Inhibition of Ca\(^{2+}\) Uptake in Freshwater Carp, *Cyprinus carpio*, During Short-Term Exposure to Aluminum

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**ABSTRACT**
In carp exposed to pH 5.2 in fresh water, the Ca\(^{2+}\) influx from the water is reduced by 31% when compared to fish in water of neutral pH. At pH 5.2, the Ca\(^{2+}\) influx but not Na\(^{+}\) uptake is decreased by aluminum (Al). Al reduces Ca\(^{2+}\) influx dose-dependently: a maximum 55% reduction was observed after 1–2 h exposure to 200 \(\mu g.l^{-1}\) (7.4 \(\mu M\)) Al. Branchial Ca\(^{2+}\) efflux is less sensitive to Al and affected only by exposure for more than 1 h to high Al concentrations. Na\(^{+}\) influx is not affected by concentrations Al up to 400 \(\mu g.l^{-1}\). Na\(^{+}\) efflux, similarly to Ca\(^{2+}\) efflux, increased when fish were exposed for more than 1 h to 400 \(\mu g.l^{-1}\). © 1992 Wiley-Liss, Inc.

Inorganic aluminum (Al) forms, especially Al-hydroxide complexes, are more toxic to fish than organic Al complexes (Driscoll et al., '80). Regardless of other ligands that may be present, Al\(^{3+}\) interacts with water. In solutions more acidic than pH 5 Al\(^{3+}\) exists as the hexahydrate Al(H\(_2\)O\(_6\))\(^{3+}\) ("free Al\(^{3+}\)"). As a solution becomes less acidic successive deprotonation of Al(H\(_2\)O\(_6\))\(^{3+}\) yields Al(OH\(_2\))\(^{2+}\) and Al(OH\(_3\))\(^{+}\). Circumneutral solutions give an Al(OH\(_3\))\(^{–}\) precipitate that dissolves in basic solutions due to formation of Al(OH\(_4\))\(^{–}\) (MacDonald and Martin, '88). The principle soluble form at neutral pH is Al(OH\(_3\))\(^{–}\) (Spry et al., '81). Aluminum toxicity to fish is pH-dependent and at pH 5.2, which is the most toxic pH for fish (Baker and Schofield, '82; Brown and Lynam, '82; Fjellheim et al., '85), Al(OH\(_2\))\(^{2+}\) and Al(OH\(_3\))\(^{2+}\) are the dominant species.

The aim of this study was to elucidate the effects of Al in acid water on Ca\(^{2+}\) and Na\(^{+}\) fluxes in freshwater fish. In these animals the primary site damaged by water-borne aluminum (Al) in acid water appears to be the branchial epithelium (Spry et al., '81), where most of the ion exchange with the environment takes place (Mayer-Gostan et al., '87; Fenwick, '89). So far only a few studies have been reported to show toxic effects of Al on branchial ion fluxes in aquatic species. In crayfish, Al at neutral pH had no effect on \(^{45}\)Ca uptake, but at pH 5.5 (200–1,000 \(\mu g.l^{-1}\), 7–37 \(\mu M\)) Al produced a small inhibition in addition to the inhibitory effect of low pH (Malley and Chang, '85). The lack of a dose-response relationship was ascribed to the limited solubility of Al salts, which would restrict an increase of the toxic monomeric species when the total Al concentration was increased. Short-term exposure of brown trout to 160 \(\mu g.l^{-1}\) Al had no effect on calcium fluxes at pH 4.5 (Reader and Morris, '88). In another study on brown trout, an Al concentration of 216 \(\mu g.l^{-1}\) at pH 4.5 reduced Na\(^{+}\) influx significantly, but Al addition at pH 5.4 (or pH 7.0) had no effect and Na\(^{+}\) efflux was not affected at any condition tested (Dalziel et al., '86). In the studies mentioned above, only the nominal Al concentrations were reported; at the end of most experiments the Al concentrations measured were markedly lower than at the start, which is most likely accounted for by binding of Al to the tank and to the fish. Hence, to establish a dose-response curve for the effects of Al on Ca-balance in freshwater fish, an exposure system is required in which Al levels are adequately controlled. Studies that fulfill this criterion are unknown to us at present. Therefore, we developed an exposure system for carp in which the Al concentration and the H\(^{+}\) concentration were kept constant during the exposure period. The short duration of the exposure was chosen to prevent transformation of Al species since we are interested in the mechanism of Al toxicity in this study more than in the long-term adaptation to Al.

