dependent stimulation of the short circuit current, $I_{sc}$, which could be abolished by amiloride (N-amidino-3,5-diamino-6-chloropyrazine-carboxamide, Sigma, Poole, Dorset) which blocks epithelial Na$^+$ channels (ENaC) probably by binding to the alpha subunit of the channel [21]. Flonta et al. [21] further refined the kinetics to conclusively demonstrate the effect of amiloride, and more
importantly, repeated the experiments successfully with amiloride-like analogues that were unlikely to chelate Cu ions in solution (e.g. with 6 chloro-3,5-diaminopyrazine-2-carboxamide). Overall, these observations on frog skin imply that Cu may interfere with Na⁺ entry into epithelial cells by binding to the external amiloride-binding site on the ENaC. This Cu binding to the channel would seem to discourage the notion of Cu²⁺ (or Cu⁺) entry into cells via nonspecific leak through Na⁺ channels, and is supported by observations on the perfused rat intestine (Table 1). Wapnir and co-workers [22,23] demonstrated that the removal of the luminal Na⁺ slows apparent Cu uptake by around 75%. If Cu and Na⁺ were competing for entry through the same channel one would expect Na⁺ removal to enhance Cu entry; and this is clearly not the case. Furthermore, simultaneous removal of both Na⁺ and Cl⁻ reduced the NaCl-sensitive component to 15% of the overall apparent Cu flux in rat intestine (Table 1), implying either a
chloride-effect or indirect effects of altered membrane potential on Cu flux. In the catfish intestine, Handy et al. [24] demonstrated that removal of luminal Cl_\text{\text{o}} completely abolished transepithelial Cu uptake. In the same experiment, intestinal tissue Na^+ concentrations tended to decline in response to Cu exposure.

Copper could interfere with Na^+ transport across epithelial cells into the blood in a number of ways (Fig. 2). One of the main concerns regarding apparent Na^-sensitive Cu uptake is that it is an artifact of inhibition of the Na^+-K^+ -ATPase. Inhibition of Na^+ uptake into the blood has clearly been demonstrated; for example, in terms of Na^+ uptake kinetics across freshwater fish gills ([25,26], Table 1). Pharmacological investigations also show that Cu^2+ noncompetitively inhibits the Na^+-K^+-ATPase by nonspecific binding to thiol groups on the subunits of the transporter, and also by binding to the Mg^{2+} binding site [27]. Regardless of the precise mechanism of inhibition, blockade of the Na^+ pump will normally result in two major effects: (a) elevation of intracellular Na^- ([Na^{+}]_\text{\text{i}}) because Na_\text{\text{i}} efflux to the blood is prevented and (b) an associated slow decline in resting membrane potential [9,13]. The consequences of these events for Cu transport are outlined below. Elevation of [Na^+]_\text{\text{i}}, would reduce the concentration gradient for Na_\text{\text{c}} entry into the cell and presumably slow the inward diffusion of Na_\text{\text{c}} through mucosal membrane (or apical membrane) ion channels. In an experiment this effect might manifest as an apparent lack of disappearance of radiolabelled Na^+ from the external medium during Cu exposure (e.g. Ref. [23]), which in turn, could be misinterpreted as evidence for competitive uptake of Cu^2+ through an ENaC. This misinterpretation can be avoided by measuring tissue [Na^+]_\text{\text{i}}. If the Cu-effect is mediated via inhibition of the Na^+ pump, then failure of Na^+ uptake across the mucosal membrane into the cell should also be accompanied by a paradoxical accumulation of Na^+ in the epithelial tissue (caused by failure of Na^+ export to the blood). Unfortunately, several authors have failed to make the latter measurements (e.g. [22,23]). Another theoretical possibility is slowed Cu uptake due to changes in the electrochemical gradient. The equilibrium potential (E_{\text{Cu}}) for Cu is about +60 mV (E_{\text{Cu}} = 30 \log [Cu^{2+}]_\text{\text{o}}/[Cu^{2+}]_\text{\text{i}}), assuming [Cu^{2+}]_\text{\text{o}} = 1 \text{mM} and [Cu^{2+}]_\text{\text{i}} = 10 \text{nM} in animal cells, and with a resting membrane potential (V_m) of −70 mV, there is a voltage of 130 mV driving passive Cu influx into the cell from the external medium (that is, E_{\text{Cu}} − V_m). However, in practice activity of the Na^+ pump is largely insensitive to voltage at the resting membrane potential of animal cells [13], suggesting that Cu-independent inhibition of Na^+-K^+ -ATPase will only dissipate V_m by a few millivolts. Clearly, this will not alter the driving force for passive Cu entry into animal cells very much. Thus, elevation of [Na^+]_\text{\text{i}} would seem the most likely reason for misinterpreting Na^-sensitive Cu uptake as evidence for competition for entry through a common pathway. Application of the logic above may resolve some of the apparently conflicting conclusions in the current literature on Na^-dependent Cu uptake by animal cells, which has until now focused on Cu or Na^+ uptake across the mucosal membrane into epithelial cell via epithelial cation channels. None of the literature to date reflects on the notion that transepithelial ion movements are usually coordinated events that involve communication (cross-talk) between the mucosal and basolateral membranes, and apart from Handy et al. [24] there is little consideration of possible NaCl-dependent pathways for Cu-flux on the basolateral/serosal membrane of epithelia.

