Cadmium inhibition of Ca\textsuperscript{2+} uptake in rainbow trout gills

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Cadmium (Cd) is known to cause a wide spectrum of toxic effects in fish (1, 3, 5, 16, 17, 27). Exposure of freshwater fish to Cd\textsuperscript{2+} in the water leads to a disturbed calcium (Ca) balance, resulting in hypocalcemia (10, 24). An important factor effecting mortality may be a serious hypocalcemia after Cd\textsuperscript{2+} exposure (24). Little is known, however, about the mechanisms of Cd\textsuperscript{2+} toxicity in fish, especially with regard to Ca homeostasis. Giles (10) proposed that the hypocalcemia after Cd\textsuperscript{2+} exposure might result from an impairment of mechanisms for Ca\textsuperscript{2+} uptake.

It is well established that freshwater fish take up most of the Ca necessary for growth and Ca homeostasis from the water via their gills (2, 6, 19, 23). In freshwater fish branchial Ca\textsuperscript{2+} influx occurs against a chemical gradient. For freshwater fish such as tilapia and eel it has been shown that Ca\textsuperscript{2+} influx is transcellular (7). Ca\textsuperscript{2+} efflux on the other hand is a passive process, Ca\textsuperscript{2+} moving down a chemical gradient via a paracellular route.

The hypocalcemia observed after Cd\textsuperscript{2+} exposure indicates a decreased or reversed net Ca\textsuperscript{2+} flux, which is defined as the difference between influx and efflux of Ca\textsuperscript{2+}. Therefore, to study the effects of Cd\textsuperscript{2+} on Ca balance in fish, one should consider the effects on both Ca\textsuperscript{2+} influx and efflux. Cd\textsuperscript{2+} could inhibit transcellular Ca\textsuperscript{2+} influx at the apical membrane, at the basolateral membrane, or both. An effect on basolateral Ca\textsuperscript{2+} transport could be predicted because it has been shown that Cd\textsuperscript{2+} accumulates in the cells of the gills (22). An effect of Cd\textsuperscript{2+} on Ca\textsuperscript{2+} efflux may be anticipated when Cd\textsuperscript{2+} would affect the junctional complexes between the cells of the epithelium. Gills are especially appropriate for this study because Cd\textsuperscript{2+} concentrations at the mucosal side can easily be manipulated.

We have studied the effects of Cd\textsuperscript{2+} on branchial influx and efflux of Ca\textsuperscript{2+} in trout by the use of the perfused-head technique (20, 23). This technique enables the in situ study of gills under defined in vitro conditions. We determined the effect of Cd\textsuperscript{2+} on transepithelial Ca\textsuperscript{2+} transport in gill preparations after prior in vivo exposure of the fish to this heavy metal or during acute in vitro exposure to different Cd concentrations.

The use of the Ca\textsuperscript{2+} channel blocker lanthanum (La) (11) allowed us to conclude that Cd\textsuperscript{2+} exerts an inhibitory action at the basolateral membrane rather than by inhibiting apical membrane Ca\textsuperscript{2+} transport.

MATERIALS AND METHODS

Fish

Rainbow trout of both sexes ranging in body weight from 140 to 190 g were purchased at a local trout farm. After transfer to the laboratory the fish were kept in 1,000-liter tanks supplied with running, filtered, and aerated Nijmegen tap water at 10°C. Fish were fed trout pellets (Trouvit), except for a 24-h period prior to experimentation.

Isolated Perfused Head Preparation

The perfused isolated head of rainbow trout was prepared according to Pärt and Svanberg (20). Briefly, 15 min after intraperitoneal injection of 5,000 IU heparin/100 g fish, the animal was decapitated 2 cm behind the pectoral fins. The ventral and dorsal aortas were cannulated with polyethylene catheters. Perfusion of the gills
was started by connecting the ventral aortic cannula to a cardiac pump (flow: 2.2 ml·min⁻¹·100 g fish⁻¹; 40 beats/min). During perfusion the gills were irrigated by recirculation of ~220 ml of tap water (pump rate: 1.2 l/min). The perfusion medium used was filtered (Whatman no. 1 and subsequently 0.45 µm Millipore) Cortland saline (28) of pH 7.6, to which 40 g/l polyvinylpyrrolidone and 2 g/l bovine serum albumin were added as plasma protein substitutes. During the experiments the perfusion medium and the ventilatory water were continuously aerated. The ionic Ca²⁺ concentration of the saline was brought to 1.3 mM, a value equivalent to Ca²⁺ levels found in trout blood; Ca²⁺ was determined with a Radiometer ionic calcium analyzer. In addition the medium contained 5.55 mM glucose as energy source, 10 μM adrenaline bitartrate to reduce capillary resistance, and 5,000 IU/l heparin to prevent blood from clotting.