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Impaired ionoregulation has been proposed as a major cause of mortality (Neville, '85) and we employed an experimental setup in which this hypothesis could be tested. Branchial Ca²⁺ and Na⁺ fluxes were found to be affected differentially by Al exposure. We conclude that Al may specifically interfere with Ca²⁺ transport sites in the branchial epithelium.

MATERIALS AND METHODS

Animals

Carp (Cyprinus carpio), weighing 70–120 g (95 ± 16 g, n = 34) were held in Nijmegen city tapwater (0.78 mM Ca²⁺, 3.15 mM HCO₃⁻, 0.61 mM Na⁺, 0.05 mM K⁺, 0.38 mM Mg²⁺, 0.66 mM Cl⁻, 0.32 mM SO₄²⁻, pH ~7.4) at 23°C. At 18 h before the experiment, fish were placed individually in opaque boxes (vol = 1.75 l) with a flow (100 ml.min⁻¹ per box) of well-aerated and thermostatted recirculating acclimation water (total vol. 140 l). This acclimation water approached tapwater in ion composition (0.8 mM CaCl₂, 0.33 mM NaHCO₃, 3.8 mM NaCl, 0.06 mM KCl, 0.20 mM MgSO₄, pH 7.2; Verbost et al., '89a); the background level of inorganic Al was less than 2 µg.1⁻¹, as determined by Inductively Coupled Plasma emission (ICP) analysis (Instrumentation Laboratory, Plasma 2000; measured at 167.1 nm in a vacuum monochromator).

Analytical techniques

For the analysis of blood plasma Ca²⁺, Na⁺, or Al content, a blood sample was taken by puncture from the caudal vessels after 4 h or exposure to Al using a heparinized syringe with a 23-gauge needle. Blood cells were removed by centrifugation (1 min, 10,000 g), and the plasma was stored at ~20°C until further analysis. Al concentration in water or plasma samples were determined as described above (ICP). All samples were acidified with 10 |xl 1 M H₂SO₄ per ml to dissolve all Al. Water and plasma total Ca content were measured colorimetrically using a calcium kit (Sigma Diagnostics). Water and plasma total Na content was determined with a flame photometer (FLM 3, Radiometer) coupled to an auto-analyzer (SA-20, Skalar). Plasma-free Ca²⁺ was determined with an Ionized Calcium Analyzer (ICA1, Radiometer). ⁴⁵Ca contents of water samples and tissue digestes were determined by liquid scintillation analysis. Aqueous samples (0.5 ml) were mixed with 4.5 ml Aqualuma scintillation fluid. ²⁴Na contents of water and tissue samples were determined directly by gamma counting and corrected for differences in counting efficiency resulting from volume differences. The protein concentration of membrand vesicles suspensions was determined with a Coomassie blue kit (Bio-Rad) using bovine serum albumine as standard.

Al exposure

Al, as an Al₂(SO₄)₃ stock solution, was continuously pumped (30 µl.min⁻¹) into the flux box using a roller pump. Concentrations of Al were empirically established such that the required Al concentrations were reached within one h and maintained at that level for at least another 3 h (Fig. 1). Water pH was maintained as described in following section.

