

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.

<http://hdl.handle.net/2066/16674>

Please be advised that this information was generated on 2019-06-16 and may be subject to change.

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

PIETER M. VERBOST, FLORIS P.J.G. LAFEVER,
FRANCISCUS A.T. SPANINGS, ELISABETH M. AARDEN, AND
SJOERD E. WENDELAAR BONGA

*Department of Animal Physiology, Faculty of Science, University of Nijmegen,
Toernooiveld, 6525 ED Nijmegen, The Netherlands*

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 $200 \mu\text{g.l}^{-1}$ ($7.4 \mu\text{M}$) 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 $400 \mu\text{g.l}^{-1}$. Na^+ efflux, similarly to Ca^{2+} efflux, increased when fish were exposed for more than 1 h to $400 \mu\text{g.l}^{-1}$ Al. © 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 $\text{Al}(\text{H}_2\text{O})_6^{3+}$ ("free Al^{3+} "). As a solution becomes less acid successive deprotonation of $\text{Al}(\text{H}_2\text{O})_6^{3+}$ yields $\text{Al}(\text{OH})^{2+}$ and $\text{Al}(\text{OH})_2^+$. Circumneutral solutions give an $\text{Al}(\text{OH})_3$ precipitate that dissolves in basic solutions due to formation of $\text{Al}(\text{OH})_4^-$ (MacDonald and Martin, '88). The principle soluble form at neutral pH is $\text{Al}(\text{OH})_4^-$ (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), $\text{Al}(\text{OH})_2^+$ and $\text{Al}(\text{OH})_3$ 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 ($200\text{--}1,000 \mu\text{g.l}^{-1}$, $7\text{--}37 \mu\text{M}$) 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 $160 \mu\text{g.l}^{-1}$ 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 $216 \mu\text{g.l}^{-1}$ 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.

Received June 7, 1991; revision accepted October 31, 1991.

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 Ca^{2+} and Na^+ fluxes were found to be affected differentially by Al exposure. We conclude that Al may specifically interfere with Ca^{2+} 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 Ca^{2+} , 3.15 mM HCO_3^- , 0.61 mM Na^+ , 0.05 mM K^+ , 0.38 mM Mg^{2+} , 0.66 mM Cl^- , 0.32 mM SO_4^{2-} , 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 CaCl_2 , 0.33 mM NaHCO_3 , 3.8 mM NaCl , 0.06 mM KCl , 0.20 mM MgSO_4 , pH 7.2; Verbost et al., '89a); the background level of inorganic Al was less than $2 \mu\text{g}\cdot\text{l}^{-1}$, 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 Ca^{2+} , 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 μl 1 M H_2SO_4 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 (FLM3, Radiometer) coupled to an auto-analyzer (SA-20, Skalar). Plasma-free Ca^{2+} was determined with an Ionized Calcium Analyzer (ICAL, Radiometer). ^{45}Ca contents of water samples and tissue digests were determined by liquid scintillation analysis. Aqueous samples (0.5 ml) were mixed with 4.5 ml Aqualuma scintillation fluid. ^{24}Na 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 membrane vesicles suspensions was determined with a Coomassie blue kit (Bio-Rad) using bovine serum albumine as standard.

Al exposure

Al, as an $\text{Al}_2(\text{SO}_4)_3$ stock solution, was continuously pumped (30 $\mu\text{l}\cdot\text{min}^{-1}$) 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 Ca^{2+} 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 $\text{H}_2\text{SO}_4/\text{HAc}$ (1 part 1 M H_2SO_4 :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 Ca^{2+} or Na^+ influxes were determined over a 1-h period. By so doing we studied the effects of Al on the ion fluxes