Ventilatory water samples were taken to monitor the water specific activity. Perfusate samples (100–50 ml) were recirculated. Ventilatory water samples were kept at 0.78 Ca²⁺, 3.8 NaCl, 0.06 KCl, 0.32 SO₄²⁻, and 3.15 HCO₃⁻ (pH 7.6).

Both the ventilatory water and the perfusion medium were continuously monitored with a pressure transducer connected to a potentiometric recorder. On stabilization of the pressure to 25–30 mmHg, the dorsal aortic outflow and anterior venous outflow (from the cut end part) were determined gravimetrically. The ratio between both flows was ~8:1, and the sum of the dorsal and venous outflow equaled ventral aortic inflow. The Na⁺ concentration of the water was recorded with a Na⁺ selectrode. A constant pressure and a decreasing input pressure was continuously monitored with a pressure meter for the first 20 min and every 5 min thereafter. Every 10 min 1-ml samples of ventilatory water were taken to monitor the water specific activity.

**Transepithelial Ca²⁺ Influx Experiments**

⁴⁵Ca⁺ (1.55 ± 0.36 MBq/l) was added to the ventilatory water [at time (t) = 0], and the tracer appearance rate in the perfusate collected from the dorsal aortic cannula was determined. Perfusate samples (100 µl) were taken at 1-min intervals for the first 20 min and every 5 min thereafter. Every 10 min 1-ml samples of ventilatory water were taken to monitor the water specific activity.

**Transepithelial Ca²⁺ Efflux Experiments**

Ca²⁺ efflux rate was determined on the basis of tracer appearance in the ventilatory water (220 ml); in this case ~50 ml saline containing 46.3 ± 1.6 MBq/l ⁴⁵Ca⁺ were recirculated. Ventilatory water samples (1 ml) were taken at 1-min intervals throughout the experiment. These samples were replaced by 1 ml tracer-free water to guarantee a constant volume of the external medium. The radioactivity of the perfusion medium was checked on 50-µl samples taken every 5 min.

**Effects of Cd²⁺ on Transepithelial Ca²⁺ Influx and Efflux**

The effects of Cd²⁺ on Ca²⁺ transport were determined both on branchial Ca²⁺ influx and efflux. In one series of experiments trout gills were exposed during perfusion to Cd (1 and 10 μM) in the ventilatory water (“in vitro exposure”). In another series of experiments rainbow trout were preexposed to Cd (0.01, 0.1, 1, and 10 μM) for 16 h, prior to perfusion (“in vivo exposure”); in the latter experiments no additional Cd²⁺ was present in the ventilatory water. Water Cd concentrations refer to dissolved cadmium as determined by atomic absorption spectrophotometry.

**Effects of La³⁺ on Transepithelial Ca²⁺ Influx**

The effect of La³⁺ on Ca²⁺ influx was studied by addition of LaCl₃ (1 μM) to the ventilatory water when ⁴⁴Ca⁺ appearance rate had reached constancy (after 20 min). The perfusion was continued until ⁴⁴Ca⁺ influx had established a new steady state. In these studies we used CO₂-free artificial tap water to prevent precipitation of La³⁺; the composition of this water was (in mM) 3.8 NaCl, 0.06 KCl, 0.2 MgSO₄, and 0.8 CaCl₂ (pH 7.6).