4. Does Cu^{2+} compete with Na^+ for binding to epithelial surfaces?

The first step in the movement of any ion across a cell membrane is the provision of a readily available supply of the solute to the membrane surface. The overall process of adsorption onto the cell membrane surface is influenced by several factors [28–32] including:

1. The free metal ion concentration in the bulk solution.
2. The number and type of solute binding ligands on the epithelial surface.
3. The rate of ion uptake and any associated replacement of surface ligands.
4. Unstirred layer formation on the extracellular surface of the cell membrane.

In the context of comparative physiology, these processes have been mostly investigated at the surface of fish gills and frog skin with respect to Na^+ transport [33–37], and in ecotoxicology for the uptake of toxic metals by fish gills [38–42]. The components that make up the surface interface on epithelia include (see Fig. 1): (i) the bulk solution which is the freely exchangeable external medium (e.g. freshwater for gills, luminal fluid for intestine), (ii) unstirred layers of waters (relatively non-mobile solvent layers adjacent to the membrane surface), (iii) mucus (secreted by the epithelia), (iv) the glyocalyx on the cell surface and associated external binding sites on membrane-bound ion transporters.

A comparison of the main factors influencing the adsorption of Na^+ and Cu^{2+} is described (Table 2). If we consider the bulk solution, in most biological systems [Na^+]_\text{\text{o}} is at least 1000 times more abundant than [Cu^{2+}]_\text{\text{o}}. The crystal-line ionic radius of Cu^{2+} is about half that of Na^+, but the large hydration shell for Cu^{2+} results in these ions having similar mobilities (\lambda) in solution (Table 2). Consequently, in a hypothetical solution with equal numbers of Cu^{2+} and Na^+, both metals would have an equal chance of diffusing to a biological membrane. However, the much higher stability constants for either Cu^{2+} or Cu^+ compared to Na^+ for anionic ligands in solution ensure that even when total Cu and Na contents are the same, the [Cu^{2+}]_\text{\text{o}} is likely to be two to three orders of magnitude lower than that for [Na^+]_\text{\text{o}}. Thus, Na^+ will easily out compete Cu^{2+} in bulk solution.
### Table 2
A comparison of factors influencing the adsorption of sodium and copper ions to cell membranes

<table>
<thead>
<tr>
<th>Factor</th>
<th>Sodium</th>
<th>Copper (II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Me(^{n+})]_{(aq)}</td>
<td>Freshwater, 1–2 mM gut lumen, 10–100’s mM</td>
<td>Freshwater, &lt;0.1 µM gut lumen, 10’s µM</td>
</tr>
<tr>
<td>Ionic radius (pm)</td>
<td>99</td>
<td>57 (199, hydrated)</td>
</tr>
<tr>
<td>Ionic mobility ((\lambda, \text{cm}^2 \text{Int. ohms}^{-1} \text{equiv}^{-1}))</td>
<td>50.1</td>
<td>53.6</td>
</tr>
<tr>
<td>Stability Constants ((- \log K, 25 , ^{\circ}C))</td>
<td>0.7 Cu(^{2+}); 6.8 Cu(^{+}); 14.7</td>
<td></td>
</tr>
<tr>
<td>Number/type of ligands</td>
<td>Numerous fixed anions</td>
<td>Numerous fixed anions</td>
</tr>
<tr>
<td>Saturation of ligands</td>
<td>Gill; 1–2 nmol cm(^{-2})</td>
<td>Gill; 30–40 nmol g(^{-1})</td>
</tr>
<tr>
<td>Mucus production</td>
<td>Non-irritant</td>
<td>Irritant</td>
</tr>
<tr>
<td>Binding Affinity (e.g. (\log K) for gills)</td>
<td>About 5.9 Binding order Na(^{+})&gt;Cu(^{3+})&gt;Cu(^{2+}) (sulfated ligands) reversed for carboxylated ligands.</td>
<td>About 7.4</td>
</tr>
</tbody>
</table>

