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THE MOVEMENT OF CADMIUM THROUGH FRESHWATER TROUT BRANCHIAL EPITHELIUM AND ITS INTERFERENCE WITH CALCIUM TRANSPORT

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Accepted 25 May 1989

Summary
Exposure of freshwater trout (Salmo gairdneri) to waterborne Cd\textsuperscript{2+} results in accumulation of the metal in the branchial epithelial cells and its appearance in the blood. Cd\textsuperscript{2+} apparently enters the cells via Ca\textsuperscript{2+} channels in the apical membrane. Transfer of Cd\textsuperscript{2+} through the basolateral membrane is probably by diffusion. Inhibition by Cd\textsuperscript{2+} of transepithelial Ca\textsuperscript{2+} influx is time- and Cd\textsuperscript{2+}-concentration-dependent. The inhibition of transepithelial Ca\textsuperscript{2+} influx is accompanied by blockage of apical Ca\textsuperscript{2+} channels. In line with the assumption that cytosolic Cd\textsuperscript{2+} inhibits Ca\textsuperscript{2+} uptake by inhibiting the basolateral Ca\textsuperscript{2+} pump, we hypothesize that the blockage of Ca\textsuperscript{2+} channels is an indirect effect of Cd\textsuperscript{2+} and results from a rise in cytosolic Ca\textsuperscript{2+} level caused by inhibition of the basolateral membrane Ca\textsuperscript{2+} pump.

Introduction
Ca\textsuperscript{2+} uptake in freshwater fish mainly occurs via the gills (Flik et al. 1985). Ca\textsuperscript{2+} transport across the gills, a tight ion-transporting epithelium, follows a transcellular route (Perry & Flik, 1988). Micromolar concentrations of cadmium (Cd) in the water inhibit branchial Ca\textsuperscript{2+} uptake (Verbost et al. 1987a; Reid & McDonald, 1988) and induce hypocalcaemia (Giles, 1984; Pratap et al. 1989). We have advanced circumstantial evidence that the inhibition of Ca\textsuperscript{2+} uptake by waterborne Cd\textsuperscript{2+} may result from a competitive inhibition by cytosolic Cd\textsuperscript{2+} of the Ca\textsuperscript{2+} pump in the basolateral membrane of the Ca\textsuperscript{2+}-transporting cells in the gills (Verbost et al. 1988).

Cd\textsuperscript{2+} uptake from the water in freshwater fish mainly occurs via the gills (Williams & Giesy, 1978). Like Ca\textsuperscript{2+}, Cd\textsuperscript{2+} enters the fish predominantly via a transcellular route because of the tight character of the branchial epithelium (Pärt, 1983). Interference of Cd\textsuperscript{2+} with the Ca\textsuperscript{2+} influx route may occur at at least three sites: the apical membrane, where Ca\textsuperscript{2+} enters the cell via Ca\textsuperscript{2+} channels (Perry & Flik, 1988), the intracellular Ca\textsuperscript{2+} buffering systems (Flik et al. 1985) and the

Key words: freshwater teleost fish, gills, calcium transport, cadmium.
basolateral membrane, where Ca$^{2+}$ is translocated to the blood by a high-affinity Ca$^{2+}$ pump (Flik et al. 1985). Levels of Cd$^{2+}$ in fresh water that cause hypocalcaemia (0.1 $\mu$mol l$^{-1}$ Cd in water containing 0.7 mmol l$^{-1}$ Ca) do not instantly inhibit Ca$^{2+}$ influx (Verbost et al. 1987a), indicating that no significant competition between waterborne Cd$^{2+}$ and Ca$^{2+}$ occurs for the cell entrance step (the concentration ratio Cd/Ca being $1.4 \times 10^{-4}$ under these conditions). The same conclusion was reached by Pärt et al. (1985), who showed that changes in water Ca$^{2+}$ concentration (0–10 mmol l$^{-1}$) had no effect on $^{109}$Cd accumulation in the gills (the concentration ratio Cd/Ca ranged from $0.7 \times 10^{-4}$ to $7.0 \times 10^{-4}$).