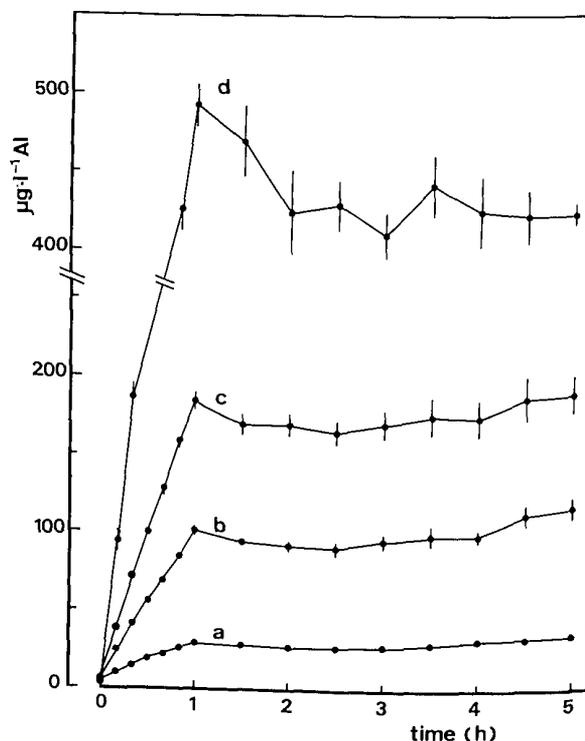


Fig. 1. The concentration of aluminum in flux boxes with fish during the gradual addition of Al (pH 5.2 in artificial freshwater). Values represent means \pm SEM ($n = 6$). The means of the Al concentrations measured from the second to fifth hour were 30.1 ± 0.7 (line a), 100.1 ± 1.9 (line b), 176.2 ± 3.3 (line c) and 438.3 ± 6.6 (line d) corresponding to 30, 100, 200, and 400 $\mu\text{g}\cdot\text{l}^{-1}$ Al, respectively, as used in the text and other figures.

after different exposure times (up to 4 h). The experiment was started by addition of tracer (^{45}Ca , 1.0 MBq.l $^{-1}$ or ^{24}Na , 0.5 MBq.l $^{-1}$). 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 anaesthetized for 5 min by addition of 0.5 g MS222 (neutralized with NaHCO_3), rinsed in acclimation water with 8 mM CaCl_2 (for ^{45}Ca uptake) or 40 mM NaCl (for ^{24}Na uptake) to remove tracer bound to the outside of the fish in ^{45}Ca and ^{24}Na 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 (~ 0.15 g weighed to the nearest 3 decimals) were processed for determination of radioactivity as described earlier (Verboost et al., '89a).

Whole body Ca^{2+} and Na^+ efflux

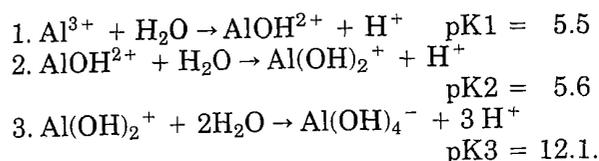
Eighteen h before the experiment, the fish received an intraperitoneal injection of 1.8 MBq ^{45}Ca ($^{45}\text{CaCl}_2$ in 0.6% NaCl ; 0.3 ml per fish) or 2 MBq ^{24}Na ($^{24}\text{Na}_2\text{CO}_3$ in 0.6% NaCl ; 0.32 ml per fish) and were placed in the flux boxes. A continuous flow of thermostatted 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 anaesthetized 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 $52 \pm 10\%$ inside-out and $39 \pm 13\%$ rightside-out orientated vesicles (Verboost 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 μM 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+} :



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 K_d 's for Al of the ligands in the buffer system ($K_d - \log[\text{Al}^{3+}]$; Martin, '86). Thus at low pH where the free Al concentration is almost equal to the total Al concentration, the K_d 's do not differ much from the uncorrected values ($K_a \text{ ATP} = 10.9$, $K_b \text{ ATP} = 6.5$, $K_a \text{ EDTA} = 16.1$, $K_a \text{ CDTA} = 17.6$). At higher pH less Al^{3+} is available and the adapted K_d 's are lower, corresponding with the seeming decrease in affinity for Al^{3+} . The adapted K_d 's and the K_d '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 ^{45}Ca or ^{24}Na , 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).

$$(1) F_{in} = \frac{q_g' \cdot 1.65}{SA_w} \cdot 100$$

F_{in} is expressed in $\mu\text{mol}\cdot\text{h}^{-1}\cdot(100\text{ g fish})^{-1}$, where q_g' is the activity per g wet weight and SA_w is the water activity per mol Ca or Na.