Ionic radii and mobilities from Ref. [67]. Stability constants Ref. [68]. Binding affinity for gills Cu [41,42] and ligand binding orders from Ref. [44].

The situation may be reversed amongst the complex mucopolysaccharides and other fixed anions of the unstirred layer. The fixed anionic ligand composition of epithelial surfaces involves a complex mixture of phosphated, sulfated, and carboxylated ligands derived for a plethora of glycosylated and sialated mucopolysaccharides [35–37,43,44]. Carboxylated ligands are particularly common and physico-chemical theory predicts that Cu\(^{2+}\) ions will bind to these at least 100 times more strongly than Na\(^{+}\). These stronger binding characteristics of Cu\(^{2+}\) in the unstirred layer should balance out the advantage of abundance that Na\(^{+}\) has in the bulk solution. Thus, if adsorption processes are experimentally measured the overall outcome for Na\(^{+}\) and Cu\(^{2+}\) should be similar. This appears to be the case. For example in gill epithelia, Handy and Eddy [34] developed a series of “rapid solution dipping” experiments to estimate radiolabelled Na\(^{+}\) adsorption to the gills of freshwater fish. This approach involves the rapid transfer of fish through a series of carefully defined electrolyte solutions in order to measure ion binding events on the gill and body surface. Fig. 3 illustrates the time course of a rapid solution dipping experiment to resolve Na\(^{+}\) adsorption time to the gills and body surface of rainbow trout (Oncorhynchus mykiss). This experiment shows that only 5 and 2 nmol (absolute amount not concentration) of Na\(^{+}\) appear in the entire blood volume and whole liver, respectively, over 45 s compared to 35 nmol associated with the fish surface/epithelial cells, an apparent adsorption of 88% of the Na\(^{+}\) compared to 12% transfer into the blood (Fig. 3). On the basis of this first experiment, an optimum adsorption time of 30 s was selected to study the concentration dependence of Na\(^{+}\) binding to gill and body surface (Fig. 4). This latter experiment indicates that Na\(^{+}\) binding ligands on the gill surface saturate when the water [Na\(^{+}\)] exceeds 1 mM, and that the body surface of the fish has a much higher capacity for Na\(^{+}\) binding compared to the gills (Fig. 4), probably arising from the thicker mucus coat (more ligands) on the latter.

This approach of “rapid solution dipping” was simultaneously developed by Reid and McDonald [45] to investigate toxic metal binding to the gills. Playle and coworkers then refined the techniques and concepts towards a practical model for predicting metal toxicity to fish on the basis of gill–metal binding affinities (e.g. for Cu ions, see Refs. [41,42,46,47]). These experiments showed that the binding affinity (log K) of the gills for Cu is about 7.4, and although precise values for Na\(^{+}\) remain to be determined, it is clear for the fish gill at least, that both Cu\(^{2+}\) and Na\(^{+}\) saturate on the gill at nanomolar concentrations (e.g. Cu 30–40 nM g\(^{-1}\) [42], and Na\(^{+}\) 1–2 nmol cm\(^{-2}\), Fig. 4). Thus, Na\(^{+}\) and Cu\(^{2+}\) ions appear to have a roughly equal chance of adsorption to epithelia, and therefore equal opportunity to influence ion availability for the next step in the overall transport process. In short, the apical/mucosal carriers see an
5. Is there a common influx pathway for Na\(^+\) and Cu ions into epithelial cells?

