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Copper toxicity in cultured human skeletal muscle cells: the involvement of Na+/K+-ATPase and the Na+/Ca2+-exchanger

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Abstract Copper (Cu2+) intoxication has been shown to induce pathological changes in various tissues. The mechanism underlying Cu2+ toxicity is still unclear. It has been suggested that the Na+/K+ -ATPase and/or a change of the membrane permeability may be involved. In this study we examined the effects of Cu2+ on the Na+ and Ca2+ homeostasis of cultured human skeletal muscle cells using the ion-selective fluorescent probes Na+-binding benzofuran isophtalate (SBFI) and Fura-2, respectively. In addition, we measured the effect of Cu2+ on the Na+/K+ -ATPase activity. Cu2+ and ouabain increase the cytoplasmic free Na+ concentration ([Na+]i). Subsequent addition of Cu2+ after ouabain does not affect the rate of [Na+]i increase. Cu2+ inhibits the Na+/K+ -ATPase activity with an IC50 of 51 μM. The cytoplasmic free Ca2+ concentration ([Ca2+]i) remains unaffected for more than 10 min after the administration of Cu2+. Thereafter, [Ca2+]i increases as a result of the Na+/Ca2+-exchanger operating in the reversed mode. The effects of Cu2+ on the Na+ homeostasis are reversed by the reducing and chelating agent dithiothreitol and the heavy metal chelator N,N′,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN). In conclusion, SBFI is a good tool to examine Na+ homeostasis in cultured human skeletal muscle cells. Under the experimental conditions used, Cu2+ does not modify the general membrane permeability, but inhibits the Na+/K+-pump leading to an increase of [Na+]i. As a consequence the operation mode of the Na+/Ca2+-exchanger reverses and [Ca2+]i rises.

Key words Human skeletal muscle cells Copper toxicity · Sodium homeostasis Calcium homeostasis · Na+/K+-ATPase Na+/Ca2+-exchanger · DTT · TPEN

Introduction

Copper (Cu2+) is essential for various enzymes, e.g. cytochrome c oxidase [1]. Its concentration is carefully controlled by homeostatic mechanisms. In plasma and cytoplasm the Cu2+-binding proteins ceruloplasmin and metallothionein, respectively, play an important role [7, 10, 17]. Studies on the toxic effects of Cu2+ on liver, kidney and smooth muscle cells have shown severe pathological changes [20, 34]. However, the mechanism underlying Cu2+ toxicity is still unclear. Kramhoft et al. [21] attributed the Cu2+-induced increase of the Na+ influx into Ehrlich ascites tumour cells to either an inhibition of the Na+/K+ -ATPase, or to an increase of the Na+ permeability of the plasma membrane. Cu2+ has also been shown to alter general membrane properties and to increase rapidly passive permeability to both cations and anions in heart mitochondria [15], gill epithelial cells [29] and renal proximal tubule cells [19].

Since the Na+/K+ -ATPase is the primary mechanism for the active transport of Na+ and K+ between the extracellular and intracellular fluids, inhibition of this pump will seriously disrupt the steady-state level of the cytoplasmic free concentrations of Na+ and K+, ([Na+]i and [K+]i, respectively). On the other hand, if Cu2+ intoxication increases the membrane permeability, then simultaneous changes of Na+, K+ as well as Ca2+ will take place, since their steep transmembrane ion gradients can no longer be maintained. To our knowledge no studies have been carried out to show the direct consequences of Cu2+ intoxication for [Na+]i and/or Cu2+ concentration ([Ca2+]i) in living cells. Several methods have been applied to measure [Na+]i, Flame photometry and 22Na+ tracer experiments quantify total instead of free Na+, and are destructive assays [13]. Na+-selective microelectrodes are limit-
ed to relatively large cells, whereas nuclear magnetic resonance of $^{23}$Na demands large quantities of tissue [13].