Efflux of Ca^{2+} or Na^+ , the ion loss to the water (F_{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).

$$(2) F_{out} = \frac{dq'_w/dt \cdot 100}{SA_p \cdot W}$$

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

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

RESULTS

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

Net Ca^{2+} uptake ($F_{net} = F_{in} - F_{out}$, where F_{in} equals Ca^{2+} intake from the water and F_{out} equals the Ca^{2+} loss to the water) in freshwater of pH 7.2 was $5.3\ \mu\text{mol}\cdot\text{h}^{-1}\cdot(100\text{ g fish})^{-1}$. 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, and Al

A 4-h treatment with $400\ \mu\text{g}\cdot\text{l}^{-1}$ 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 $400\ \mu\text{g}\cdot\text{l}^{-1}$ Al in the water) on whole body Ca^{2+} intake and loss (hatched bars indicate calculated net uptake). Thirty $\mu\text{g}\cdot\text{l}^{-1}$ Al had no effect on Ca^{2+} influx during a 4 h Al exposure. Exposure to $100\ \mu\text{g}\cdot\text{l}^{-1}$ 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 $400\ \mu\text{g}\cdot\text{l}^{-1}$ 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%.

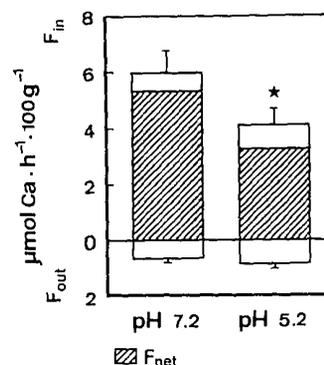


Fig. 2. Effects of water acidification on carp whole body Ca^{2+} uptake ($n = 4$). Hatched bars represent the net flux (influx minus efflux) of Ca^{2+} .

Ca^{2+} effluxes had increased significantly in the animals exposed to $400\ \mu\text{g}\cdot\text{l}^{-1}$ Al for 2 to 4 h (up to 72% after 4 h of exposure).

The net result is that Ca^{2+} uptake was reduced by Al in a dose-dependent way mainly because of the inhibitory action of the metal on the influx of Ca^{2+} . A maximum of 81% reduction in net Ca^{2+} uptake was observed after 4 h exposure to $400\ \mu\text{g}\cdot\text{l}^{-1}$ Al.

Effects of Al on Na^+ balance (pH 5.2)

Na^+ influx was not affected by 4 h of exposure to $400\ \mu\text{g}\cdot\text{l}^{-1}$ (or less) Al in the water (Fig. 4); however, Na^+ efflux had increased by 95% in the second hour of exposure and by 117% in the following 2 h. There was no effect in the first hour of exposure. Exposure to $200\ \mu\text{g}\cdot\text{l}^{-1}$ Al for 4 h had no effect on Na^+ -efflux.

Thus carp show a net uptake of Na^+ from the water at pH 5.2 ($9\ \mu\text{mol}\cdot\text{h}^{-1}\cdot(100\text{ g fish})^{-1}$) and a net loss of Na^+ ($23\text{--}27\ \mu\text{mol}\cdot\text{h}^{-1}\cdot(100\text{ g fish})^{-1}$) when exposed to $400\ \mu\text{g}\cdot\text{l}^{-1}$ Al for 2 to 4 h as a result of an increased Na^+ efflux.

Effect of Al^{3+} on Ca^{2+} -transport across erythrocyte membranes

In Table 2 the maximum solubility of Al^{3+} at various H^+ concentrations is listed showing that

TABLE 1. Plasma Na, Ca, and Al of fish exposed to $400\ \mu\text{g}\cdot\text{l}^{-1}$ Al for 4 h¹

	Control	$400\ \mu\text{g}\cdot\text{l}^{-1}$ Al
Na (mM)	148 ± 6	138 ± 11
Ca (mM)	2.67 ± 0.14	2.58 ± 0.20
Ca^{2+} (mM)	1.40 ± 0.07	1.35 ± 0.04
Al ($\mu\text{g}\cdot\text{l}^{-1}$)	283 ± 40	265 ± 35

¹Values represent means of 5 to 6 animals (\pm S.D.). The Al exposure did not significantly change any of the parameters.