The transcellular uptakes of both Na\(^+\) and Cu ions into the blood are compared in Fig. 2. It is clear that events at the mucosal membrane are central to possible interactions between Na\(^+\) and Cu uptake. Early experiments on frog skin started with the notion that Cu\(^{2+}\) and Na\(^+\) ions may compete for entry through ENaC. However, as the pharmacology of ENaC become available it became apparent that simple competition for a common influx pathway (except in dilute medium such as freshwater) was an unlikely explanation. Instead Cu might modulate the function of ENaC. A more detailed account of these developments follows below.

The frog skin literature (see above) initially focussed attention on whether or not Na\(^+\) and Cu\(^{2+}\) ions enter the cell through a common ENaC. This idea was based on a logical progression of physical chemistry. The Cu\(^{2+}\) (not hydrated) would presumably fit the Na\(^+\) channel since it has a smaller crystalline ionic radius than Na\(^+\) (Table 2). However, as Li et al. [27] and Flonta et al. [21] suggest, Cu\(^{2+}\) is highly reactive with proteins, and will bind to –SH groups on the exterior of the channel rather than go through it. This is supported by the observation in frog skin that Cu\(^{2+}\) does not stop the Na\(^+\) current [21]. However, most of the frog skin experiments have been performed under short circuit conditions where [Na\(^+\)]\(_o\) is over 100 mM. It might therefore not be surprising if the Na\(^+\) influx continues in the face of a relatively low [Cu\(^{2+}\)]\(_o\), (around 50 \(\mu\)M or less). Interestingly, Grosell and Wood [72] have recently shown Na\(^+\)\(_o\)-dependent Cu uptake from the water by the gills of freshwater fish. This suggests that, provided the [Na\(^+\)]\(_o\) is around 1 mM or less, Cu\(^{2+}\) entry into the epithelia can be accommodated by nonspecific leak through an ENaC.

Our laboratory has recently explored Na\(^+\)\(_o\)-dependent Cu uptake in the isolated perfused catfish intestine (Tables 3 and 4), using the perfusion protocol described by Handy et al. [24]. In these experiments intestines were perfused with 20 \(\mu\)M total Cu in the luminal solution (mucosal side), with or without 2 mM amiloride hydrochloride (Sigma) added to the luminal solution (normal Na\(^+\) both sides, 140 mM). A second series of experiments explored the effect of low luminal [Na\(^+\)] on Cu uptake (NaCl replaced by choline chloride, so that luminal [Na\(^+\)] was 3 mM). Serosal Na\(^+\) was normal (140 mM) in these experiments. In all experiments the luminal solution, serosal perfusate and intestinal tissue were monitored for metal composition and Cu uptake over 4 h.

The addition of 2 mM amiloride inhibited Cu absorption, particularly in the middle region of the intestine, so that Cu accumulation in the tissue was reduced by 50% overall (Table 3) and net Cu flux to the serosal compartment was almost abolished in 4 h (Table 4). The failure of external Cu to cross the mucosal membrane into the tissue can be explained by the effects of amiloride which can cause a 20% closure of ENaCs during Cu exposure [21]. Interestingly, the amiloride sensitivity of the middle intestine (Table 3) is coincident with a region of high chloride dependence for Cu uptake [24]. It is likely that chloride depletion in our previous experiments [24] slowed Cl\(^-\)/Co conductance through the cystic fibrosis transmembrane conductance regulator (CFTR). A loss of CFTR conductance is a potent down regulator of ENaCs [48]. Therefore, it is possible that chloride depletion in our previous experiments [24] would not only slow putative basolateral Cu–Cl symport but also mask Na\(^+\)\(_o\)-sensitive components of Cu uptake into the intestinal epithelial cells.