Intracellular Ca$^{2+}$ interacts with a series of more-or-less Ca$^{2+}$-specific ligands, such as calmodulin, Ca$^{2+}$-binding proteins (CaBPs) and Ca$^{2+}$-ATPase. Calmodulin is a Ca$^{2+}$-dependent regulator protein with a high Ca$^{2+}$ affinity and is present in all eukaryotic cells. The affinity of calmodulin for Cd$^{2+}$ is comparable to that for Ca$^{2+}$ (Chao et al. 1984; Flik et al. 1987). The affinities of the CaBPs in fish gills for Cd$^{2+}$ and Ca$^{2+}$ have not been determined so far, but the vitamin-D-dependent CaBPs from rat kidney and pig duodenal mucosa share with calmodulin the property of having the same high affinity for Cd$^{2+}$ and Ca$^{2+}$ (Richardt et al. 1986). To affect the Ca$^{2+}$ buffering capacity of these proteins in the cell, the intracellular concentration of Cd$^{2+}$ must reach that of the cytosolic Ca$^{2+}$ concentration. In comparison with calmodulin or CaBPs, the Ca$^{2+}$ site of the plasma membrane Ca$^{2+}$-ATPase in fish gills has an affinity for Cd$^{2+}$ at least 100 times higher than that for Ca$^{2+}$ (Verbost et al. 1988). It follows that the Ca$^{2+}$ extrusion pump is a very sensitive target of Cd$^{2+}$ in fish gills. Although an inhibition of the basolateral Ca$^{2+}$ pumps by Cd$^{2+}$ could explain the diminished branchial Ca$^{2+}$ uptake following exposure to Cd$^{2+}$, the possibility that cytosolic Cd$^{2+}$ also impedes the movement of Ca$^{2+}$ through apical membrane Ca$^{2+}$ channels cannot be excluded.

The experiments described here were designed to test the hypothesis that Cd$^{2+}$ enters the Ca$^{2+}$-transporting cell via Ca$^{2+}$ channels, and that cytosolic Cd$^{2+}$ may affect apical Ca$^{2+}$ channels. Two types of experiments were performed. (i) Short-term accumulation of $^{45}$Ca and $^{109}$Cd from the water into the branchial epithelium was determined to evaluate the movements of Ca$^{2+}$ and Cd$^{2+}$ through the apical membranes into the epithelium. (ii) Branchial influx of Ca$^{2+}$ and Cd$^{2+}$ was measured to evaluate the movement of these ions through both apical and basolateral plasma membranes. In these two types of experiments, apical membrane permeability for Ca$^{2+}$ was manipulated by adding exogenous La$^{3+}$ and by injections of the hormone hypocalcin. Recently, it was concluded that this hormone, produced in the Stannius corpuscles of fish (Wendelaar Bonga & Pang, 1986), controls apical membrane permeability to Ca$^{2+}$ (Lafeber, 1988).

**Materials and methods**

**Fish**

Freshwater rainbow trout (*Salmo gairdneri*) ranging in mass from 20 to 40 g were kept indoors and acclimated to city of Nijmegen tapwater ([Ca] = 0.70 ±
Cadmium and calcium uptake in fish

0.02 mmol l\(^{-1}\), \(N = 20\) under the conditions described previously (Verbost et al. 1987a). In experiments with La\(^{3+}\), carbonate-free artificial tapwater was used to prevent precipitation of La\(_2\)(CO\(_3\))\(_3\). The composition of the artificial tapwater was (in mmol l\(^{-1}\)): CaCl\(_2\), 0.7; MgCl\(_2\), 0.2; NaCl, 3.8; and KCl, 0.06 in demineralized water (pH 7.6). In experiments with Co\(^{2+}\), CoCl\(_2\) was added to normal tapwater. The ionic content of tapwater was (in mmol l\(^{-1}\)): Ca\(^{2+}\), 0.7; Mg\(^{2+}\), 0.38; Na\(^+\), 0.61; K\(^+\), 0.05; Cl\(^-\), 0.66; SO\(_4^{2-}\), 0.32; and HCO\(_3^-\), 3.15 (pH 7.6).

**Analytical methods**

The total calcium content of water was determined with a calcium kit (Sigma Chemical). Water total cadmium content was determined by atomic absorption spectrophotometry. Protein was measured with a reagent kit (BioRad) using bovine serum albumin as reference. Tracer content of water samples and tissue digests was determined by liquid scintillation analysis. Aqueous samples (0.5 ml) were mixed with 4.5 ml of Aqualuma scintillation fluid.

**Treatments**

**Cadmium exposure**

Fish were pre-exposed to Cd\(^{2+}\) (nominal 1.0 or 0.1 µmol l\(^{-1}\)) up to 16 h before experimentation. Water Cd\(^{2+}\) [added as Cd(NO\(_3\))\(_2\)] concentrations were carefully monitored, and a maximum 10% deviation from the calculated concentrations was accepted. Pre-exposure was followed by a 1 h flux period in which the Cd\(^{2+}\) concentration in the water was kept constant (see radiotracer techniques).

