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

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Summary

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

Introduction

Ca^{2+} uptake in freshwater fish mainly occurs *via* the gills (Flik *et al.* 1985). Ca^{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^{2+} uptake (Verbost *et al.* 1987*a*; Reid & McDonald, 1988) and induce hypocalcaemia (Giles, 1984; Pratap *et al.* 1989). We have advanced circumstantial evidence that the inhibition of Ca^{2+} uptake by waterborne Cd^{2+} may result from a competitive inhibition by cytosolic Cd^{2+} of the Ca^{2+} pump in the basolateral membrane of the Ca^{2+}-transporting cells in the gills (Verbost *et al.* 1988).

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

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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 ($0{\cdot}1\,\mu mol\,l^{-1}$ Cd in water containing $0{\cdot}7\,mmol\,l^{-1}$ Ca) do not instantly inhibit Ca^{2+} influx (Verbost *et al.* 1987*a*), indicating that no significant competition between waterborne Cd^{2+} and Ca^{2+} occurs for the cell entrance step (the concentration ratio Cd/Ca being $1{\cdot}4\times10^{-4}$ 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 $0{\cdot}7\times10^{-4}$ to $7{\cdot}0\times10^{-4}$). 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 ±

0·02 mmol l^{-1}, $N = 20$) under the conditions described previously (Verbost *et al.* 1987*a*). 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.* (1988*b*). 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 (MS 222, 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}Cd and six to ^{45}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 Cd^{2+} or Ca^{2+} was calculated from the total body radioactivity after 1 h of exposure to ^{109}Cd or ^{45}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 (Cd^{2+}) or micromoles (Ca^{2+}) per hour per 100 grams of fish (Lafeber *et al.* 1988*a*).

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

The amount of 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}Cd in the water (SA_w) using the equation: Cd^{2+} accumulated in 1 h $= q_g'/SA_w$ (in nanomoles 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 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 Ca^{2+} tracer (Borle, 1981). Therefore, to compare the accumulation of ^{109}Cd and ^{45}Ca in

gill tissue (Fig. 1A), relative values are presented (q_g'/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 $P \leq 0{\cdot}05$.

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

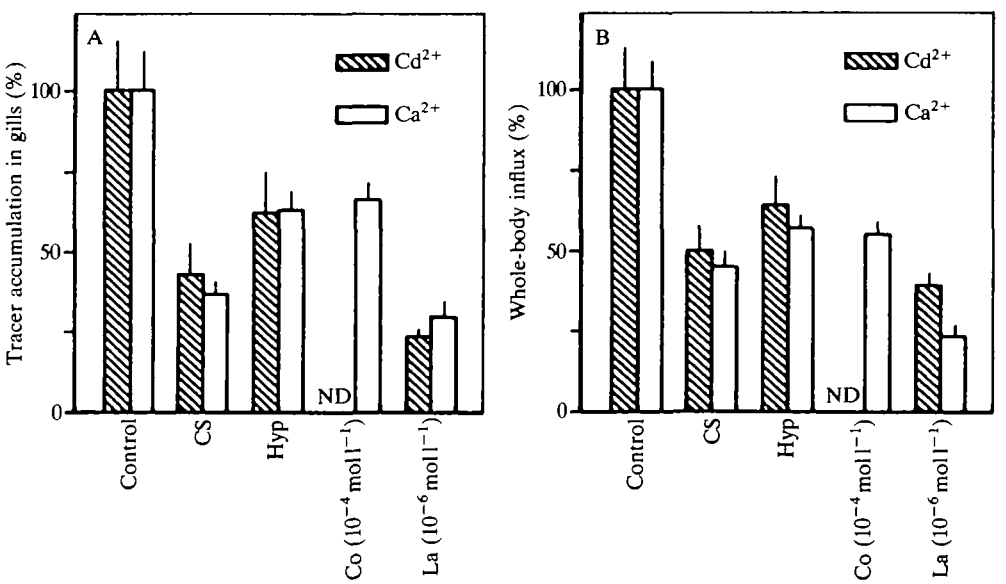

Fig. 1. (A) ^{45}Ca and ^{109}Cd accumulation in gill soft tissue after manipulation of apical membrane permeability. Values represent means ± S.E. ($N = 6$). All values were significantly different from their respective controls. CS, intraperitoneal injection of homogenate of corpuscles of Stannius; 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