The latter may be important in Na\(^+\)\(_o\)-replacement experiments which tend to lower tissue chloride as well, and may therefore down-regulate Na\(^+\) channel opening. This notion is supported by the observation that removal of Na\(^+\)\(_o\) tends to slow Cu absorption in catfish intestine (Tables 3 and 4). The response to Na\(^+\)\(_o\) removal was more variable than the effect of amiloride, although all fish responded to some extent, three out of the six fish showed much strong responses to Na\(^+\)\(_o\) replacement. Similar to observations on the rat intestine (Table 1), the data argue against simple competition between Na\(^+\) and Cu for entry into the cell through an ENaC.
Table 3
Ionic composition of perfused African Catfish intestine after addition of 2 mM amiloride, or Na\textsuperscript{o} removal, from the luminal solution

<table>
<thead>
<tr>
<th>Tissue ion concentration (µM g\textsuperscript{-1} dry weight)</th>
<th>Treatment</th>
<th>Region of the intestine</th>
<th>Anterior</th>
<th>Middle</th>
<th>Hind</th>
<th>Whole intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Cu]</td>
<td>Control</td>
<td>0.28±0.10</td>
<td>0.30±0.10</td>
<td>0.45±0.21</td>
<td>0.34±0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amiloride</td>
<td>0.14±0.06</td>
<td>0.08±0.02*</td>
<td>0.29±0.10</td>
<td>0.17±0.02*</td>
<td></td>
</tr>
<tr>
<td>[Na\textsuperscript{+}]</td>
<td>Control</td>
<td>331.3±85.6</td>
<td>563.6±107.3</td>
<td>476.3±166.9</td>
<td>457.0±119.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amiloride</td>
<td>297.6±97.1</td>
<td>284.6±92.2</td>
<td>445.0±106.3</td>
<td>342.4±98.5</td>
<td></td>
</tr>
<tr>
<td>[K\textsuperscript{+}]</td>
<td>Control</td>
<td>259.9±63.2</td>
<td>304.7±77.1</td>
<td>422.6±83.8</td>
<td>325.0±76.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amiloride</td>
<td>204.9±63.2</td>
<td>194.5±60.7</td>
<td>241±61.9</td>
<td>213±61.9</td>
<td></td>
</tr>
<tr>
<td>Water (%)</td>
<td>Control</td>
<td>81.2±0.8</td>
<td>85.5±1.0</td>
<td>85.6±0.7</td>
<td>84.0±0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amiloride</td>
<td>83.2±0.9*</td>
<td>85.0±1.0</td>
<td>86.6±1.1</td>
<td>82.8±0.8</td>
<td></td>
</tr>
</tbody>
</table>

Series 2: low Na\textsuperscript{+} experiments

| [Cu]                                                          | Control   | 0.10±0.03                | 0.12±0.04 | 0.20±0.06 | 0.12±0.04 |
|                                                               | Low sodium| 0.07±0.02                | 0.05±0.02 | 0.06±0.03 | 0.05±0.02 |
| [Na\textsuperscript{+}]                                       | Control   | 267.0±29.3               | 264.3±30.8| 360±63.9  | 335.1±28.3 |
|                                                               | Low sodium| 132.0±12.6*              | 169.7±16.8*| 173.4±50.4*| 158.0±24.3* |
| [K\textsuperscript{+}]                                        | Control   | 259.9±43.7               | 286.1±35.8| 254.9±21.8| 277.6±37.6 |
|                                                               | Low sodium| 201.7±20.0               | 232.6±22.6| 221.8±32.6| 218.7±23.4 |
| Water (%)                                                     | Control   | 79.3±0.5                 | 82.2±0.4  | 83.6±0.6  | 81.6±0.4* |
|                                                               | Low sodium| 78.9±0.2                 | 79.9±0.2  | 79.5±0.7  | 79.4±0.19* |

Everted intestines from African catfish (Clarias gariepinus) were serosally perfused for 4 h at 22 °C in the presence or absence of 2 mM amiloride hydrochloride (series 1 experiments), or with NaCl substituted for choline chloride (“Low sodium”, series 2 experiments) so that the luminal [Na\textsuperscript{+}] is 3 mM compared to a nominal 140 mM in normal Na\textsuperscript{+} controls. Note, the fish in series 2 were older animals on growing from the same stock used in series 1. Fish in series 1 and 2 weighted (mean ± S.E.) 674±5 g (n=16) and 1355±165 g (n=12) respectively. Serosal [Na\textsuperscript{+}] is 140 mM (normal) in all experiments. All intestines were perfused in the presence of a nominal 20 µM Cu added as CuSO\textsubscript{4} to the luminal bath, to give measured [Cu\textsubscript{o}], of around 10 µM (optimum for Cu uptake).

Date are mean±S.E. Series 1, n=6 for control, n=8 amiloride treated. In series 2, n=6/treatment.