In cultured human skeletal muscle cells we determined the effect of Cu$^{2+}$ on [Na$^+$], and [Ca$^{2+}$], by means of fluorescent indicators specific for Na$^+$ or Ca$^{2+}$, i.e. Na$^+$-binding benzofuran isophthalate (SBFI) [25] and Fura-2 [12], respectively. We were unable to study ion homeostasis in separate fibres from human muscle, because it is impossible to isolate them intact. However, cultured skeletal muscle cells exhibit many characteristics which resemble the in vivo situation, e.g. signal transduction mechanism [6] and oxidative metabolism [4, 41]. Na$^+$/K$^+$-ATPase is abundantly synthesized in these cells. Its location in the plasma membrane is comparable to the in vivo situation [5]. Since the Cu$^{2+}$-induced increase of [Ca$^{2+}$], was delayed with respect to the [Na$^+$], increase, we investigated a possible involvement of the Na$^+$/Ca$^{2+}$-exchanger. Moreover, we examined the effect of dithiothreitol (DTT) on the Cu$^{2+}$-induced change of [Na$^+$], since DTT protects against heavy metal intoxication, by either preventing oxidation of thiol groups or by chelating the heavy metals from membranes and proteins [19, 33, 39]. To discriminate between both possibilities we also investigated the effect of $N,N',N''$-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a specific membrane-permeable heavy metal chelator without reducing properties [2].

Materials and methods

Materials. SBFI/AM, Fura-2/AM and pluronic F-127 were purchased from Molecular Probes, Eugene, Ore., USA; and gramicidin D, nigericin, monensin, ouabain and TPEN from Sigma, St Louis, Mo., USA.

Human skeletal muscle cell cultures. Muscle biopsies from m. quadriceps, m. biceps or m. rectus abdominis were obtained from individuals without any known muscular disorder, as approved by the Committee on Medical Ethics. Samples were dissociated and the isolated satellite cells were cultured on glass coverslips (10x30 mm, fluorescence ratio measurement; diameter 22 mm, digital imaging) in serum-containing media as described by Jacobs et al. [16]. All experiments were performed with myotubes obtained after 7 days of differentiation.

Fluorescence ratio measurement and digital imaging. [Na$^+$] or [Ca$^{2+}$], were determined by fluorescence ratio measurement [12, 25] and digital imaging [26]. Myotubes were washed with physiological salt solution (PSS) containing in mM: 125 NaCl, 10 NaHCO$_3$, 1 NaH$_2$PO$_4$, 5 KCl, 2 MgSO$_4$, 1.8 CaCl$_2$, 10 HEPES and 10 glucose; pH 7.4 and loaded in 1 ml PSS with 10 mM SBFI/AM and 50 j.tM pluronic F-127, or in 1 ml PSS with 5 j.tM Fura-2/AM for 60-90 min at 37°C in the presence of 5% CO$_2$ and 95% air. Excess of dye was removed by washing thrice with PSS.

Coverslips were mounted into a thermostatically controlled (37°C) cuvet (ratio measurement) or heating chamber (imaging). During ratio measurements fluorescence was recorded with a Shimadzu RF-5000 spectrofluorophotometer at an emission wavelength of 492 nm (bandwidth 5 nm) and alternating excitation wavelengths of 340 and 380 nm (bandwidth 3 nm). Digital imaging was performed using the MagiCal hardware and TARDIS software of Joyce-Loebl (Gateshead, UK). A Nikon Diaphot epifluorescence microscope was used to focus the cells onto a charge-coupled device camera (Photonics Science, Robertsbridge, UK). The excitation light was passed alternately through filters of 340 and 380 nm (10 and 13 nm bandwidths, respectively) and the emission light was collected through a 400-nm dichroic mirror and a 492-nm bandpass filter. Video images were hardware averaged eight times, to reduce noise of the camera. The 340-to-380 ratio was calculated on a pixel-by-pixel basis. All fluorescence signals were corrected for background fluorescence and autofluorescence. Cu$^{2+}$ (added as sulphate), ouabain, DTT as well as TPEN, at concentrations as mentioned in the results, do not introduce autofluorescence or modify the fluorescence characteristics of SBFI or Fura-2. During the measurements the cells were superfused with PSS (2.5 ml/min; 37°C) with additions as indicated in the Results.

[Na$^+$], was calibrated by using PSS with varying Na$^+$ concentrations. Cells were clamped with gramicidin D, monensin and nigericin (5 j.tM each) at 5, 10, 15, 20, 30, 40 and 50 mM Na$^+$; [Ca$^{2+}$], was calibrated with 4 j.tM ionomycin in the presence of 10 mM Cu$^{2+}$ (pH 7.7) or 20 mM EGTA (pH 8.5).

Na$^+$/K$^+$-ATPase activity. The effect of Cu$^{2+}$ on the Na$^+$/K$^+$-ATPase activity in muscle cell homogenates was determined as described by Benders et al. [5]. Homogenates were incubated with 0.4 mg saponin per mg protein and the Na$^+$/K$^+$-ATPase activity was assayed in the presence of different Cu$^{2+}$ concentrations in the range of 0–100 j.tM.