Whole body Ca²⁺ and Na⁺ influx

One h prior to the start of the Al exposure, the water flow was stopped. The water in the flux boxes was slowly and stepwise acidified over a 45-min period with H₂SO₄/HAc (1 part 1 M H₂SO₄:1.3 parts 1 M HAc pH 5.0). Water pH was maintained between 5.1 and 5.3 and measured every 30 min throughout the experiment. The Ca²⁺ or Na⁺ influxes were determined over a 1-h period. By so doing we studied the effects of Al on the ion fluxes.
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after different exposure times (up to 4 h). The experiment was started by addition of tracer (45Ca, 1.0 MBq, 1 \(^{-1}\) or 24Na, 0.5 MBq, 1 \(^{-1}\)). Instantaneous mixing of the tracer with the water was guaranteed by aeration. The water-specific activity was determined on samples taken at the beginning, and after 30 min and 60 min tracer exposure. Fish exposed to low pH plus Al were run simultaneously with those exposed to low pH alone. After 1 h tracer exposure fish were anesthetized for 5 min by addition of 0.5 g MS222 (neutralized with NaHCO\(_3\)) or 40 mM NaCl (for 24Na uptake) to remove tracer bound to the outside of the fish in 45Ca and 24Na uptake measurements, respectively. Fish bodies were microwave-cooked (3 min) and homogenized in a blender with demineralized water (water volume: 65% of body weight). Quadruple samples of the homogenate (\(\sim 0.15 \text{ g} \) weighed to the nearest 3 decimals) were processed for determination of radioactivity as described earlier (Verbost et al., '89a).

**Whole body Ca\(^{2+}\) and Na\(^{+}\) efflux**

Eighteen h before the experiment, the fish received an intraperitoneal injection of 1.8 MBq 45Ca (45CaCl\(_2\) in 0.6% NaCl; 0.3 ml per fish) or 2 MBq 24Na (24Na\(_2\)CO\(_3\) in 0.6% NaCl; 0.32 ml per fish) and were placed in the flux boxes. A continuous flow of thermostatted tapwater (100 ml min\(^{-1}\) to each fish) for 18 h secured that all activity released by the fish was removed from the box. In this period the specific activity of the blood stabilized to a virtually constant level (Lafeber et al., '88). One h before the addition of Al to the water, the water pH was brought to 5.2 as described for the influx experiments. At zero time the gradual addition of Al was started. Here, too, control fish were run concurrently with Al-exposed fish. Water samples were taken hourly. After 3 to 4 h the fish were anesthetized as described above. Blood was taken as described above and plasma collected. Plasma radioactivity values were determined in duplicate. Plasma total Ca or Na concentrations were determined in triplicate.

**Preparation of membrane vesicles**

Resealed inside-out membrane vesicles were prepared from freshly collected human blood, according to Sarkadi et al. ('80). Sodium-heparin was used as an anticoagulant (100 IU.ml\(^{-1}\)). The membranes were 90% resealed with 52 % inside-out and 39 % rightside-out orientated vesicles (Verbost et al., '89b).

**In vitro Ca\(^{2+}\)-transport assay**

Plasma membrane vesicles were resuspended in 150 mM KCl, 1.5 mM MgCl\(_2\), 20 mM Hepes/Tris, pH 7.4 (or pH as indicated). ATP-dependent Ca\(^{2+}\)-transport was determined using a rapid filtration technique (Van Heeswijk et al., '84). Incubation media contained 150 mM KCl, 20 mM Hepes/Tris, 1 mM EDTA, and 1 mM CDTA as metal-ion buffers, 3 mM ATP, 1.5 mM Mg\(^{2+}\), 0.4 \(\mu\)M Ca\(^{2+}\), and Al\(^{3+}\) as indicated. The free Al\(^{3+}\) concentration along with the Ca\(^{2+}\) and Mg\(^{2+}\) concentration was controlled in this metal-ion buffer system. In this medium Al\(^{3+}\) is bound in two ways. First, in aqueous solutions Al\(^{3+}\) forms hydroxide complexes. Regardless of the other ligands present equilibria 1-3 must be considered in all solutions containing Al\(^{3+}\):

1. Al\(^{3+}\) + H\(_2\)O \(\rightarrow\) AlOH\(^{2+}\) + H\(^+\) \(\text{pK}_1 = 5.5\)
2. AlOH\(^{2+}\) + H\(_2\)O \(\rightarrow\) Al(OH)\(_2^+\) + H\(^+\) \(\text{pK}_2 = 5.6\)
3. Al(OH)\(_2^+\) + 2H\(_2\)O \(\rightarrow\) Al(OH)\(_4^-\) + 3 H\(^+\) \(\text{pK}_3 = 12.1\).