**Hypocalcin injections**

Purified hypocalcin and crude extract of Stannius corpuscles were prepared as described by Lafeber et al. (1988b). The hypocalcin content of extracts of Stannius corpuscles, determined by ELISA (Kaneko et al. 1988) was 120–150 µg mg\(^{-1}\) protein. The dose of hypocalcin injected (intraperitoneally) was 2.2–3.0 nmol hypocalcin 100 g\(^{-1}\) fish. Hormone or extract was injected 1 h before tracer exposure. Injection of saline, used as vehicle, served as control.

**Radiotracer techniques**

**Gill tracer accumulation**

Fish were transferred to 3.0 l of aerated recirculating water containing 1.0 MBq l\(^{-1}\) \(^{45}\)CaCl\(_2\), 0.9 MBq l\(^{-1}\) \(^{109}\)CdCl\(_2\) or 0.2 MBq l\(^{-1}\) \(^{22}\)NaCl in tapwater. After 30 min (\(^{22}\)Na) or 1 h (\(^{45}\)Ca, \(^{109}\)Cd) of tracer exposure the fish were quickly (2 min) anaesthetized in bicarbonate-buffered methane sulphonate salt (MS222, 0.5 g l\(^{-1}\), pH 7.4) and injected intraperitoneally with 5000 i.u. of sodium heparin per 100 g of fish. The gills were rapidly cleared of blood by perfusion with saline (10 ml 0.9% NaCl) via the ventral aorta. The perfusate was collected by suction after opening the atrium to determine its tracer content. Next, the gill arches were excised, rinsed for 5 s in demineralized water and blotted on wet tissue paper. Gill
epithelium was carefully scraped off onto a glass plate with a microscope slide (approx. 0.4 g wet mass, weighed to ±0.001 g) and dissolved overnight at 60°C in tissue dissolver (NCS, Amersham). The digested tissue was neutralized with glacial acetic acid, scintillation fluid (9 vols) was added, and the radioactivity determined. The remainder of the fish was processed for the determination of total body radioactivity (see below).

**Flux determinations**

Influx was determined on the basis of total body radioactivity. Fish bodies and scraped gill arches were microwave-cooked (2 min) and homogenized in a blender with distilled water (volume: 65% of body mass). Triplicate samples of the homogenate (approx. 0.4 g weighed to ±0.001 g) were processed for determination of radioactivity, as described above for the gill scrapings. Total body tracer content included the combined activities of homogenate, gill scrapings and perfusate.

To check whether the tracer associated with the gill scrapings reflected accumulation into the epithelium, and not adsorption to the external side of the epithelium, six trout were exposed to \(^{109}\text{Cd}\) and six to \(^{45}\text{Ca}\) for 1 h and the gills perfused and excised as described above. Then, the left gill arches were rinsed in 1 mmol l\(^{-1}\) EDTA and those from the right side in demineralized water (control). No difference in radioactivity was found between samples from either side for either isotope, indicating that the tracers were associated with the internal face of the epithelial cells.

**Calculations**

Influx of \(\text{Cd}^{2+}\) or \(\text{Ca}^{2+}\) was calculated from the total body radioactivity after 1 h of exposure to \(^{109}\text{Cd}\) or \(^{45}\text{Ca}\), respectively, and the respective mean tracer specific activities of the water. Fluxes were normalized to fish mass by linear extrapolation and expressed in nanomoles (\(\text{Cd}^{2+}\)) or micromoles (\(\text{Ca}^{2+}\)) per hour per 100 grams of fish (Lafeber et al. 1988a).

\(\text{Na}^{+}\) influx was calculated from the plasma \(^{22}\text{Na}\) activity (blood samples were taken directly after anaesthetizing the fish), the apparent radiospace at 30 min for \(\text{Na}^{+}\) (approx. 125 ml kg\(^{-1}\)) and the specific activity of the water (Payan & Maetz, 1973; Bath & Eddy, 1979).

The amount of \(\text{Cd}^{2+}\) accumulated in gill soft tissue was calculated from the tissue tracer content (activity per gram wet mass, \(q_g'\)) and the mean specific activity of \(^{109}\text{Cd}\) in the water (SA\(_w\)) using the equation: \(\text{Cd}^{2+}\) accumulated in 1 h = \(q_g'\)/SA\(_w\) (in nanomoles \(\text{Cd}^{2+}\) per gram wet mass). For this calculation we have assumed that during the experiment no significant transflux or backflux from blood to cells or from cells to water occurred. The \(\text{Ca}^{2+}\) accumulation in the cellular compartment could not be calculated from the tracer accumulation because of the multicompartment behaviour of cells when exposed to the \(\text{Ca}^{2+}\) tracer (Borle, 1981). Therefore, to compare the accumulation of \(^{109}\text{Cd}\) and \(^{45}\text{Ca}\) in
Cadmium and calcium uptake in fish

Cadmium and calcium uptake in fish 189
gill tissue (Fig. 1A), relative values are presented ($q_s'/q_w'$; where $q_w'$ is the water activity per millilitre). Values found for control fish were designated as 100%.