1 Hind gut different from whole gut within treatment (ANOVA, P<0.05).
2 Significant difference between control and experimental treatment (amiloride or low Na\textsuperscript{+} as appropriate) within region of intestine (t-test, P<0.05; 3-value where P=0.06).
3 Significant difference from anterior gut within treatment (t-test, P<0.05 in series 1; ANOVA, P<0.05 in series 2).

The major electrolytes showed the expected changes in response to amiloride or Na\textsuperscript{o} removal, with intestinal tissue Na\textsuperscript{+} and K\textsuperscript{+} declining (Table 3). Declining tissue Na\textsuperscript{+} will reduce Na\textsuperscript{+}K\textsuperscript{+}-ATPase activity [9,10], and perhaps Cu directly inhibited the reuptake of K\textsuperscript{+} on the Na\textsuperscript{+}K\textsuperscript{+}-ATPase, to decrease tissue K\textsuperscript{+}. Tissue moisture content was largely unaffected (Table 3), and water fluxes were variable with a tendency to decline and become negative during experiments (as expected, [24]). Interestingly, there is evidence of a fish weight (age)-effect in the control data, with Cu fluxes being lower in the larger (older) fish used in the second series of experiments. This is well known for Cu homeostasis in animals where whole body Cu declines with age [49].

An alternative hypothesis to Cu entry through ENaCs is for Cu to enter epithelial cells through a Cu-specific channel (Fig. 2b). A gene encoding a membrane protein with channel characteristics has been identified (CTR1) with homologues in yeasts and mammals [50,51]. Recently, Lee et al. [52] attempted the first physiological characterization of this transporter using an oocyte expression system. The Cu flux pathway was Cu-specific, of high affinity, and energy independent. The Cu flux was also stimulated by high [K\textsuperscript{+}], indicating the pathway could be opened by membrane depolarisation, a typical voltage-sensitive channel characteristic [52]. This also implies that transient hyperpolarisation (for example via [Na\textsuperscript{+}], substitution) might increase the probability of channel closure; thus causing an apparently Na\textsuperscript{+}-sensitive decrease in Cu uptake as observed in rat (Table 1) and catfish intestine (Table 4). The pharmacology of the newly discovered Cu channel is unknown; if CTR1 is amiloride-sensitive, then we should rethink the notion of Cu leak through ENaCs. CTR1 is ubiquitously expressed in mammalian tissue [52], and co-expression of CTR1 and ENaCs seems likely. This offers a combination of pathways for Cu entry into epithelial cells depending on [Na\textsuperscript{o}]. For example, when [Na\textsuperscript{o}] is 10–100 mM (e.g. intestinal epithelia) CTR1 may be the likely route of Cu entry across the mucosal membrane into cells, whilst in epithelia routinely exposed to low [Na\textsuperscript{o}] (e.g. freshwater fish gills or frog skin) then Cu leak through an ENaC may suffice. Interestingly, this notion is supported by observations on rat intestine, where the Na\textsuperscript{o} dependence of Cu uptake is lost in animals fed a high salt diet [53]. Grosell and Wood [72] have demonstrated both Na\textsuperscript{o}-dependent (probably ENaC) and a high-affinity Na\textsuperscript{o}-independent Cu uptake mechanism on the apical membrane of fish gills. The latter might be CTR1. Clearly, determination of the Na\textsuperscript{o} depend-
Table 4
Effect of amiloride or removal of external Na⁺ on net flux rates for Cu, K⁺, and water across perfused catfish intestine

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Flux rate (mM g⁻¹ h⁻¹)</th>
<th>Initial</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jₙet Cu</td>
<td>Control</td>
<td>Control 0.53 ± 0.20</td>
<td>0.11 ± 0.03⁴</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amiloride</td>
<td>Amiloride 0.15 ± 0.05*</td>
<td>0.03 ± 0.004*</td>
<td></td>
</tr>
<tr>
<td>Jₙet Na⁺</td>
<td>Control</td>
<td>Control N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amiloride</td>
<td>Amiloride N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Jₙet K⁺</td>
<td>Control</td>
<td>Control 1.67 ± 0.40</td>
<td>1.29 ± 0.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amiloride</td>
<td>Amiloride 0.99 ± 0.2</td>
<td>0.89 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Jₙet H₂O</td>
<td>Control</td>
<td>Control 45.20 ± 17.2</td>
<td>24.53 ± 39.6⁹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amiloride</td>
<td>Amiloride 2.80 ± 9.20</td>
<td>-49.80 ± 26.6⁹</td>
<td></td>
</tr>
</tbody>
</table>