**Ca²⁺ and Cd²⁺ Accumulation in Branchial Epithelium**

Accumulation of ⁴⁵Ca or ¹¹⁵mCd in gill epithelium was determined after a 45-min exposure to either tracer in the ventilatory water: the left and right first two gill arches were removed, rinsed for 30 s in tracer-free water, and blotted on wet filter paper. The epithelium was scraped off, weighed, and digested in 40% H₂O₂ overnight at 50°C. After addition of 900 μl distilled water to 100 μl of tissue digest, the ⁴⁴Ca²⁺ or ¹¹⁵mCd²⁺ content was determined. Values were expressed in becquerels per gram wet weight of gill epithelium. The effects of La³⁺ on ⁴⁴Ca²⁺ or ¹¹⁵mCd²⁺ accumulation in the gills were studied by the addition of 1 μM La at zero time of tracer exposure. On completion of the tracer exposure, tissue tracer content was determined as described above.

**Calculations**

**Influx.** The calculation of Ca²⁺ influx rates [F_in(Ca)] was based on the ratio of tracer content of the perfusate (qₚ) to the specific activity of the water at time t [SA_w(t)]. Figure 1 shows tracer appearance in the perfusate of perfused trout gills on addition of ⁴⁴Ca to the ventilatory water. Tracer appearance rates stabilized 12–15 min after addition of tracer to the ventilatory water. At steady state, q₀ values were used to calculate F_in(Ca) according to

\[ F_{in}(Ca) = \frac{q_p \cdot f_i \cdot 60 \cdot 100 \cdot W^{-1}}{S_{A_w}(t)} \]

F_in(Ca) is expressed in nmol·h⁻¹·100 g fish⁻¹ where
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\[ (q'_p/q'_w) \times 10^{-3} \]

FIG. 1. Appearance rate of \(^{45}\)Ca in perfusion medium from perfused trout gills. \(^{45}\)Ca was added to ventilatory water at zero time. \(q'_p\), perfusate tracer content (dpm/ml); \(q'_w\), ventilatory water tracer content (dpm/ml). Values are means ± SD for 12 experiments.

\[ \Sigma q'_w \times 10^{-3} \]

FIG. 2. Cumulative appearance of \(^{45}\)Ca (\(\Sigma q'_w\)) in ventilatory water on perfusion of trout gills. \(^{45}\)Ca was added to perfusion fluid at zero time. Values are means ± SD for 8 experiments. See text for further definition.

\(q'_p\) is perfusate tracer content (dpm/ml), \(SA_w(t)\) is specific activity of water (dpm/nmol) at time \(t\), \(f_i\) is individual perfusion flow (ml/min), and \(W\) is fish weight (g).

Efflux. The efflux rates of Ca\(^{2+}\) \([F_{\text{out}}(\text{Ca})]\) were calculated on the basis of the time curves of the cumulative tracer appearance in the water after addition of \(^{45}\)Ca to the perfusate. Figure 2 shows tracer appearance in the water on perfusion of trout gills with \(^{45}\)Ca-containing saline. After 15–20 min an apparent linear tracer appearance rate in the water was observed. The slope of the line (\(dq'_w/dt\)) fitted by linear regression through these points was used to calculate \(F_{\text{out}}(\text{Ca})\) according to

\[ F_{\text{out}}(\text{Ca}) = \frac{(dq'_w/dt) \cdot 60 \cdot 100 \cdot W^{-1}}{SA_p(t)} \]

\(F_{\text{out}}(\text{Ca})\) is expressed in nmol/h\(^{-1}\)·100 g fish\(^{-1}\) where \(SA_p(t)\) is specific activity of perfusate (dpm/nmol) at time \(t\) and \(dq'_w/dt\) is slope of linear part of \(^{45}\)Ca\(^{2+}\) efflux curve.

Statistics

Results are presented as means ± SE. The Wilcoxon rank sum test and Student's \(t\) test were used for statistical analysis where appropriate; 5% was taken as the fiducial limit of significance.

Materials

Materials and suppliers are as follows: Cd(NO\(_3\))\(_2\) (UCB, Brussels); LaCl\(_3\) (BDH Chemicals, UK); Na\(_2\) heparin (Organon, The Netherlands); \(^{45}\)Ca and \(^{115m}\)Cd\(^{2+}\) (Amersham International, UK); polyethylene catheters (Intramedic PE-90 and PE-190).