Statistics. Data represent means ± SD. Statistical analysis was performed by means of the unpaired Student’s t-test and significance was set at P<0.01. Curve fittings were performed by (non-)linear regression analysis using SlideWrite Plus 5.00 (Advanced Graphics Software, Carlsbad, Calif., USA).

Results

Effects of Cu$^{2+}$ and ouabain on [Na$^+$], and [Ca$^{2+}$],

The relation between the ratio of the SBFI fluorescence and [Na$^+$], in cultured human skeletal muscle cells is shown in Fig. 1. The mean basal [Na$^+$], in the muscle cells is 12.4±3.2 mM (n = 7). Exposure of the cells to 50 j.tM Cu$^{2+}$ increases [Na$^+$], at a rate of about 2 mM/min (Fig. 2A). Inhibition of the Na$^+$/K$^+$-pumps with 500 nM...
Effects of Cu²⁺ and ouabain on [Na⁺]j and the dose/response curve of Cu²⁺. [Na⁺]j is given as a function of time upon addition of 50 μM Cu²⁺ (A), 500 nM ouabain (B) or both successively (C). D The dose/response curve of Cu²⁺ and the rate of increase of [Na⁺]j relative to the increase rate at 50 μM Cu²⁺. The correlation of the regression curve is 0.994 (P<0.01). For comparison, the effect of 500 nM ouabain is given (bar). The number of experiments is given between parentheses.

Ouabain affects [Na⁺]j in a similar way (Fig. 2B), while subsequent addition of 50 μM Cu²⁺ after 500 nM ouabain does not alter the rate of change of [Na⁺]j (Fig. 2C). The rate of increase of [Na⁺]j is half maximal at a Cu²⁺ concentration (EC₅₀) of approximately 25 μM (Fig. 2D). The average rates of [Na⁺]j change are presented in Table 1.

The resting [Ca²⁺]j, as measured with Fura-2 in cultured human skeletal muscle cells is 130±27 nM (n = 7) and is in line with published data [6, 31]. After addition of 50 μM Cu²⁺, [Ca²⁺]j remains unchanged for more than 10 min. Thereafter, [Ca²⁺]j increases at about 50 nM/min and reaches a plateau near to 500 nM, which is main-

Table 1 Rates of change of [Na⁺]j induced by various additions to cultured human skeletal muscle cells

<table>
<thead>
<tr>
<th>Series of experiments (n)</th>
<th>Addition</th>
<th>Subsequent addition</th>
<th>Rate of change of [Na⁺]j (mM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (7)</td>
<td>50 μM Cu²⁺</td>
<td></td>
<td>2.18±0.41</td>
</tr>
<tr>
<td>2 (7)</td>
<td>500 nM Ouabain</td>
<td></td>
<td>1.94±0.33</td>
</tr>
<tr>
<td>3 (5)</td>
<td>500 nM Ouabain</td>
<td>50 μM Cu²⁺</td>
<td>1.87±0.37</td>
</tr>
<tr>
<td>4 (4)</td>
<td>141 mM NMDG</td>
<td>PSS</td>
<td>-0.78±0.19a</td>
</tr>
<tr>
<td>5 (5)</td>
<td>50 μM Cu²⁺</td>
<td>100 μM DTT</td>
<td>2.22±0.45</td>
</tr>
<tr>
<td>6 (4)</td>
<td>50 μM Cu²⁺</td>
<td>100 μM TPEN</td>
<td>-1.38±0.22</td>
</tr>
</tbody>
</table>

Values represent means ± SD of the number of individual cultures (in parentheses)

a Extracellular Na⁺ and K⁺ are replaced by NMDG and the Na⁺/Ca²⁺-exchanger lowers [Na⁺]j. The Na⁺/Ca²⁺-exchanger becomes inhibited when [Na⁺]j reaches values near 0 nM.

b Basal [Na⁺]j is restored upon the reintroduction of extracellular Na⁺ and K⁺ by perfusion with PSS, NMDG, N-Methyl-D-glucamine; DTT, dithiothreitol; TPEN, N,N,N',N'-tetraakis(2-pyridylmethyl)ethylenediamine; PSS, physiological salt solution
Table 2  Rates of change of 
[Ca\(^{2+}\)]\(_{i}\) induced by various 
additions to cultured human 
skeletal muscle cells