Second, the ligands ATP, EDTA, and CDTA bind Al\(^{3+}\). We developed a computer program that first calculates the Al\(^{3+}\) mole fraction and uses this information for adaptation of the Kd's for Al of the ligands in the buffer system (Kd - log[Al\(^{3+}\); Martin, '86). Thus at low pH where the free Al concentration is almost equal to the total Al concentration, the Kd's do not differ much from the uncorrected values (Ka ATP = 10.9, Kb ATP = 6.5, Ka EDTA = 16.1, Ka CDTA = 17.6). At higher pH less Al\(^{3+}\) is available and the adapted Kd's are lower, corresponding with the seeming decrease in affinity for Al\(^{3+}\). The adapted Kd's and the Kd's of the ligands for Ca\(^{2+}\) and Mg\(^{2+}\) (from Sillen and Martell, '64) were used in a matrix program (according to Van Heeswijk et al., '84) to calculate the free ion concentrations in the medium. The first and second protonations of the respective ligands were taken into account.

**Flux calculations**

Influx of Ca\(^{2+}\) or Na\(^{+}\) was calculated from the total body radioactivity after exposure to 45Ca or 24Na, respectively, and the mean tracer specific activities of the water. The value for whole body activity, corrected for the 65% water added and divided by the specific activity of the water, gives the whole body ion influx, also named the ion intake from the water (\(F_{in}\) see equation 1).

\[
F_{in} = \frac{q_\text{g} \cdot 1.65}{S \text{A}_w} \cdot 100
\]
Fin is expressed in (μmol h \(^{-1}\) (100 g fish) \(^{-1}\)), where \(q'\) is the activity per g wet weight and \(S_{AW}\) is the water activity per mol Ca or Na.

Efflux of Ca\(^{2+}\) or Na\(^{+}\), the ion loss to the water \((F_{\text{out}})\), was calculated from the tracer appearance rate (thus independent of binding of "effluxed" \(^{45}\)Ca) in the water \((dq'_{W}/dt)\) and the specific activity of the blood (see equation 2).

\[
F_{\text{out}} = \frac{dq'_{W}/dt \times 100}{S_{Ap} \times W}
\]

\(F_{\text{out}}\) is expressed in (μmol h \(^{-1}\) (100 g fish) \(^{-1}\)), where \(dq'_{W}/dt\) is the cumulative water activity per h, \(S_{Ap}\) is the plasma radioactivity per mol Ca or Na, and \(W\) is the fish wet weight in g.

Results are presented as means ± SD (unless otherwise stated). For statistical evaluation the Mann-Whitney U-test was used. Significance was accepted for \(P < 0.05\).

RESULTS

Effects of water acidification on Ca\(^{2+}\) balance

Net Ca\(^{2+}\) uptake \((F_{\text{net}} = F_{\text{in}} - F_{\text{out}})\), where \(F_{\text{in}}\) equals Ca\(^{2+}\) intake from the water and \(F_{\text{out}}\) equals the Ca\(^{2+}\) loss to the water) in freshwater of pH 7.2 was 5.3 μmol h \(^{-1}\) (100 g fish) \(^{-1}\). At pH 5.2 the Ca\(^{2+}\) uptake was reduced by 39% as a result of a decreased influx of Ca\(^{2+}\); Ca\(^{2+}\) efflux was not significantly affected (Fig. 2).

Effects of Al exposure on plasma Na\(^{+}\), Ca\(^{2+}\), and Al

A 4-h treatment with 400 μg l \(^{-1}\) Al (or less) in the water did not result in a change in plasma total Na, Ca, or Ca\(^{2+}\), nor did we observe an increase in total plasma Al concentration (Table 1).