RESULTS

The TEP determined in isolated head preparations was \(-0.5 ± 1.9\) mV (\(n = 20\)). Addition of Cd\(^{2+}\) to the ventilatory water had no influence on this value.

Figure 3 shows the effects of in vitro and in vivo Cd\(^{2+}\) exposure of trout gills on Ca\(^{2+}\) influx, efflux, and net flux. In vitro Cd\(^{2+}\) exposure (Cd\(^{2+}\) added during gill perfusion) did not influence Ca\(^{2+}\) influx. In vivo exposure (fish preexposed to Cd\(^{2+}\) for 16 h prior to gill perfusion) to Cd concentrations >0.01 µM inhibited Ca\(^{2+}\) influx significantly. Ca\(^{2+}\) efflux, too, was only affected after in vivo exposure. The Ca\(^{2+}\) efflux rates had increased significantly after in vivo exposure to 1 µM Cd but remained at control levels after preexposure to lower Cd concentrations.

\[ F_{\text{Ca}}/\text{nmol} \cdot \text{h}^{-1} \cdot (100 \text{g fish})^{-1} \]

FIG. 3. Effect of Cd\(^{2+}\) on Ca\(^{2+}\) influx \((F_{\text{in}})\) and efflux \((F_{\text{out}})\). Fish were exposed to Cd\(^{2+}\) in vitro (Cd\(^{2+}\) added to ventilatory water from beginning of perfusion) or in vivo (16 h prior to perfusion). Water-dissolved Cd was determined by atomic absorption spectrophotometry. Values are means ± SD for 5–8 experiments per condition.
Preparations made after in vivo exposure up to 1 μM Cd met our criteria for viability. Only after in vivo exposure to Cd concentrations of 10 μM or more did the isolated head preparations show Na+ loss and an unstable perfusion pressure. Data on this group were, therefore, not included.

The effect of La3+ on Ca2+ influx is shown in Fig. 4. La3+ inhibited the Ca2+ influx instantaneously (inset Fig. 4). This inhibitory effect proved dose dependent; from a log dose-response curve 4.6 x 10^-6 M La3+ was found to give 50% inhibition of Ca2+ influx.

Table 1 shows the effects of La3+ and Cd2+ on tissue accumulation of 115mCd or 45Ca, after a 45-min period of exposure to tracer. No effect of Cd (1–10 μM) was found on the 45Ca content of the tissue after 45 min, whereas La (1 μM) almost completely blocked the tracer accumulation in the tissue (95% decrease). Also, La3+ decreased 115mCd accumulation in the gill epithelium by 76%.

**DISCUSSION**

Five major conclusions can be drawn from the data presented in this study. 1) Cd2+ (0.01 < Cd2+ < 1.0 μM) is able to disturb Ca2+ fluxes in the gills of trout without affecting Na+ balance in these fish. 2) Cd2+ (0.01 < Cd2+ < 0.1 μM) inhibits Ca2+ influx via the gills at concentrations close to those occurring in polluted freshwaters (18). 3) Cd2+ does not interfere with the entrance of Ca2+ across the apical membranes into the cell, but rather inhibits Ca2+ extrusion via the basolateral membrane because the tissue Ca2+ content is not reduced on Cd exposure via the water. 4) Ca2+ and Cd2+ seem to enter the epithelium via the same mucosal La3+-sensitive Ca2+ channels. 5) Branchial Ca2+ influx is more sensitive to Cd2+ than Ca2+ efflux.