<table>
<thead>
<tr>
<th>Series of</th>
<th>Addition</th>
<th>Subsequent</th>
<th>Rate of change of [Ca(^{2+})](_{i})</th>
</tr>
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<tbody>
<tr>
<td>experiments</td>
<td></td>
<td>addition</td>
<td>(nM/min)</td>
</tr>
<tr>
<td>1 (3)</td>
<td>50 (\mu)M Cu(^{2+})</td>
<td></td>
<td>50±10</td>
</tr>
<tr>
<td>2 (3)</td>
<td>500 nM ouabain</td>
<td></td>
<td>645±12</td>
</tr>
<tr>
<td>3 (4)</td>
<td>141 mM NMDG</td>
<td></td>
<td>590±118</td>
</tr>
<tr>
<td></td>
<td>50 (\mu)M Cu(^{2+})</td>
<td></td>
<td>-435±129(^a)</td>
</tr>
</tbody>
</table>

Values represent means ± SD of the number of individual cultures (in parentheses). If the additions do not instantaneously affect [Ca\(^{2+}\)]\(_{i}\), the data presented are the initial rate changes of [Ca\(^{2+}\)]\(_{i}\) at onset of the response.

* The NMDG-induced increase of [Ca\(^{2+}\)]\(_{i}\) activates the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase, which restores [Ca\(^{2+}\)]\(_{i}\) to the basal level and functioning of the Na\(^{+}\)/Ca\(^{2+}\)-exchanger becomes concomitantly inhibited.

Fig. 4 Dose/response curve of Cu\(^{2+}\) and the Na\(^{+}\)/K\(^{+}\)-ATPase activity. Values are means ± SD of 5 experiments. The correlation of the regression line is 0.994 (\(P<0.01\)). The Na\(^{+}\)/K\(^{+}\)-ATPase activity without Cu\(^{2+}\) was 22.1±3.2 mU/mg protein.

Discussion

We could demonstrate well the disturbing effect of Cu\(^{2+}\) on Na\(^{+}\) and Ca\(^{2+}\) homeostasis in cultured human skeletal muscle cells using the fluorescent probes SBFI and Fura-2, respectively. Cu\(^{2+}\) exposure to these cells resulted in a rapid increase of [Na\(^{+}\)], by inhibition of the Na\(^{+}\)/K\(^{+}\)-ATPase activity, rather than by a general increase of the membrane permeability. We conclude this from five observations. First, the rates of change of [Na\(^{+}\)], as induced by Cu\(^{2+}\) or ouabain are similar. Second, addition of Cu\(^{2+}\) after inhibition of the Na\(^{+}\)/K\(^{+}\)-pump by ouabain does not affect the rate of increase of [Na\(^{+}\)]. Third, the activity of Na\(^{+}\)/K\(^{+}\)-ATPase is inhibited by Cu\(^{2+}\). These observations agree with the Cu\(^{2+}\)-induced increase of Na\(^{+}\) and decrease of K\(^{+}\) concentration in erythrocytes [3, 14]. Furthermore, the IC\(_{50}\) of the Cu\(^{2+}\)-inhibited Na\(^{+}\)/K\(^{+}\)-ATPase

Na\(^{+}\)/K\(^{+}\)-ATPase activity

In the cultured muscle cells the activity of Na\(^{+}\)/K\(^{+}\)-ATPase is 22.1±3.2 mU/mg protein (\(n=5\)), which is in line with previous observations [5]. Cu\(^{2+}\) reduces the Na\(^{+}\)/K\(^{+}\)-ATPase activity in a dose-dependent manner (Fig. 4). The half-maximal inhibiting concentration (IC\(_{50}\)) is 51 \(\mu\)M.

Effects of DTT and TPEN on the Cu\(^{2+}\)- and ouabain-induced increase of [Na\(^{+}\)]

Addition of the reducing agent DTT (100 \(\mu\)M) reverses the Cu\(^{2+}\)-induced [Na\(^{+}\)] increase in cultured human skeletal muscle cells back to its resting level (Fig. 5A). An identical effect is established by the heavy metal chelator TPEN, at a concentration of 100 \(\mu\)M (Fig. 5B). The rate of decrease of [Na\(^{+}\)] as achieved by DTT or TPEN is about 1.3 mM/min (Table 1). After inhibition of Na\(^{+}\)/K\(^{+}\)-ATPase by ouabain, neither DTT nor TPEN are able to reverse the enhanced [Na\(^{+}\)] (Fig. 5C).