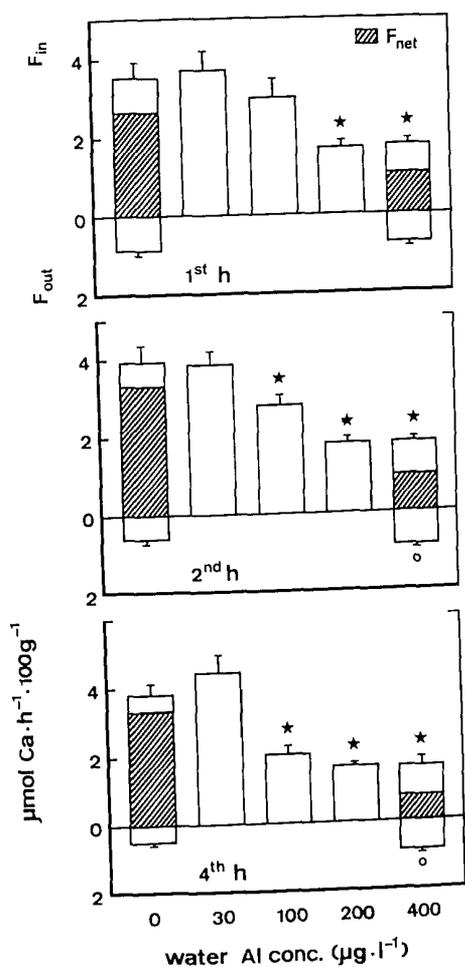


Fig. 3. Effects of Al on carp whole body Ca^{2+} -influx and efflux at pH 5.2 in artificial freshwater. Bars represent means \pm SEM ($n = 6$). The hatched bars indicate the net Ca^{2+} uptake. Influx and efflux values that are significantly different from their respective controls are marked with an asterisk and a small closed circle, respectively.

in a cytosolic medium Al^{3+} concentrations higher than $3\cdot 10^{-12}$ 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 $2\cdot 10^{-11}$ 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 ($100\ \mu\text{g}\cdot\text{l}^{-1}$ or more) mainly because of inhibitory effects on Ca^{2+} influx, although high doses of Al ($400\ \mu\text{g}\cdot\text{l}^{-1}$ 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

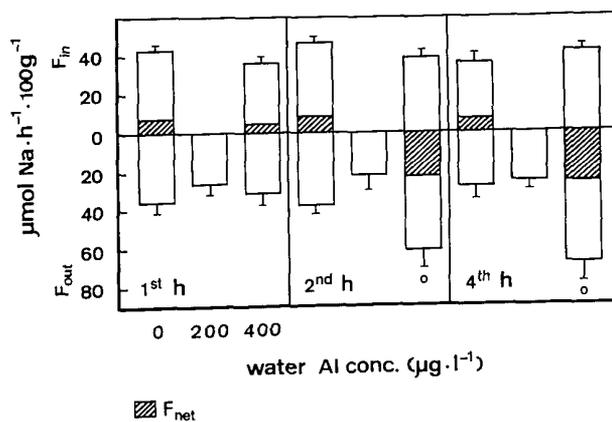


Fig. 4. Effects of Al on carp whole body Na^{+} influx and efflux in artificial freshwater of pH 5.2. Bars represent means \pm SEM ($n = 6$ for the first hour, $n = 5$ for the hours following). The hatched bars indicate the net Na^{+} uptake. Efflux values that are significantly different from their respective controls are marked with a small circle.

Na^{+} loss by $400\ \mu\text{g}\cdot\text{l}^{-1}$ 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 $400\ \mu\text{g}\cdot\text{l}^{-1}$ Al had no effect on either plasma calcium or sodium, Ca^{2+} influx was already significantly inhibited after 2 h of exposure to $100\ \mu\text{g}\cdot\text{l}^{-1}$ Al. Concentrations of Al in natural fresh waters easily rise to $400\ \mu\text{g}\cdot\text{l}^{-1}$ 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-

TABLE 2. Effect of Al^{3+} on ATP-dependent Ca^{2+} -transport in erythrocyte plasma membrane vesicles¹

pH medium	7.4	7.0	6.6	6.0	5.5
Max. Al^{3+} sol. ²	$3.2 \cdot 10^{-12}$	$5.0 \cdot 10^{-11}$	$7.9 \cdot 10^{-10}$	$5.0 \cdot 10^{-8}$	$1.6 \cdot 10^{-6}$
Highest $[Al^{3+}]$ tested (M)	$0.8 \cdot 10^{-12}$	$0.8 \cdot 10^{-12}$	$1.9 \cdot 10^{-12}$	$5.7 \cdot 10^{-12}$	$1.6 \cdot 10^{-11}$
Ca^{2+} -transport ³	4.69 ± 0.58	3.72 ± 0.25	3.40 ± 0.20	2.91 ± 0.23	1.64 ± 0.08
Control Ca^{2+} -transport ²	4.54 ± 0.32	3.60 ± 0.21	3.14 ± 0.89	2.65 ± 0.25	1.75 ± 0.25

¹The 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+} .