Effects of Al on Ca\(^{2+}\)-balance (pH 5.2)

Figure 3 shows the effects of Al exposure (0, 30, 100, 200, or 400 μg l \(^{-1}\) Al in the water) on whole body Ca\(^{2+}\) intake and loss (hatched bars indicate calculated net uptake). Thirty μg l \(^{-1}\) Al had no effect on Ca\(^{2+}\) influx during a 4 h Al exposure. Exposure to 100 μg l \(^{-1}\) inhibited Ca\(^{2+}\) influx progressively with time. The inhibition was not significant for the first hour, but was at later times. In the second hour of exposure Ca\(^{2+}\) influx was reduced to 71% and in the fourth h to 53%. With 200 and 400 μg l \(^{-1}\) Al in the water Ca\(^{2+}\) influx was already significantly inhibited in the first hour of exposure (40–50%); no difference in the degree of influx inhibition occurred between these two Al-concentrations. The average influx inhibition in the second and fourth hour was 55%.

\[
\begin{array}{c|c|c}
\text{Al (μg l}^{-1}) & \text{Ca (mM)} & \text{Na (mM)} \\
\hline
0 & 2.67 ± 0.14 & 148 ± 6 \\
30 & 2.58 ± 0.20 & 138 ± 11 \\
100 & 1.35 ± 0.04 & 2.65 ± 35 \\
200 & 1.00 ± 0.07 & 1.40 ± 0.07 \\
400 & 283 ± 40 & \end{array}
\]

Values represent means of 5 to 6 animals (± S.D.). The Al exposure did not significantly change any of the parameters.
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Fig. 3. Effects of Al on carp whole body Ca\(^{2+}\)-influx and efflux at pH 5.2 in artificial freshwater. Bars represent means ± SEM (n = 6). The hatched bars indicate the net Ca\(^{2+}\) uptake. Influx and efflux values that are significantly different from their respective controls are marked with an asterisk and a small closed circle, respectively.

in a cytosolic medium Al\(^{3+}\) concentrations higher than 3.2 \times 10^{-12} M are unobtainable. By lowering the pH of the medium, it was possible to test the effect of higher concentrations of the metal. The decrease in pH on itself reduces the Ca\(^{2+}\) pump activity. However, Al\(^{3+}\) up to 2 \times 10^{-11} had no additional effect.

DISCUSSION

The major conclusion from this study is that Al exposure at pH 5.2 has differential effects on net Ca\(^{2+}\) and net Na\(^{+}\) uptake from the water. Ca\(^{2+}\) uptake was inhibited by Al (100 \mu g.l\(^{-1}\) or more) mainly because of inhibitory effects on Ca\(^{2+}\) influx, although high doses of Al (400 \mu g.l\(^{-1}\) or more) also affect Ca\(^{2+}\) efflux. The influx inhibition was found to be dose-dependent. Conversely, Na\(^{+}\) influx was not affected: net Na\(^{+}\) uptake is reversed to a net Na\(^{+}\) loss by 400 \mu g.l\(^{-1}\) Al through an increase in Na\(^{+}\) efflux.

It is furthermore concluded that the ion fluxes prove a much more sensitive parameter for the toxic effect of Al than blood ion content. Whereas exposure for 4 h to 400 \mu g.l\(^{-1}\) Al had no effect on either plasma calcium or sodium, Ca\(^{2+}\) influx was already significantly inhibited after 2 h of exposure to 100 \mu g.l\(^{-1}\) Al. Concentrations of Al in natural fresh waters readily rise to 400 \mu g.l\(^{-1}\) in situations where acid leaching of aluminum from soils occurs (Driscoll et al., '80; Spry et al., '81).

Ca\(^{2+}\) and Na\(^{+}\) influx

The observation that Na\(^{+}\) influx was not affected at the Al concentrations used demonstrates that the inhibition of the Ca\(^{2+}\) influx is not caused by a general damage inflicted upon the ion-transporting cells, but by a specific effect on the calcium uptake and transport route across these cells. The trans-epithelial Ca\(^{2+}\) uptake route has been elucidated for several fish species. It comprises entrance of Ca\(^{2+}\) via voltage independent Ca\(^{2+}\) channels in the apical membrane, transfer of calcium through the cytoplasm to the basolateral membrane, and extrusion of the ions across this membrane in the blood, via high-affinity Ca\(^{2+}\)-ATPase activity (Flik et al., '85a,b; Perry and Flik, '88). Al may cause inhibition of Ca\(^{2+}\) passage of the apical membrane by closure of the Ca\(^{2+}\) channels or it may cause a decrease in Ca\(^{2+}\) uptake by inhibition of the basolateral Ca\(^{2+}\) pumps. Blockage of Ca\(^{2+}\) channels by Al\(^{3+}\) at the Ca\(^{2+}\) binding site within the channels appears unlikely to us because of the large dif-
The erythrocyte membrane vesicles were preincubated 1 to 2 h at 4°C in KCl medium of the desired pH. Ca$^{2+}$-transport was measured at 0.4 μM Ca$^{2+}$.