Statistics

Results are presented as means ± s.e. For statistical evaluation the Mann-Whitney U-test was used; significance was accepted for $P \leq 0.05$.

Results

Manipulation of apical membrane

Fig. 1A summarizes the effects of several treatments that affect apical Ca$^{2+}$ permeability on $^{45}$Ca and $^{109}$Cd accumulation in the gills. La$^{3+}$ (1 μmol l$^{-1}$) and Co$^{2+}$ (100 μmol l$^{-1}$) in the water reduced the Ca$^{2+}$ accumulation in gill soft tissue by 71% and 33%, respectively, compared with controls. Because of the limited availability of purified hypocalcin we used both Stannius corpuscle extract and purified hypocalcin. Injection of Stannius extract or hypocalcin reduced $^{45}$Ca accumulation in gills by 63% and 37%, respectively.

To evaluate the effects of the aforementioned treatments on Cd$^{2+}$ accumulation in the gills, a water Cd concentration had to be established that did not influence

Fig. 1. (A) $^{45}$Ca and $^{109}$Cd accumulation in gill soft tissue after manipulation of apical membrane permeability. Values represent means ± s.e. ($N = 6$). All values were significantly different from their respective controls. CS, intraperitoneal injection of homogenate of corpuscles of Stannius; hyp, intraperitoneal injection of isolated hypocalcin; Co ($10^{-4}$ mol l$^{-1}$) and La ($10^{-6}$ mol l$^{-1}$), nominal concentration of Co$^{2+}$ or La$^{3+}$ in the water during the flux measurement; ND, not determined. (B) Whole-body Ca$^{2+}$ and Cd$^{2+}$ influx after manipulation of apical membrane permeability.
the apical permeability to Cd$^{2+}$ during the 1 h $^{109}$Cd accumulation measurement. Since even a 4 h exposure to 0·1 μmol l$^{-1}$ Cd had no effect on the Cd$^{2+}$ (or Ca$^{2+}$) accumulation (see Figs 2, 3), this concentration was used. The results showed that treatments that decreased $^{45}$Ca accumulation had a similar, and proportional, effect on $^{109}$Cd accumulation in the gills (Fig. 1A).

Fig. 1B summarizes the effects of the treatments on Ca$^{2+}$ and Cd$^{2+}$ influx. Ca$^{2+}$ influx decreased by 77% when LaCl$_3$ was added to the water. Injections of Stannius extracts and of hypocalcin reduced Ca$^{2+}$ influx by 55% and 43%, respectively. Comparable effects were also found on the Cd$^{2+}$ influx (Fig. 1B). These results show that for both Ca$^{2+}$ and Cd$^{2+}$ a reduction in tracer accumulation in the gills is accompanied by a reduction in whole-body influx of the ions.

**Effects of Cd$^{2+}$ exposure on Ca$^{2+}$ influx**

Fig. 2A shows the effects of time of exposure to exogenous Cd$^{2+}$ on the $^{45}$Ca accumulation rate in gill epithelium. A time-related inhibition of $^{45}$Ca accumulation was found, with a significant 25% inhibition after 6 h of exposure to 0·1 μmol l$^{-1}$ Cd, and with 60% and 70% inhibition after 9 and 17 h, respectively, of exposure to 0·1 μmol l$^{-1}$ Cd (exposure time to Cd$^{2+}$ included pre-exposure and the 1 h flux period). When fish were exposed to 1·0 μmol l$^{-1}$ Cd, however, $^{45}$Ca accumulation was inhibited by 25% within 1 h (without pre-exposure), whereas a maximum inhibition of 60% was observed after 3–4 h of exposure. The same maximum inhibition of $^{45}$Ca accumulation was reached for 0·1 and 1·0 μmol l$^{-1}$ Cd. Exposure of trout for 16·5 h to 1·0 μmol l$^{-1}$ Cd did not affect $^{22}$Na accumulation in branchial tissue.