²Calculated using the formula $K_{sol} = [Al^{3+}]/[H^+]^3 = 10^{10.7}$ (Martin, '86).

³Transport activity in $nmol Ca^{2+} \cdot min^{-1} \cdot mg prot.^{-1}$

ferences in ion size. However, a reduction of Ca^{2+} influx could result from occupation of a regulatory site. The presence of a regulatory, high affinity Ca^{2+} binding site has been suggested in voltage dependent Ca^{2+} channels (Kostyuk et al., '83). Aluminium may also cause inhibition of transcellular movement of Ca^{2+} after it has accumulated in the chloride cells. The lagtime of inhibition (as seen with 100 $\mu g \cdot l^{-1}$ Al) may be indicative of such an Al accumulation. Once in the cytosol, Al^{3+} could disturb the Ca^{2+} extrusion over the basolateral membrane or upset the Ca^{2+} channels in the apical membrane from the cytosolic side. The in vitro studies with isolated plasma membranes showed no effect on the Ca^{2+} pump of Al^{3+} in concentrations even 10 times higher than maximally obtainable at cell-physiological pH. In these in vitro studies we used membrane vesicles prepared from human erythrocytes. The Ca^{2+} pump in these membranes has the same characteristics as the Ca^{2+} pump in the basolateral membrane from fish gills (Verbost et al., '89b), but the erythrocyte membrane preparation has some important advantages. It is not contaminated with intracellular membranes, it has a high specific activity for the Ca^{2+} transporting enzyme, and vesicles are easy to prepare. The effect of Al^{3+} ions was tested since for cellular enzymes this is the most clearly defined toxic species of Al (MacDonald and Martin, '88). The free Al^{3+} concentration was controlled with a metal-ion buffer, which is a necessity in such enzyme studies (Martin, '86). When Al^{3+} does not affect the basolateral Ca^{2+} pump, an indirect effect on the Ca^{2+} channels from Al^{3+} in the cytosol seems the most reasonable explanation. The Ca^{2+} channels are second messenger operated (Flik et al., '89) and Al^{3+} could have effects on the Ca^{2+} channels by acting on the second messenger(s) involved. Inhibition of adenylate cyclase and subsequently a decrease in cellular cAMP levels may be correlated with closing of Ca^{2+} channels in the apical membrane (Flik et al., '89). Since adenylate cyclase

depends very much on Mg^{2+} for its activity (Bockaert et al., '84; Maguire, '84) and because Al^{3+} has a high affinity for Mg^{2+} binding sites, which may lead to Mg^{2+} substitution (Martin, '86), it is tempting to suggest that Al has a specific effect on Ca^{2+} influx by disturbing the cAMP branch in the second messenger controlled Ca^{2+} influx. An effect of Al in the blood plasma on the serosal Ca^{2+} extrusion pump seems unlikely to us. During the short-term experiments, no significant amounts of Al enter the blood (Table 1). The plasma Al concentration in nonexposed animals (around 10 $\mu M = 0.27 mg \cdot l^{-1}$) may seem rather high. However, when taking into account the high natural total Al level in fish (2–5 mg/kg^{-1} wet weight; Sidwell et al., '78), the value is not surprising.

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 $\mu g \cdot l^{-1}$ 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 aluminium species (at pH 5.2: 340 $\mu g \cdot l^{-1}$) was exceeded only with 400 $\mu g \cdot l^{-1}$. Thus the inhibitory effects of Ca^{2+} and Na^+ fluxes in our experiments cannot be explained by the suggestion of Playle et al. ('89) that disturbing effects of Al on ion fluxes in fish are caused by Al-precipitation at the gill surface.

At $400 \mu\text{g}\cdot\text{l}^{-1}$ the formation of a solid precipitate (microcrystalline gibbsite) was not observed. However, the precipitation of $\text{Al}(\text{OH})_3$ (solid) is notoriously slow, particularly at low degrees of oversaturation and in the absence of suitable nuclei (Turner, '76).