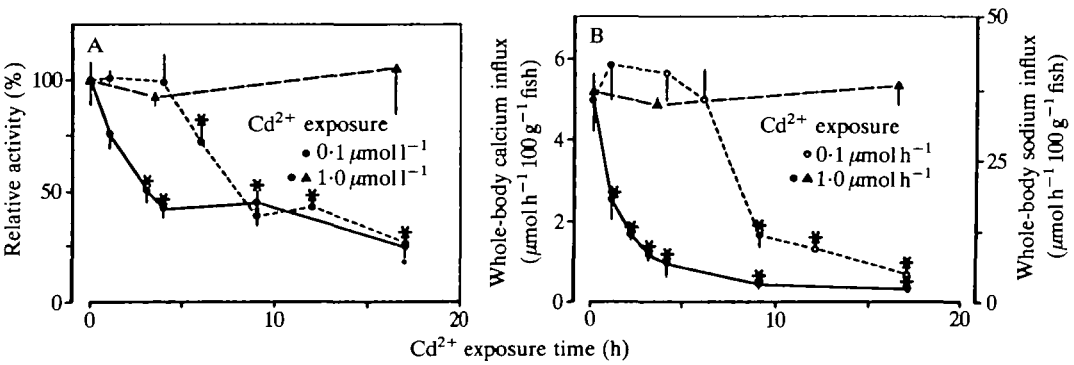

Fig. 2. (A) Effects of Cd^{2+} exposure time on ^{45}Ca (○,●) and ^{22}Na (▲) accumulation in gill soft tissue. Tracer accumulation was determined after 1 h of exposure. The nominal Cd concentrations were 0·1 μmol l^{-1} or 1·0 μmol l^{-1}, both during (pre-)exposure to Cd^{2+} and during exposure to 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.

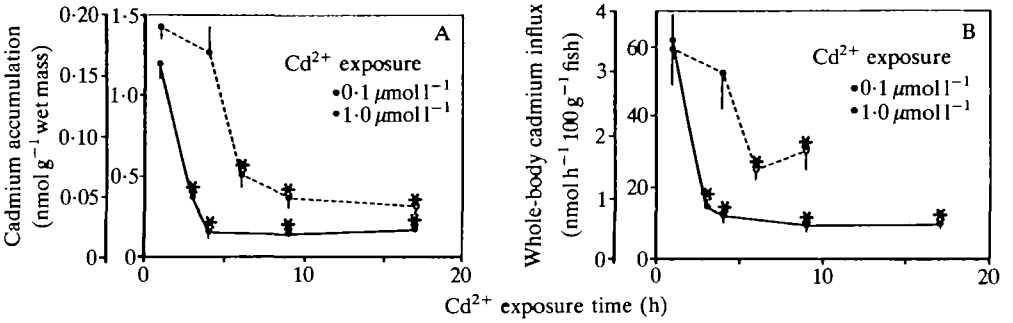

Fig. 3. (A) Effects of exposure to Cd^{2+} on the accumulation of ^{109}Cd in gill soft tissue. Points represent means ± S.E. ($N = 6$). Points marked with an asterisk are significantly different from their respective values at 1 h of exposure. (B) Effects of exposure to Cd^{2+} on whole-body Cd^{2+}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

Ca^{2+} influx, but after 9 and 17 h Ca^{2+} influx was inhibited by 67 % and 87 %, respectively. With 1 μmol l^{-1} exogenous Cd, Ca^{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 μmol l^{-1} Cd for 16·5 h had no effect on Na^+ influx.

Effects of Cd^{2+} exposure on Cd^{2+} influx

The effects of exogenous Cd^{2+} on the ^{109}Cd accumulation in gill epithelium are shown in Fig. 3A. Exposure to 0·1 μmol l^{-1} Cd for up to 4 h had no significant effect, but ^{109}Cd accumulation decreased by 64 % after 6 h and by 75 % after 17 h of exposure to Cd^{2+}. With 1·0 μmol l^{-1} Cd in the water, six times more Cd^{2+} accumulated in gill soft tissue than with 0·1 μmol l^{-1} Cd in the water. If we designate Cd^{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^{2+} on whole-body Cd^{2+} influx are shown. The Cd^{2+} influx at 0·1 μmol l^{-1} external Cd was around 3·4 nmol h^{-1} 100 g^{-1} fish. Exposure to 0·1 μmol l^{-1} Cd for 4 h did not significantly affect Cd^{2+} influx. However, after 6 h or more Cd^{2+} influx decreased by 55 %. In fish exposed to 1·0 μmol l^{-1} Cd for 1 h, Cd^{2+} influx was around 60 nmol h^{-1} 100 g^{-1} fish, 18 times higher than in those exposed to 0·1 μmol l^{-1} Cd. Cd^{2+} influx during the second hour of exposure to 1 μmol l^{-1} Cd and thereafter decreased by 80 %.