The effects of exogenous Cd$^{2+}$ on whole-body Ca$^{2+}$ and Na$^+$ influx are shown in Fig. 2B. Exposure to 0·1 μmol l$^{-1}$ Cd for up to 6 h had no significant effect on

![Fig. 2. (A) Effects of Cd$^{2+}$ exposure time on $^{45}$Ca (○,●) and $^{22}$Na (▲) accumulation in gill soft tissue. Tracer accumulation was determined after 1 h of exposure. The nominal Cd concentrations were 0·1 μmol l$^{-1}$ or 1·0 μmol l$^{-1}$, both during (pre-)exposure to Cd$^{2+}$ and during exposure to the tracer. Points represent means ± s.e. ($N = 6$). Points marked with an asterisk are significantly different from their respective controls at zero time. (B) Effects of exposure to Cd$^{2+}$ on whole-body Ca$^{2+}$ (○,●) and Na$^+$ (▲) influx.](image-url)
Cadmium and calcium uptake in fish

Fig. 3. (A) Effects of exposure to Cd\textsuperscript{2+} on the accumulation of \textsuperscript{109}Cd in gill soft tissue. Points represent means ± s.e. (N = 6). Points marked with an asterisk are significantly different from their respective values at 1 h of exposure. (B) Effects of exposure to Cd\textsuperscript{2+} on whole-body Cd\textsuperscript{2+} influx.

Ca\textsuperscript{2+} influx, but after 9 and 17 h Ca\textsuperscript{2+} influx was inhibited by 67% and 87%, respectively. With 1 \(\mu\)mol l\(^{-1}\) exogenous Cd, Ca\textsuperscript{2+} influx was significantly (49%) inhibited within 1 h of exposure, and after 3–4 h of exposure a maximum 80% inhibition was observed. Exposure of fish to 1 \(\mu\)mol l\(^{-1}\) Cd for 16.5 h had no effect on Na\textsuperscript{+} influx.

**Effects of Cd\textsuperscript{2+} exposure on Cd\textsuperscript{2+} influx**

The effects of exogenous Cd\textsuperscript{2+} on the \textsuperscript{109}Cd accumulation in gill epithelium are shown in Fig. 3A. Exposure to 0.1 \(\mu\)mol l\(^{-1}\) Cd for up to 4 h had no significant effect, but \textsuperscript{109}Cd accumulation decreased by 64% after 6 h and by 75% after 17 h of exposure to Cd\textsuperscript{2+}. With 0.0 \(\mu\)mol l\(^{-1}\) Cd in the water, six times more Cd\textsuperscript{2+} accumulated in gill soft tissue than with 0.1 \(\mu\)mol l\(^{-1}\) Cd in the water. If we designate Cd\textsuperscript{2+} accumulation after 1 h as 100%, an 80% inhibition occurred during the second hour of incubation, which was almost the maximum inhibition observed after longer exposure (up to 17 h).

In Fig. 3B the effects of exogenous Cd\textsuperscript{2+} on whole-body Cd\textsuperscript{2+} influx are shown. The Cd\textsuperscript{2+} influx at 0.1 \(\mu\)mol l\(^{-1}\) external Cd was around 3.4 nmol h\(^{-1}\) 100 g\(^{-1}\) fish. Exposure to 0.1 \(\mu\)mol l\(^{-1}\) Cd for 4 h did not significantly affect Cd\textsuperscript{2+} influx. However, after 6 h or more Cd\textsuperscript{2+} influx decreased by 55%. In fish exposed to 1.0 \(\mu\)mol l\(^{-1}\) Cd for 1 h, Cd\textsuperscript{2+} influx was around 60 nmol h\(^{-1}\) 100 g\(^{-1}\) fish, 18 times higher than in those exposed to 0.1 \(\mu\)mol l\(^{-1}\) Cd. Cd\textsuperscript{2+} influx during the second hour of exposure to 1 \(\mu\)mol l\(^{-1}\) Cd and thereafter decreased by 80%.

**Discussion**

Two major conclusions are drawn from this study. Cd\textsuperscript{2+} in the water at a concentration that provokes a specific hypocalcaemia inhibits both Ca\textsuperscript{2+} and Cd\textsuperscript{2+} influx, but not Na\textsuperscript{+} influx. The inhibitory effect of Cd\textsuperscript{2+} is concentration- and time-dependent. Treatments that inhibit branchial Ca\textsuperscript{2+} influx (exposure to La\textsuperscript{3+} in the water or hypocalcin injections) inhibit branchial Cd\textsuperscript{2+} influx from the water.
The main purpose of our study was to examine how Cd\(^{2+}\) passes across the branchial epithelium. We tested the hypothesis that Cd\(^{2+}\) follows the Ca\(^{2+}\) route through the epithelium. This idea was prompted by the similarity in charge and ionic radius of Ca\(^{2+}\) and Cd\(^{2+}\).