The described effects of Cu\(^{2+}\) and TPEN could also be visualized at the single cell level (Fig. 5D). The spatial distribution of [Na\(^{+}\)], in response to Cu\(^{2+}\) or TPEN, shown in pseudocolour representation, is homogeneous. All cultured skeletal muscle cells have a comparable sensitivity for Cu\(^{2+}\) as well as TPEN. The cell-to-cell variance is less than 10%.

Na\(^{+}\)/K\(^{+}\)-ATPase activity

[464]
**Fig. 5A–D** Effects of DTT and TPEN on the Cu$^{2+}$- or ouabain-induced increase of [Na$^+$]. Time function of [Na$^+$], upon subsequent addition of 100 μM DTT or TPEN after 50 μM Cu$^{2+}$ (A, B) or 500 nM ouabain (C). D Effects of Cu$^{2+}$ and TPEN on the temporal and spatial distribution of [Na$^+$], at the single cell level. [Na$^+$], is shown in false colours as indicated by the calibration bar at the bottom right of the figure. Cu$^{2+}$ (50 μM) or TPEN (100 μM) were added after 600 and 1200 s, respectively. Bar at the bottom right represents 100 μm.

activity is comparable to the EC$_{50}$ of the Cu$^{2+}$-induced increase of [Na$^+$]. These values are of the same order of magnitude as described for preparations of brain [37], gills [35], kidney and blood vessels [20]. Fourth, a general increase of the membrane permeability is not probable, since the time courses for the rises of [Na$^+$], and [Ca$^{2+}$], are quite different. [Na$^+$], increases instantaneously, whereas [Ca$^{2+}$], remains unaffected for more than 10 min upon the addition of Cu$^{2+}$. If an increase of the membrane permeability was involved, an abrupt increase of [Ca$^{2+}$], would be expected, because of the enormous Cu$^{2+}$ gradient across the plasma membrane. Moreover, a general change of membrane permeability caused by Cu$^{2+}$ would be monitored as an increased loss of SBFI or Fura-2 fluorescence [30], which was never observed. The delayed [Ca$^{2+}$], increase can be explained by the action of the Na$^+/Ca^{2+}$-exchanger operating in the reversed mode resulting from the Cu$^{2+}$-induced rise of [Na$^+$]. Fifth, after inhibition of the Na$^+/Ca^{2+}$-exchanger, i.e. when [Na$^+$], has become nearly 0 mM as provoked by NMDG, [Ca$^{2+}$], remains unchanged upon Cu$^{2+}$ addition. Besides, this implies that the membrane permeability of intracellular Ca$^{2+}$-stores, i.e. sarcoplasmic reticulum, has not been affected by Cu$^{2+}$ either.

Unlike other tissues, relatively little is known about the Na$^+/Ca^{2+}$-exchanger in skeletal muscle. Minor activities of this exchanger have been detected in skeletal muscle sarcolemma [11, 40]. Our data demonstrate the presence of a Na$^+/Ca^{2+}$-exchanger in human skeletal muscle cells. The Na$^+/Ca^{2+}$-exchanger is electrogenic and sensitive to the membrane potential and uses the energy from the Na$^+$ electrochemical potential gradient ($\Delta\mu_{Na^+}$) to extrude Ca$^{2+}$ from the cell against a large Cu$^{2+}$ gradient [36]. However, the antiporter can also operate in the reversed mode, i.e. Na$^+$ influx and Ca$^{2+}$ efflux by $\Delta\mu_{Ca^{2+}}$ [28, 32]. Whether the Na$^+/Ca^{2+}$-exchanger operates in the forward or the reversed mode depends on the intra- and extracellular concentrations of Na$^+$ and Ca$^{2+}$ as well as on the membrane potential difference, $\Delta\psi$. In cultured muscle cells the membrane potential ranges between -45 and -75 mV [9, 16, 18]. Accepting a [Na$^+$], of 12.4 mM, a [Ca$^{2+}$], of 130 mM, extracellular concentrations of Na$^+$ and Ca$^{2+}$ of 136 and 1.8 mM, respectively, and a membrane potential of -60 mV the Nernst equilibrium potential and electrochemical potential gradient of Na$^+$ and
Ca\textsuperscript{2+} can be calculated.\textsuperscript{1} \(\Delta t_i\text{Na}^+\) is found to be 12.0 kJ/mol and \(\Delta t_i\text{Ca}^{2+}\) 36.1 kJ/mol. Assuming a stoichiometry of 3 Na\textsuperscript{+} versus 1 Ca\textsuperscript{2+} [36], the two electrochemical potential gradients are balanced, i.e. \(3\Delta t_i\text{Na}^+ = \Delta t_i\text{Ca}^{2+}\), which implies that the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger will not be functioning when the muscle cells are at rest. However, if [Na\textsuperscript{+}] increases, as induced by Cu\textsuperscript{2+}, then the Nernst equilibrium potential and the electrochemical potential gradient for Na\textsuperscript{+} decrease and \(\Delta t_i\text{Ca}^{2+}\) exceeds \(3\Delta t_i\text{Na}^+\) (Fig. 6A). Consequently, the Na\textsuperscript{+}/Ca\textsuperscript{2+}-antiporter will operate as a Ca\textsuperscript{2+} influx pathway, i.e. the reversed mode.