Net flux rates are calculated from the cumulative appearance of the relevant metal ion or water in the serosal compartment according to Ref. [24]. Initial rates and overall rates are for the first 10 min (except Na⁺, 20 min) and 4 h of the experiment, respectively. Negative values indicate a net loss from the serosal solution, whilst positive values indicate a net influx to the serosal compartment.

* Significant difference from initial rate within treatment (F-test, P < 0.05 series 1; t-test series 2, P < 0.05).

** Significant difference between control and treated (2 mM amiloride or low Na⁺ as appropriate) within region of intestine (t-test, P < 0.05). N.D., no data. Other details as in Table 3.

ence of CTR1 is critical to this debate, and to our knowledge this experiment has not been performed.

Another theoretical possibility is for Cu entry on the apical Na⁺K⁺2Cl⁻ cotransporter or related NaCl and KCl cotransporters. The small amount of available evidence is conflicting. In the rat gut, furicoside has no effect on Cu uptake in the presence of [Na⁺], [23], but in catfish intestine the strong dependence of Cu uptake on luminal [Cl⁻]₀ does not exclude Cu entry on Na⁺K⁺2Cl⁻ cotransporter [24]. Copper entry into epithelial cells on the proton-coupled divalent metal ion transporter 1 (DMT1, [54]) is possible where acidosis of the external medium enables this transporter to operate. The latter would seem unlikely in functionally normal intestine, but could be an important route of Cu entry for animals in slightly acidic freshwater environments.

6. Intracellular trafficking of Na⁺ and Cu

The intracellular traffickings of Na⁺ and Cu²⁺ (or more likely Cu⁺) are vastly different. Cu ions are always chaperoned, whilst Na⁺ ions are not (Fig. 2). Consider first the movement of Na⁺ through the cell. The intracellular movement of Na⁺ is not just a simple matter of diffusion. The spatial organization of the subcellular compartments (nucleus, organelles, cytoplasm) is carefully regulated by the cytoskeleton (e.g. Ref. [55]), to ensure that solutes arrive at the right compartment at the correct time to efficiently serve cellular biochemistry. The presence of mucoids and other large non-diffusible polyanions in the cell suggest that ion-exchange phenomena will greatly influence ion distributions in the cytoplasm and on the surface of organelles (review Ref. [56]), so that local differences in ion content occur. This ion exchange phenomena might enable very localized competitive ion exchange phenomena between Cu²⁺/Cu⁺ and Na⁺ for fixed anionic ligands. This competitive ion exchange will be particularly important on membrane surfaces next to metal transporters, since this may locally control the supply of free ions for transport across the membrane. For example, coincidental local delivery of Cu ions to membranes adjacent to a Cu transporter might displace Na⁺ ions from fixed anions on the membrane surface, depending on the fixed anions present and the relative free ion concentrations of Cu²⁺/Cu⁺ and Na⁺. If Cu ions successfully out-compete Na⁺ for binding sites on the membrane, then the transporter may see a sudden local increase in [Na⁺]. There is some evidence for ion exchange events producing local alternations in the membrane transport of Cu ions. Davis-Kaplan et al. [57] report an allosteric effect involving intracellular chloride channels, which alters apparent Cu delivery to the vesicular Cu-ATPase in yeasts. Such interactions indicate that local changes in [Cl⁻] may alter the activity of Cu-ATPase. This might involve altered ion exchange processes on the accessible surfaces of the Cu-ATPase where [Cl⁻] competes with fixed anions for Na⁺ and other major cations to modify the charge screening and so tertiary structure of the protein. Further research showing time lapse confocal imaging of [Na⁺]ₙ, [Cl⁻]ₙ and [Cu²⁺]ₙ is needed to investigate these possibilities.