Whole-body Cd\(^{2+}\) influx amounted to 3.4 nmol h\(^{-1}\) 100 g\(^{-1}\) fish at 0.1 \(\mu\)mol l\(^{-1}\) external Cd and 61.9 nmol h\(^{-1}\) 100 g\(^{-1}\) fish at 1.0 \(\mu\)mol l\(^{-1}\) Cd. These values for whole-body Cd\(^{2+}\) influxes are of the same order as the values found by Pärt & Svanberg (1981) for Cd\(^{2+}\) influx in isolated head preparations of trout. These results extend the observations of Williams & Giesy (1978) that the gills are the predominant site of Cd\(^{2+}\) influx. Our finding that inhibition of Cd\(^{2+}\) influx is accompanied by a decrease in Cd\(^{2+}\) accumulation in gill soft tissue establishes that the Cd\(^{2+}\) influx is transcellular.

Cd\(^{2+}\) accumulation in branchial epithelium

Our earlier experiments with the perfused isolated head technique indicated that Cd\(^{2+}\) may be transferred across the apical membrane via La\(^{3+}\)-inhibitable Ca\(^{2+}\) channels (Verbost et al. 1987a). The present results on intact trout confirm and extend these earlier findings: both La\(^{3+}\) treatment and hypocalcin treatment decrease Ca\(^{2+}\) and Cd\(^{2+}\) accumulation in the gills. These observations indicate an effect of hypocalcin on apical membrane Ca\(^{2+}\) channels, and thus also extend the data of Lafeber et al. (1988a), who showed that hypocalcin inhibits transepithelial Ca\(^{2+}\) influx. Moreover, the observation that both Ca\(^{2+}\) and Cd\(^{2+}\) accumulation are inhibited by these treatments strongly suggests that these ions enter the cells via the same pathway.

Whole-body Cd\(^{2+}\) and Ca\(^{2+}\) influx

As the decrease in tissue accumulation was proportional to the decrease in transcellular Ca\(^{2+}\) and Cd\(^{2+}\) flux, both whole-body Ca\(^{2+}\) and whole-body Cd\(^{2+}\) influx depend on passage of the ions through the apical membranes of the epithelium. Ca\(^{2+}\) and Cd\(^{2+}\) transfer across the epithelium should not be expected to be the same, because of the differences in tissue status of the ions (at the beginning of an experiment the gill cells contain millimolar concentrations of Ca and no Cd). However, if epithelial cells were unable to discriminate between Ca\(^{2+}\) and Cd\(^{2+}\), \(^{109}\)Cd could be used as Ca\(^{2+}\) tracer (and \(^{45}\)Ca as Cd\(^{2+}\) tracer). The tracer uptake studies show, however, that the epithelial cells do discriminate between Ca\(^{2+}\) and Cd\(^{2+}\) (\(^{109}\)Cd being differentially accumulated in the gills compared with \(^{45}\)Ca, using similar amounts of radioactivity), which indicates a difference in cellular buffering and/or a difference in basolateral transfer.

To understand transepithelial Cd\(^{2+}\) uptake it is important to know how Cd\(^{2+}\) passes through the basolateral membrane. Because we obtained no evidence for an ATP-driven translocation from experiments with basolateral membrane vesicles in our laboratory (results not shown), and because the transepithelial Cd\(^{2+}\)
Cadmium and calcium uptake in fish

influx is too high to be explained by diffusion through a pure lipid bilayer, some mechanism of facilitated diffusion seems likely. A similar conclusion was drawn for Cd\(^{2+}\) transfer through basolateral membranes in rat duodenum (Foulkes, 1986).

Short-term regulation of transepithelial Ca\(^{2+}\) influx takes place at the level of the apical membrane, which is under the control of fast-acting hypocalcin (Lafeber et al. 1988a). Indeed, the transepithelial influx of Ca\(^{2+}\) proved to be proportionally related to Ca\(^{2+}\) accumulation in the gills. The rate of transport of Cd\(^{2+}\) via the gills appears to depend on the permeability of the apical membrane to Cd\(^{2+}\), as well as on passage through the cytosol and/or the basolateral membrane of the cell.

In the calculations of Ca\(^{2+}\) accumulation and Cd\(^{2+}\) influx, we have assumed that no backflux from cytosol to water or from blood to cytosol occurred. This assumption seems justified as the influx of Cd\(^{2+}\) is very low (62 nmol h\(^{-1}\) 100 g\(^{-1}\) fish in water containing 1.0 \(\mu\)mol l\(^{-1}\) Cd and 0.7 mmol l\(^{-1}\) Ca) and the Cd\(^{2+}\) space is large. Also, backflux of Cd\(^{2+}\) through the apical membrane will be negligible because of the electrical potential difference between cytosol and water (the cytosol being negative; Perry & Flik, 1988).