In water of circumneutral pH, $400 \mu\text{g}\cdot\text{l}^{-1}$ Al did not affect Ca^{2+} influx from the water (results not shown). In neutral solutions of Al most Al occurs as $\text{Al}(\text{OH})_3$ precipitate and $\text{Al}(\text{OH})_4^-$ 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 $\text{Al}(\text{OH})_3$ and $\text{Al}(\text{OH})_4^-$ are non-toxic or $\text{Al}(\text{OH})_3$ is nontoxic and the $\text{Al}(\text{OH})_4^-$ concentration is too low to be toxic.

Our experiments are the first to show dose-dependent inhibition of transepithelial Ca^{2+} uptake by Al. In a study on crayfish in tapwater of pH 5.5 Ca^{2+} influx was inhibited for 35% by Al, irrespective of the dose in a range from 200 to $1,000 \mu\text{g}\cdot\text{l}^{-1}$ 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 ($20\text{--}30 \mu\text{g}\cdot\text{l}^{-1}$), 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 Ca^{2+} by 39%. Reader and Morris acclimated trout for 5 days to low pH and measured large net Ca^{2+} 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 Ca^{2+} -transporting cells.

ACKNOWLEDGMENTS

This research was supported through Grant EV4V-0117-NL (GDF) of the European Communities. We thank Mr. J. Eygensteyn for his excellent technical assistance in the absorption spectrophotometry. We are grateful to Mr. P. van der Zandt

for able assistance during pilot studies. Dr. G. Flik is acknowledged for many stimulating discussions and critical reading of the manuscript.

LITERATURE CITED

- Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J.D. Watson (1989) Cell junctions. In: *Molecular Biology of the Cell*, 2nd ed. M. Robertson, ed. Garland Publishing, New York, pp. 792-802.
- Baker, J.P., and C.L. Schofield (1982) Aluminum toxicity to fish in acidic waters. *Water Air Soil Pollut.*, **18**:289-309.
- Bockaert, J., B. Cantau, and M. Sebben-Perez (1984) Hormonal inhibition of adenylate cyclase, a crucial role for Mg^{2+} . *Mol. Pharmacol.*, **26**:180-186.
- Brown, D.J.A., and S. Lynam (1982) The effects of calcium and aluminum concentrations on the survival of brown trout (*Salmo trutta*) at low pH. TPRD/L/2347/n82, Central Electricity Generating Board, Leatherhead, England, p. 14.
- Dalziel, T.R.K., R. Morris, and D.J.A. Brown (1986) The effects of low pH, low calcium concentrations and elevated aluminum concentrations on sodium fluxes in brown trout, *Salmo trutta* L. *Water Air Soil Pollut.*, **30**:569-577.
- Driscoll, C.T., Jr., J.P. Baker, Jr., J.J. Bisgoni, Jr., and C.L. Schofield (1980) Effects of aluminum speciation on fish in dilute acidified waters. *Nature*, **284**:161-164.
- Fenwick, J.C. (1989) The gill. In: *Vertebrate Endocrinology, Fundamental and Biomedical Implications. Regulation of Ca^{2+} and Phosphate*, Vol. 3. P.K.T. Pang and M.B. Schreibman, eds. Academic Press, New York.
- Fjellheim, A., G.G. Raddum, and T. Sagen (1985) Effect of aluminum at low pH on the mortality of elvers (*Anguilla anguilla* L.), a laboratory experiment. *Verh. Internat. Verein. Limnol.*, **22**:2544-2547.
- Flik, G., J.H. van Rijs, and S.E. Wendelaar Bonga (1985a) Evidence for high affinity Ca^{2+} -ATPase activity and ATP-driven Ca^{2+} transport in membrane preparations of the gill epithelium of the cichlid fish *Oreochromis mossambicus*. *J. Exp. Biol.*, **119**:335-347.
- Flik, G., S.E. Wendelaar Bonga, and J.C. Fenwick (1985b) Active Ca^{2+} transport in plasma membranes of branchial epithelium of the North-American eel, *anguilla rostrata* Le Sueur. *Biol. Cell*, **55**:265-272.
- Flik, G. (1989) Hypocalcin Physiology. In: *Progress in Comparative Endocrinology*, A. Eppe, C.G. Scanes, and M.H. Stetson, eds. Wiley-Liss, New York, pp. 578-585.
- Kostyuk, P.G., S.L. Mironov, P.A. Doroshenko, and V.N. Ponomarev (1983) Surface changes on the outer side of mollusk neuron membrane. *J. Membr. Biol.*, **70**:171-180.
- Lafeber, F.P.J.G., and S.F. Perry (1988) Experimental hypercalcemia induces hypocalcin release and inhibits branchial Ca^{2+} influx in freshwater trout. *Gen. Comp. Endocrinol.*, **72**:136-143.
- MacDonald, T.L., and R.B. Martin (1988) Aluminum in biological systems. *Trends Biochem. Sci.*, **13**:15-19.
- Maguire, M.E. (1984) Hormone-sensitive magnesium transport and magnesium regulation of adenylate cyclase. *Trends Pharmacol. Sci.*, **5**:73-77.
- Malley, D.F., and P.S.S. Chang (1985) Effects of aluminum and acid on calcium uptake by the crayfish *Orconectes virilis*. *Arch. Environ. Contam. Toxicol.*, **14**:739-747.
- Martin, R.B. (1986) The chemistry of aluminum as related to biology and medicine. *Clin. Chem.*, **32**:1797-1806.
- Mayer Gostan, N., S.E. Wendelaar Bonga, and P. Balm (1987) Mechanism of hormone actions on gill transport. In: *Vertebrate*