Discussion

Two major conclusions are drawn from this study. Cd^{2+} in the water at a concentration that provokes a specific hypocalcaemia inhibits both Ca^{2+} and Cd^{2+} influx, but not Na^+ influx. The inhibitory effect of Cd^{2+} is concentration- and time-dependent. Treatments that inhibit branchial Ca^{2+} influx (exposure to La^{3+} in the water or hypocalcin injections) inhibit branchial Cd^{2+} influx from the water.

Transepithelial Cd^{2+} influx

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 μmol l^{-1} external Cd and 61·9 nmol h^{-1} 100 g^{-1} fish at 1·0 μmol 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.* 1987*a*). 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.* (1988*a*), 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+}

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.* 1988*a*). 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 $1{\cdot}0\,\mu mol\,l^{-1}$ Cd and $0{\cdot}7\,mmol\,l^{-1}$ 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 basolaferal 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 $0{\cdot}25\,\mu mol\,l^{-1}$ 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^{2+} target in the gills

Our data show that Ca^{2+} and Cd^{2+} enter the gills *via* the same route. This implies that Cd^{2+} is concentrated in the ion-transporting cells of the gills, the chloride cells, since these cells account for the branchial Ca^{2+} transport (Fenwick, 1989). As a consequence, inhibition of Ca^{2+} transport will occur as soon as Cd^{2+} has accumulated to a level sufficient to inhibit the Ca^{2+}-ATPase transport system (Verbost *et al.* 1988). However, the present results also show a reduction in the rate of accumulation of Ca^{2+} in gills after prolonged Cd^{2+} exposure. We suggest that this effect is caused by a decrease in the permeability of the apical membrane to Ca^{2+}, possibly by an indirect blockage of Ca^{2+} channels by cytosolic Cd^{2+}. This hypothesis is supported by several observations. First, the inhibition by Cd^{2+} is not acute, in contrast to the inhibition by La^{3+} (Verbost *et al.* 1987*a*). Moreover, short-term exposure to Cd^{2+} ($0{\cdot}1\,\mu mol\,l^{-1}$ Cd) has no effect on the rate of accumulation of Ca^{2+} in the tissue, whereas long-term exposure (17 h) decreases the rate of accumulation of Ca^{2+}. Second, long-term exposure to Cd^{2+} decreases the rate of accumulation of Cd^{2+} in the gills, whereas short-term exposure to Cd^{2+} has no effect. These observations indicate that Ca^{2+} channels become blocked when Cd^{2+} accumulates in the epithelial cells, as happens after a prolonged exposure to waterborne Cd^{2+}. These findings also support our conclusion that external Cd^{2+} cannot cause the inhibition of Ca^{2+} influx by competition with Ca^{2+} at mucosal sites, because the inhibition of influx is not instantaneous.

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

To test the hypothesis that Cd^{2+} closes Ca^{2+} channels by increasing $[Ca^{2+}]_i$ would require examination with Ca^{2+} fluorochromes to show the predicted rise in $[Ca^{2+}]_i$. However, this was not feasible because Cd^{2+} quenches Quin2 fluorescence and interferes with Ca^{2+}-dependent fluorescence of Fura-2 and Indo-1 (P. M. Verbost & G. Visser, personal observations). The Ca^{2+} binding sites of Fura-2 and Indo-1 are EGTA-type sites (Tsien, 1980) with a much higher affinity for Cd^{2+} than for Ca^{2+} (Sillen & Martell, 1964). There is, however, indirect evidence for a rise in $[Ca^{2+}]_i$ upon exposure to Cd^{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 $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$ (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.* 1987*b*) confirm the applicability of the gill model.

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References

AVELLA, M., MASONI, A., BORNACIN, M. & MAYER-GOSTAN, N. (1987). Gill morphology and sodium influx in the rainbow trout (*Salmo gairdneri*) acclimated to artificial freshwater environments. *J. exp. Zool.* **241**, 159–169.