**La3+ Influx**

Integumental Ca2+ exchange involves transcellular and paracellular routes (6). For transcellular Ca2+ uptake in fish gills, Flik et al. (7) recently proposed a model on the basis of studies on Ca2+-transporting adenosine triphosphatase (ATPase) activity in plasma membranes of branchial epithelium. According to this model, Ca2+ to be transported from the water to the blood enters the cell passively down an electrochemical gradient, is buffered in the cytosol by Ca-binding proteins, and subsequently pumped to the blood by an active Ca2+ transport mechanism. We now suggest that Cd2+ exerts its inhibitory action on Ca2+ uptake by an inhibition of the basolateral-active Ca2+-extrusion mechanism (cell-to-blood step). Several observations substantiate this conclusion and we will discuss them on the basis of the forementioned model. In the model of Flik et al. (7) branchial Ca2+ uptake depends on an active process. Under our experimental conditions the calculated Ca2+ equilibrium potential (E_{Nernst} = −5.9 mV) and measured TEP (−0.5 mV) show that the driving force for passive diffusion of Ca2+ is directed outward. Nevertheless we demonstrated an inwardly directed net flux of Ca2+ under control conditions. This finding is indicative for an active Ca2+ uptake in line with the model of Flik et al. (7). Further evidence that this model applies to trout was recently obtained in our laboratory by the demonstration of high-affinity Ca2+-ATPase activity in trout gills, with characteristics similar to that of tilapia and eel gills (unpublished observations).

Two findings substantiate our conclusion that Cd2+ inhibition of Ca2+ influx occurs via inhibition of the branchial Ca2+ pump. First, 45Ca accumulation in the gill tissue and therefore entrance of Ca2+ from the water into the cells is not affected by Cd2+ exposure. Moreover, the fact that significant Cd2+ accumulation occurs in a 45-min period, and that over the same time period Ca2+ accumulation in the presence of Cd2+ in the tissue is not affected, seems to exclude an inhibitory action of Cd2+ on the Ca2+ channel at the internal face of the apical cell membrane. La3+ addition to the water, on the other hand,
decreased Ca$^{2+}$ accumulation significantly. Second, inhibition of Ca$^{2+}$ influx is observed only after in vivo exposure for sufficiently long periods. After a 16-h preexposure period to Cd concentrations $>0.01$ $\mu$M, Ca$^{2+}$ influx was significantly inhibited. In another experiment it was observed that a minimum period of 12- to 16-h exposure to Cd$^{2+}$ is required to induce hypocalcemia in freshwater tilapia (results to be published elsewhere). La$^{3+}$, on the other hand, inhibited transepithelial Ca$^{2+}$ influx instantaneously, and under our experimental conditions the inhibitory concentration at 50% was calculated to be $4.6 \times 10^{-5}$ M La. La$^{3+}$ is known to block the Ca$^{2+}$ channels (11), and electron microscopic studies of fish gills exposed to La$^{3+}$ showed that La$^{3+}$ does not enter the epithelial cells. This excludes, in our experimental setup, an inhibition by La$^{3+}$ of the basolateral Ca$^{2+}$-ATPase.

Because Cd$^{2+}$ apparently does not influence Ca$^{2+}$ entrance via the apical membrane, it probably interferes with the basolateral-active Ca$^{2+}$ extrusion mechanism. In Cd$^{2+}$ accumulation studies it was shown that Cd$^{2+}$ enters the epithelial cell. The delay in producing an inhibitory effect could be due to Cd$^{2+}$ buffering by cytosolic proteins such as metallothionein, glutathione, and calmodulin (sulfhydryl- and carboxylate-rich proteins that bind Cd$^{2+}$ tightly) (8, 13, 27). This Cd$^{2+}$ buffering could prevent inhibition of the Ca$^{2+}$ transport ATPase by sequestering Cd$^{2+}$ to no-effect levels during early exposure. Ca$^{2+}$ transport in gill plasma membrane vesicles proved extremely sensitive to Cd$^{2+}$ (unpublished observations). We suggest that once the Cd$^{2+}$ concentration surpasses saturation levels of the cytosolic buffers it might inhibit the basolateral Ca$^{2+}$ pump and thus the transepithelial Ca$^{2+}$ transport.