**Transepithelial Na\(^{+}\) influx**

Chloride cells are generally considered as the sites of Na\(^{+}\) uptake in the gills (Avella et al. 1987). The Na\(^{+}\)/K\(^{+}\)-ATPase activity, located in the basolateral membrane of these cells, is regarded as the most important driving force for transepithelial Na\(^{+}\) uptake in fish gills (deRenzis & Bornacin, 1984). Branchial Na\(^{+}\) influx is not affected by Cd\(^{2+}\) exposure, in contrast to Ca\(^{2+}\) influx, which also depends on ATPase activity (Ca\(^{2+}\)-ATPase; Flik et al. 1985) colocalized with Na\(^{+}\)/K\(^{+}\)-ATPase. This suggests that Na\(^{+}\)/K\(^{+}\)-ATPase activity is not inhibited by Cd\(^{2+}\) concentrations that inhibit Ca\(^{2+}\)-ATPase. This is in agreement with in vitro studies, which indicate a much lower sensitivity of the Na\(^{+}\)/K\(^{+}\)-ATPase than the Ca\(^{2+}\)-ATPase for Cd\(^{2+}\). The concentration of Cd\(^{2+}\) causing 50% inhibition in vitro of Na\(^{+}\)/K\(^{+}\)-ATPase from various origins, for instance rabbit proximal tubule (Diezi et al. 1988), cultured vascular smooth muscle cells (Tokushige et al. 1984) or rat brain synaptosomes (Lai et al. 1980) is in the micromolar range. We found for trout gills, that the branchial para-nitrophenyl phosphatase (pNPPase) activity, which reflects the K\(^{+}\)-dependent dephosphorylation step in the Na\(^{+}\)/K\(^{+}\)-ATPase reaction cycle, was inhibited by 50% by 0.25 \(\mu\)mol l\(^{-1}\) Cd\(^{2+}\) (G. Flik, unpublished results). These data indicate that the concentration of free cytosolic Cd\(^{2+}\) that causes inhibition of Ca\(^{2+}\) influx must be below the micromolar range, as Na\(^{+}\) influx was not affected. Such a low cytosolic Cd\(^{2+}\) concentration also excludes significant binding of Cd\(^{2+}\) to calmodulin, as the \(K_m\) of calmodulin for Cd\(^{2+}\) is in the micromolar range (Flik et al. 1987). The formation of Cd\(^{2+}\)-calmodulin complexes has been proposed as the primary cause of cellular Cd\(^{2+}\) toxicity (e.g. Suzuki et al. 1985). The present results do not support such a calmodulin-mediated toxicity mechanism for Cd\(^{2+}\).
Localization of the primary Cd\textsuperscript{2+} target in the gills

Our data show that Ca\textsuperscript{2+} and Cd\textsuperscript{2+} enter the gills via the same route. This implies that Cd\textsuperscript{2+} is concentrated in the ion-transporting cells of the gills, the chloride cells, since these cells account for the branchial Ca\textsuperscript{2+} transport (Fenwick, 1989). As a consequence, inhibition of Ca\textsuperscript{2+} transport will occur as soon as Cd\textsuperscript{2+} has accumulated to a level sufficient to inhibit the Ca\textsuperscript{2+}-ATPase transport system (Verbost et al. 1988). However, the present results also show a reduction in the rate of accumulation of Ca\textsuperscript{2+} in gills after prolonged Cd\textsuperscript{2+} exposure. We suggest that this effect is caused by a decrease in the permeability of the apical membrane to Ca\textsuperscript{2+}, possibly by an indirect blockage of Ca\textsuperscript{2+} channels by cytosolic Cd\textsuperscript{2+}. This hypothesis is supported by several observations. First, the inhibition by Cd\textsuperscript{2+} is not acute, in contrast to the inhibition by La\textsuperscript{3+} (Verbost et al. 1987a). Moreover, short-term exposure to Cd\textsuperscript{2+} (0.1 \mu mol l\textsuperscript{-1} Cd) has no effect on the rate of accumulation of Ca\textsuperscript{2+} in the tissue, whereas long-term exposure (17 h) decreases the rate of accumulation of Ca\textsuperscript{2+}. Second, long-term exposure to Cd\textsuperscript{2+} decreases the rate of accumulation of Cd\textsuperscript{2+} in the gills, whereas short-term exposure to Cd\textsuperscript{2+} has no effect. These observations indicate that Ca\textsuperscript{2+} channels become blocked when Cd\textsuperscript{2+} accumulates in the epithelial cells, as happens after a prolonged exposure to waterborne Cd\textsuperscript{2+}. These findings also support our conclusion that external Cd\textsuperscript{2+} cannot cause the inhibition of Ca\textsuperscript{2+} influx by competition with Ca\textsuperscript{2+} at mucosal sites, because the inhibition of influx is not instantaneous.