The situation for Cu trafficking contrasts with that of [Na⁺], in that Cu is carried (mainly as Cu⁺) almost exclusively by specific metal-ion chaperones (Fig. 2b) and cytosolic [Cu²⁺], is probably nanomolar or less [58]. Thus, for the most part, Cu will be unlikely to interact with other electrolytes by chance. Measurements of Cu content in various membrane fractions from cells suggest that Cu is mostly located in the cytoplasmic and nuclear fractions (e.g. in liver cells, 20% nucleus, 65% cytosolic fraction, 10% mitochondria, 5% lysosomal fraction, [59]). Immunoflourescent staining of Cu-ATPase suggests a large portion of the cellular Cu is moved across the cell by a vesicular pathway involving the Golgi system [60], although other anion-dependent pathways exist [24]. Copper is also essential for the function of several key enzymes and specific chaperones may deliver Cu to these enzymes (Fig. 2b). For example, Cox17, an oligomer with three Cu binding domains, delivers to the inner mitochondrial membrane, where other carriers (Sco1, Cox1 and Cox2) may
facilitate loading of cytochromes with Cu [61–63]. Another Cu carrier called “copper chaperone to superoxide dismutase” (CCS) apparently delivers Cu specifically to cytosolic superoxide dismutase [64]. A family of peptides (Ato1 homologues) also deliver Cu to the ATPase in the Golgi network [65].

Copper trafficking is clearly highly regulated, which raises the question why an import solute such as Na⁺ is apparently not so controlled. Perhaps the much higher Na⁺ content of cells (20–30 mM), or [Na⁺], in cells (e.g. around 10 mM, [66]), compared to Cu content or [Cu²⁺], is sufficient to ensure Na⁺ delivery to cell compartments without specific carriers. Alternatively, perhaps carriers are not required given that Na⁺ will not overtly react with thiol groups on proteins, unlike Cu²⁺ where a chaperone is essential to avoid Cu²⁺-induced injury to the cell.

7. Na⁺ and Cu efflux from epithelial cells into the blood

Currently, there is no evidence that Na⁺ and Cu²⁺ share a common efflux pathway from epithelial cells into the blood (Fig. 2). Efflux of either ion into the blood is against the electrochemical gradient, and both metals use specific ATPases for this task. For Na⁺ the basolaterally located Na⁺K⁺-ATPase serves to export Na⁺ from the cell. There is no evidence that Cu ions can leak through this Na⁺ pump; indeed, the nonspecific inhibition of Na⁺K⁺-ATPase by Cu²⁺ [27] suggests this is extremely unlikely. The traditional view is that the vesicular Cu-ATPase serves Cu efflux from cells to the blood, e.g. in gut enterocytes [15] (Fig. 2b). Intracellular Cu⁺ loads into Golgi vesicles via the Cu-ATPase, and these vesicles are trafficked to the serosal membrane where the Cu is externalized to the blood by exocytosis. Thus, these ATPases are neither functionally nor spatially coincident in the cell and there is no logic for communication between Na⁺ and Cu⁺ here. However, Handy et al. [24] did demonstrate that Cu efflux to the blood from the intestinal epithelium was partly dependent on both luminal and serosal chloride concentration. This raises the possibility of anion-dependent Cu symport for Cu efflux from the cell to the blood, and with this, the chance of indirect effects of Na⁺ on Cl⁻ transport. For example, the influx of Cl⁻ into the intestinal cell from the gut lumen via the mucosal Na⁺K⁺2Cl⁻ cotransporter would depend on luminal Na⁺, which could indirectly alter Cl⁻ dependent Cu efflux from the cell to the blood.

8. Conclusion

The most logical place for interactions between Na⁺ and Cu transport are in the initial adsorption of these metals onto the epithelia, and the apical/mucosal pathways used for Na⁺ and Cu²⁺ entry into epithelial cells. At present there is evidence for both a Cu-specific channel (CTR1) and Cu leak through ENaCs. The latter may be more significant in situations where the external Na⁺ is only a few millimolar or less (fish gills, frog skin). However, this interpretation assumes that CTR1 will not show sensitivity to Na⁺ channel blocking agents. Interactions between Na⁺ and Cu ions during intracellular trafficking or export from the cell are unlikely. However, allosteric effects on intracellular chloride on the Cu-ATPase in yeast, and chloride-dependent Cu efflux in fish intestine raise the possibility that Na⁺ might indirectly alter Cu flux via effects on intracellular chloride.

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References

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