From the fact that La$^{3+}$ inhibits accumulation of Ca$^{2+}$ and Cd$^{2+}$ tracer in the tissue to a similar extent, we conclude that Ca$^{2+}$ and Cd$^{2+}$ enter the epithelial cell via the same La$^{3+}$-sensitive Ca$^{2+}$ channels. The similarity in ion radius [0.99 and 0.97 Å for Ca$^{2+}$ and Cd$^{2+}$, respectively (5)] may underlie this behavior. The smaller percentage of inhibition by La$^{3+}$ of Cd$^{2+}$ accumulation compared with Ca$^{2+}$ accumulation may be explained by the fact that substantial binding of Cd$^{2+}$ occurs to mucopolysaccharides (21). Cd$^{2+}$ will not be washed off from the gills as easily as Ca$^{2+}$ during the rinsing step prior to the determination of the tracer content. One could argue that, if Ca$^{2+}$ and Cd$^{2+}$ enter the cell via the same channels, competition between both ions occurs and that, therefore, external Cd$^{2+}$ should inhibit Ca$^{2+}$ influx. However, this competition would appear insignificant at the Cd concentrations used: the $^{45}$Ca content was not significantly influenced by Cd (1-10 $\mu$M), not even after 16-h exposure to Cd$^{2+}$ (results not shown). Apparently the difference in concentrations of Cd (1 $\mu$M) and of Ca (800 $\mu$M) could explain the lack of substantial competition.

An interesting question is whether the gill epithelium differentiates between the essential Ca$^{2+}$ ion and the toxic Cd$^{2+}$ ion. Both ions seem to enter the epithelium via the same Ca$^{2+}$ channels. Next both ions are supposed to bind to cytosolic proteins (12) that are rich in sulfhydryl groups (metallothioneins) and carboxyl groups (calcium-binding proteins). Ingersoll and Wasserman (12) reported that cytosolic fractions of rat enteroctyes show comparable affinity for Cd$^{2+}$ and Ca$^{2+}$. If Ca$^{2+}$ and Cd$^{2+}$ pass the apical membrane just as easily and if these ions are similarly buffered in the cytosol, discrimination between the two ions seems to occur at the basolateral membrane of the cell, where the Ca$^{2+}$ pump appears to become inhibited by Cd$^{2+}$.

Ca$^{2+}$ Efflux

Two conclusions are drawn from the efflux studies. First, the transepithelial Ca$^{2+}$ efflux appears less sensitive to Cd$^{2+}$ than the Ca$^{2+}$ influx. Significant stimulation of Ca$^{2+}$ efflux was found only at the highest Cd concentration tested (1 $\mu$M), whereas Ca$^{2+}$ influx is abolished almost completely at a tenfold lower Cd concentration. Second, these findings demonstrate that it is essential to study both influx and efflux of Ca$^{2+}$ for evaluation of Cd$^{2+}$ inhibition of Ca$^{2+}$ uptake via fish gills.

The question arises as to which mechanism underlies this increased Ca$^{2+}$ efflux on Cd$^{2+}$ exposure. As stated above, Ca$^{2+}$ efflux is probably a paracellular event, and we assume that Cd$^{2+}$ interacts with the junctional complex proteins in a way that causes a Ca$^{2+}$ leak.

In one series of experiments we tested the effects of in vivo exposure to 10 $\mu$M Cd on the Ca$^{2+}$ fluxes. Ca$^{2+}$ influx was strongly inhibited, and the preparations showed an extremely high Ca$^{2+}$ efflux associated with a net sodium loss. This indicates that the structural integrity of the branchial epithelium is lost when fish are exposed to 10 $\mu$M Cd (which was confirmed by histological observations) (results not shown). In an estuarine teleost exposed to similar Cd concentrations structural damage and hyperplasia of the gills have been reported (9).

In conclusion it seems that levels of 0.1 $\mu$M Cd (11 parts per billion), which are close to those found in Cd-polluted freshwaters (18), disturb the fish’s Ca balance by a specific effect on the branchial Ca$^{2+}$ uptake mechanism. Insight in the development of hypocalcemia that seems inherent to the toxic effects of sublethal amounts of Cd$^{2+}$ may be of primary importance for the understanding of Cd$^{2+}$ toxicity in fish. As has been stated before the branchial epithelium comprises the same mechanisms for cellular Ca$^{2+}$ transport (i.e., apical membrane Ca$^{2+}$ channels and basolateral membrane Ca$^{2+}$ transport ATPase) (7) as those found in, for example, mammalian intestine (26) and kidney tubules (25). Clearly, the gill model, in which mucosal and serosal media may be easily controlled, provides a powerful tool in the study of Cd$^{2+}$ toxicity toward the transepithelial Ca$^{2+}$ transport process.

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