Which mechanism underlies the blockage by Cd\textsuperscript{2+} of the permeability of the apical membrane to Ca\textsuperscript{2+}? Once Cd\textsuperscript{2+} has entered the Ca\textsuperscript{2+}-transporting cell it could affect the Ca\textsuperscript{2+} channels in several ways. The basolateral Ca\textsuperscript{2+} pump has an extremely high affinity for Cd\textsuperscript{2+} \((K_{50} = 3 \text{ nmol l}\textsuperscript{-1}; \text{Verbost et al. 1988})\) and, therefore, nanomolar intracellular Cd\textsuperscript{2+} concentrations inhibit Ca\textsuperscript{2+} extrusion. We suggest that inhibition of the Ca\textsuperscript{2+} pump leads to an increased intracellular Ca\textsuperscript{2+} concentration, \([\text{Ca}^{2+}]_i\). Possibly, \([\text{Ca}^{2+}]_i\) rises to levels that close the apical membrane Ca\textsuperscript{2+} channels. Thus, \([\text{Ca}^{2+}]_i\) serves as a feedback signal to control Ca\textsuperscript{2+} entry at the apical membrane. An analogous model was proposed for rat small intestine enterocytes (Van Os, 1987). Both an overcapacity of Ca\textsuperscript{2+} pumps and buffering of Cd\textsuperscript{2+} by cytosolic binding proteins could explain the delay in the inhibition of Ca\textsuperscript{2+} uptake by Cd\textsuperscript{2+}.

To test the hypothesis that Cd\textsuperscript{2+} closes Ca\textsuperscript{2+} channels by increasing \([\text{Ca}^{2+}]_i\), would require examination with Ca\textsuperscript{2+} fluorochromes to show the predicted rise in \([\text{Ca}^{2+}]_i\). However, this was not feasible because Cd\textsuperscript{2+} quenches Quin2 fluorescence and interferes with Ca\textsuperscript{2+}-dependent fluorescence of Fura-2 and Indo-1 (P. M. Verbost & G. Visser, personal observations). The Ca\textsuperscript{2+} binding sites of Fura-2 and Indo-1 are EGTA-type sites (Tsien, 1980) with a much higher affinity for Cd\textsuperscript{2+} than for Ca\textsuperscript{2+} (Sillen & Martell, 1964). There is, however, indirect evidence for a rise in \([\text{Ca}^{2+}]_i\) upon exposure to Cd\textsuperscript{2+} of various other cell types: erythrocytes show an acceleration in age-related changes (Kunimoto et al. 1985), protein phosphorylation in human platelets is increased (Pezzi & Cheung, 1987), water absorption in rat duodenum is reduced (Toraason & Foulkes, 1984) and in rat
skeletal muscle cells the degradative enzymes phosphorylase-b kinase, phospholipases and proteases are activated (Toury et al. 1985). All these phenomena are known to be mediated by a rise in $[Ca^{2+}]$. In human lymphocytes $Cd^{2+}$ produces an increase in the accumulation of $^{45}Ca$ and in the rate of mitogenesis (Parker, 1974). The latter effect is also dependent on a rise in $[Ca^{2+}]$ (Parker, 1974).

Although apical membrane $Ca^{2+}$ channels may also be affected in fish exposed to $Cd^{2+}$, we conclude that $Ca^{2+}$ transport in the gills becomes inhibited primarily because the basolateral membrane $Ca^{2+}$ pump has such an extremely high sensitivity to $Cd^{2+}$. This conclusion for fish gills may be extended to explain intoxication of mammalian $Ca^{2+}$-transporting epithelia, such as the kidneys and the intestine, by $Cd^{2+}$. Recent data on $Cd^{2+}$ inhibition of $Ca^{2+}$ pumps from rat kidney and rat intestinal tissues (Verbost et al. 1987b) confirm the applicability of the gill model.

This study was supported by a grant from the Foundation for Fundamental Biological Research (BION), which is subsidized by the Dutch Organisation for Scientific Research (NWO). Mr F. A. T. Spanings is thanked for his excellent fish husbandry.

References


Cd$^{2+}$ inhibit Ca$^{2+}$ transport systems in plasma membranes and intracellular Ca$^{2+}$ stores in 