BATH, R. N. & EDDY, F. B. (1979). Ionic and respiratory regulation in rainbow trout during rapid transfer to seawater. *J. comp. Physiol.* **134**, 351–357.

BORLE, A. B. (1981). Control, modulation, and regulation of cell calcium. *Rev. Physiol. Biochem. Pharmac.* **90**, 13–153.

CHAO, S.-H., SUZUKI, Y., ZYSK, J. R. & CHEUNG, W. Y. (1984). Activation of calmodulin by various metal cations as a function of ionic radius. *Molec. Pharmac.* **26**, 75–82.

DERENZIS, G. & BORNACIN, M. (1984). Ion transport and gill ATPase. In *Fish Physiology*, vol. XB (ed. W. S. Hoar & D. J. Randall), pp. 65–104. Orlando: Academic Press.

DIEZI, J., HAUSEL, P. & FELLEY-BOSCO, E. (1988). Effects of cadmium ions on Na/K-ATPase activity in a defined segment of the rabbit proximal tubule. *Arch. Toxicol.* (Suppl.) **12**, 125–128.

FENWICK, J. C. (1989). The gill. In *Vertebrate Endocrinology, Fundamental and Biomedical Implications. Regulation of Ca^{2+} and Phosphate* (ed. P. K. T. Pang & M. B. Schreibman). New York: Academic Press (in press).

FLIK, G., VAN DE WINKEL, J. G. J., PÄRT, P., WENDELAAR BONGA, S. E. & LOCK, R. A. C. (1987). Calmodulin mediated cadmium inhibition of phosphodiesterase activity in vitro. *Arch. Toxicol.* **59**, 353–359.

FLIK, G., VAN RIJS, J. H. & WENDELAAR BONGA, S. E. (1985). 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.

FOULKES, E. C. (1986). Absorption of cadmium. In *Cadmium. Handbook of Experimental Pharmacology*, vol. 80 (ed. E. C. Foulkes), pp. 75–100. Berlin: Springer-Verlag.

GILES, M. A. (1984). Electrolyte and water balance in plasma and urine of rainbow trout (*Salmo gairdneri*) during chronic exposure to cadmium. *Can. J. Fish. aquat. Sci.* **41**, 1678–1685.

KANEKO, T., FRASER, R. A., LABEDZ, T., HARVEY, S., LAFEBER, F. P. J. G. & PANG, P. K. T. (1988). Characterization of antisera raised against hypocalcin (teleocalcin) purified from corpuscles of Stannius of rainbow trout, *Salmo gairdneri*. *Gen. comp. Endocr.* **69**, 238–245.

KUNIMOTO, M., MIURA, T. & KUBOTA, K. (1985). An apparent acceleration of age-related changes of red blood cells by cadmium. *Toxicol. appl. Pharmac.* **77**, 451–457.

LAFEBER, F. P. J. G. (1988). Comparison of PTH and hypocalcin in mammalian and teleost bioassays. PhD thesis, University of Nijmegen. ISBN 90-9002131-0.

LAFEBER, F. P. J. G., FLIK, G., WENDELAAR BONGA, S. E. & PERRY, S. F. (1988*a*). Hypocalcin from Stannius corpuscles inhibits gill calcium uptake in trout. *Am. J. Physiol.* **254**, R891–R896.

LAFEBER, F. P. J. G., HANSSEN, R. G. J. M., CHOY, Y. M., FLIK, G., HERMANN-ERLEE, M. P. M., PANG, P. K. T. & WENDELAAR BONGA, S. E. (1988*b*). Identification of hypocalcin (teleocalcin) isolated from trout Stannius corpuscles. *Gen. comp. Endocr.* **69**, 19–30.

LAI, J. C. K., GUEST, J. F., LEUNG, T. K. C., LIM, L. & DAVISON, A. N. (1980). The effects of cadmium, manganese and aluminum on sodium–potassium-activated and magnesium-activated adenosine triphosphatase activity and choline uptake in rat brain synaptosomes. *Biochem. Pharmac.* **29**, 141–146.

PARKER, C. W. (1974). Correlation between mitogenicity and stimulation of calcium uptake in human lymphocytes. *Biochem. biophys. Res. Commun.* **61**, 1180–1186.

PÄRT, P. (1983). Cadmium uptake in perfused rainbow trout gills. Doctoral thesis at Uppsala University, Sweden. ISBN 91-554-1474-5.