- brate Endocrinology, Fundamental and Biomedical Implications, Vol. II. P.K.T. Pang and M.B. Schreiber, eds. Academic Press, New York, pp. 211-238.
- Perry, S.F., and G. Flik (1988) Characterization of branchial transepithelial calcium in fluxes in freshwater trout, *Salmo gairdneri*. *Am. J. Physiol.*, 254:R491-R498.
- Playle, R.C., G.G. Goss, and C.M. Wood (1989) Physiological disturbances in rainbow trout (*Salmo gairdneri*) during acid and aluminum exposures in soft water of two calcium concentrations. *Can. J. Zool.*, 67:314-324.
- Reader, J.P., and R. Morris (1988) Effects of aluminum and pH on calcium fluxes, and effects of cadmium and manganese on calcium and sodium fluxes in brown trout (*Salmo trutta* L.) *Comp. Biochem. Physiol.*, 91C(2):449-457.
- Reid, S.D., and D.G. McDonald (1988) Effects of cadmium, copper, and low pH on ion fluxes in the rainbow trout, *Salmo gairdneri*. *Can. J. Fish. Aquat. Sci.*, 45:244-253.
- Sarkadi, B., I. Szasz and G. Gardos, G. (1980) Characteristics and regulation of active calcium transport in inside-out red cell membrane vesicles. *Biochim. Biophys. Acta*, 598: 326-338.
- Sidwell, V.D., A.L. Loomis, P.R. Foncannon, and D.H. Buzzel (1978) Composition of the edible portions of raw, fresh or frozen crustaceans, finfish and molluscs. *Mar. Fish. Rev.*, 40 (12):1-16.
- Sillen, L.G., and A.E. Martell (1964) Stability constants of metal ion complexes. Chemical Society, Spec. Publ. no. 17, London.
- Spry, D.J., C.M. Wood, and P.V. Hodson (1981) The effects of environmental acid on freshwater fish with particular reference to the softwater lakes of Ontario and the modifying effects of heavy metals. A review. *Can. Tech. Rep. Fish. Aquat. Sci.*, 999:1-144.
- Turner, R.C. (1976) Effect of aging on properties of polynuclear hydroxylaluminum cations *Can. J. Chem.*, 54:1528-1534.
- Van Heeswijk, M.P.E., J.A.M. Geertsen, and C.H. Van Os (1984) Kinetic properties of the ATP-dependent Ca^{2+} pump and the Na^+/Ca^{2+} exchange system in basolateral membranes from rat kidney cortex. *J. Membrane Biol.*, 79:19-31.
- Verbost, P.M., J. van Rooij, G. Flik, R.A.C. Lock, and S.E. Wendelaar Bonga (1989a) The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. *J. Exp. Biol.*, 145:185-197.
- Verbost, P.M. G. Flik, P.K.T. Pang, R.A.C. Lock, and S.E. Wendelaar Bonga (1989b) Cadmium inhibition of the erythrocyte Ca^{2+} -pump. *J. Biol. Chem.*, 264:5613-5615.
- Wendelaar Bonga, S.E. G.Flik, P.H.M. Balm, and J.C.A. van der Meij (1990) The ultrastructure of chloride cells in the gills of the teleost *Oreochromis massambicus* during exposure to acidified water. *Cell Tissue Res.*, 259:575-585.