The rise of [Ca\textsuperscript{2+}], is directly related to the driving force for Ca\textsuperscript{2+} influx: \(\Delta t_i\mu\text{Ca}^{2+} = 3\Delta t_i\mu\text{Na}^+\) [32] and becomes significant if the driving force exceeds 9 kJ/mol (Fig. 6B). On the other hand, it can be derived that the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger operates in the reversed mode too, if extracellular Na\textsuperscript{+} is replaced by NMDG. Moreover, the antipporter is not functioning when [Na\textsuperscript{+}] reaches values near to 0 mM, or extracellular Ca\textsuperscript{2+} is omitted.

Thus, the Cu\textsuperscript{2+}-induced [Na\textsuperscript{+}], rise reverses the operational mode of the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger and causes an elevation of [Ca\textsuperscript{2+}]. During the plateau phase an equilibrium is installed between the Ca\textsuperscript{2+}-influx and -uptake as mediated by the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger and sarcoplasmatic reticulum Ca\textsuperscript{2+}-ATPase, respectively. The resulting Ca\textsuperscript{2+} overload probably contributes to the Cu\textsuperscript{2+} toxicity. Sustained increases of [Ca\textsuperscript{2+}], can activate cytotoxic mechanisms as Ca\textsuperscript{2+}-dependent proteases and Ca\textsuperscript{2+}-mediated phospholipases, which result in disruption of cytoskeletal organization, impairment of mitochondrial function and cessation of ATP synthesis in muscle [24].

The biochemical mechanism underlying Cu\textsuperscript{2+} toxicity has mainly been related to the oxidation of sulphphydryl groups in membrane proteins [14, 23]. Na\textsuperscript{+}/K\textsuperscript{+}-ATPase contains many functional sulphphydryl groups and is vulnerable to Cu\textsuperscript{2+} and other heavy metal ions [20, 22, 29, 37]. DTT has widely been used to protect against damage of biological functions caused by Cu\textsuperscript{2+}, e.g. Cu\textsuperscript{2+}-induced haemolysis of erythrocytes [33]. In the present study we show that the increase of [Na\textsuperscript{+}], following inhibition of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase by Cu\textsuperscript{2+} is reversible by DTT. The reducing capacity of DTT has been thought as the main mechanism of protection for cells against heavy metal injury [34]. However, DTT is also able to chelate heavy metal ions, including Cu\textsuperscript{2+} [34]. To distinguish whether protection against Cu\textsuperscript{2+} intoxication is prevented by either reduction of thiol groups or chelation of Cu\textsuperscript{2+}, we have also studied the effect of TPEN, which lacks reducing properties [2]. Our results conclusively illustrate that TPEN restores the Cu\textsuperscript{2+}-induced [Na\textsuperscript{+}], elevation to the basal level at the same rate as DTT. This indicates that in the intact cells Na\textsuperscript{+}/K\textsuperscript{+}-ATPase is reactivated by internal reducing agents, e.g. glutathione, after chelation of Cu\textsuperscript{2+}. In vivo Cu\textsuperscript{2+}-chelating drugs as n-penicillamine or tetrathiomolybdate suppress abnormal histological changes in the liver due to Cu\textsuperscript{2+} accumulation [8, 38].

In conclusion, SBFI is a useful probe to investigate Na\textsuperscript{+} homeostasis and to assess net and unidirectional Na\textsuperscript{+} fluxes in cultured muscle cells. Cu\textsuperscript{2+} initially induces a [Na\textsuperscript{+}], rise by inhibition of the Na\textsuperscript{+}/K\textsuperscript{+}-pump without a concomitant general change of the membrane permeability. Following the increase of [Na\textsuperscript{+}], the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger operates in the reversed mode evoking a [Ca\textsuperscript{2+}], increase, which could contribute to the toxic action of Cu\textsuperscript{2+}.

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