Ca$^{2+}$ and Na$^{+}$ efflux

A possible explanation for the increased Ca$^{2+}$ and Na$^{+}$ effluxes during exposure to high Al concentrations may be that Al influences the junctional complexes of the epithelium. These junctions form a barrier for the paracellular movement of ions (i.e., Ca$^{2+}$, Na$^+$, and Cl$^-$. Ca$^{2+}$ is required for optimal functioning of junctional complexes (Alberts et al., '89). We hypothesize that high levels of Al affect the integrity of the junctional complexes by competition with Ca$^{2+}$ for binding sites.

Al solubility

A further increase in the Al concentration from 200 to 400 μg.l$^{-1}$ had no additional effect on Ca$^{2+}$ influx. This finding argues against an effect of extracellular Al on Ca$^{2+}$ channels. Calculations based on solubility constants of Martin ('86) show that under our conditions the maximum solubility of aluminum species (at pH 5.2: 340 μg.l$^{-1}$) was exceeded only with 400 μg.l$^{-1}$. Thus the inhibitory effects of Ca$^{2+}$ and Na$^{+}$ fluxes in our experiments cannot be explained by the suggestion of Payle et al. ('89) that disturbing effects of Al on ion fluxes in fish are caused by Al-precipitation at the gill surface.
At 400 μg L⁻¹ the formation of a solid precipitate (microcrystalline gibbsite) was not observed. However, the precipitation of Al(OH)₃(solid) is notoriously slow, particularly at low degrees of oversaturation and in the absence of suitable nuclei (Turner, ’76).

In water of circumneutral pH, 400 μg L⁻¹ Al did not affect Ca²⁺ influx from the water (results not shown). In neutral solutions of Al most Al occurs as Al(OH)₃ precipitate and Al(OH)₄⁻ is the only soluble Al species (Martin, ’86). There are two possible explanations for the absence of effect of Al at pH 7.2. Either both Al(OH)₃ and Al(OH)₄⁻ are nontoxic or Al(OH)₃ is nontoxic and the Al(OH)₄⁻ concentration is too low to be toxic.

Our experiments are the first to show dose-dependent inhibition of transepithelial Ca²⁺ uptake by Al. In a study on crayfish in tapwater of pH 5.5 Ca²⁺ influx was inhibited for 35% by Al, irrespective of the dose in a range from 200 to 1,000 μg L⁻¹ Al (Malley and Chang, ’85). This discrepancy may be related to the high initial Al concentration peak in such experiments and further to background levels of Al in tapwater (20–30 μg L⁻¹), and pH-dependent speciations of Al. In pilot studies we, too, found that the effects of Al were not dose-related when the metal was added at once at the beginning of the experiment. The present experiments performed with water of well-defined and controlled composition gave clear and reproducible results. Our results also seem incompatible with the finding of Reader and Morris (’88) that at low pH Al had no effect on calcium fluxes in brown trout. This could be caused by a difference in sensitivity between species but also by a difference in the pH adaptation. We adjusted the pH gradually, shortly before the flux experiment and this reduced the net influx of Ca²⁺ by 39%. Reader and Morris acclimated trout for 5 days to low pH and measured large net Ca²⁺ losses. During the first week of adaptation to pH 4.5, the iontransporting cells (chloride cells) in the gills are literally renewed and subsequently increase in amount (Wendelaar Bonga et al., ’90). In tilapia kept at pH 4.5 for 7 days, mainly degenerated (apoptotic) and immature chloride cells were present. If trout have a similar adaptation to low pH as tilapia, the 5 days of acclimation to pH 4.5 might have obscured a possible effect of Al on the Ca²⁺-transporting cells.

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