PÄRT, P. & SVANBERG, O. (1981). Uptake of cadmium in perfused rainbow trout (*Salmo gairdneri*) gills. *Can. J. Fish. aquat. Sci.* **38**, 917–924.

PÄRT, P., SVANBERG, O. & KIESSLING, A. (1985). The availability of cadmium to perfused rainbow trout gills in different water qualities. *Water Res.* **19**, 427–434.

PAYAN, P. & MAETZ, J. (1973). Branchial sodium transport mechanisms in *Scyliorhinus canicula*: evidence for Na^+/NH_4^+ and Na^+/H^+ exchanges and for a role of carbonic anhydrase. *J. exp. Biol.* **58**, 487–502.

PERRY, S. F. & FLIK, G. (1988). A characterization of branchial transepithelial calcium fluxes in the freshwater trout (*Salmo gairdneri*). *Am. J. Physiol.* **254**, R491–R498.

PEZZI, L. & CHEUNG, W. Y. (1987). Effects of cadmium on human platelet reactions. *Arch. Toxicol.* **61**, 120–125.

PRATAP, H. B., FU, H., LOCK, R. A. C. & WENDELAAR BONGA, S. E. (1989). Effects of waterborne and dietary cadmium on plasma ions of the teleost *Oreochromis massambicus* in relation to water calcium levels. *Arch. environ. Contam. Toxicol.* (in press).

REID, S. D. & MCDONALD, D. G. (1988). Effects of cadmium, copper and low pH on ion fluxes in rainbow trout, *Salmo gairdneri*. *Can. J. Fish. aquat. Sci.* **45**, 244–253.

RICHARDT, G., FEDEROLF, G. & HABERMANN, E. (1986). Affinity of heavy metal ions to intracellular Ca^{2+}-binding proteins. *Biochem. Pharmacol.* **35**, 1331–1335.

SILLEN, L. G. & MARTELL, A. E. (1964). *Stability Constants of Metal Ion Complexes*. Special Publication no. 17. London: The Chemical Society.

SUZUKI, Y., CHAO, S. H., ZYSK, J. R. & CHEUNG, W. Y. (1985). Stimulation of calmodulin by cadmium ion. *Arch. Toxicol.* **57**, 205–211.

TOKUSHIGE, A., HIGASHINO, H., SEARLE, B. M., TAMURA, H., KINO, M., BOGDEN, J. D. & AVIV, A. (1984). Cadmium effect on the Na,K-ATPase system in cultured vascular smooth muscle cells. *Hypertension* **6**, 20–26.

TSIEN, R. Y. (1980). New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry, N.Y.* **19**, 2396–2404.

TORAASON, M. & FOULKES, E. C. (1984). Interaction between calcium and cadmium in the 1,25-dihydroxyvitamin D3 stimulated rat duodenum. *Toxicol. appl. Pharmac.* **75**, 98–104.

TOURY, R., STELLY, N., BOISSONNEAU, E. & DUPUIS, Y. (1985). Degenerative processes in skeletal muscle of Cd^{2+} treated rats and Cd^{2+} inhibition of mitochondrial Ca^{2+} transport. *Toxicol. appl. Pharmac.* **77**, 19–35.

VAN OS, C. H. (1987). Transcellular calcium transport in intestinal and renal epithelial cells. *Biochim. biophys. Acta* **906**, 195–222.

VERBOST, P. M., FLIK, G., LOCK, R. A. C. & WENDELAAR BONGA, S. E. (1987*a*). Cadmium inhibition of Ca^{2+} uptake in rainbow trout gills. *Am. J. Physiol.* **253**, R216–R221.

VERBOST, P. M., FLIK, G., LOCK, R. A. C. & WENDELAAR BONGA, S. E. (1988). Cadmium inhibits plasma membrane calcium transport. *J. Membr. Biol.* **102**, 97–104.

VERBOST, P. M., SENDEN, M. H. M. N. & VAN OS, C. H. (1987*b*). Nanomolar concentrations of Cd^{2+} inhibit Ca^{2+} transport systems in plasma membranes and intracellular Ca^{2+} stores in intestinal epithelium. *Biochim. biophys. Acta* **902**, 247–252.

WENDELAAR BONGA, S. E. & PANG, P. K. T. (1986). Stannius corpuscles. In *Vertebrate Endocrinology, Fundamental and Biomedical Implications*, vol. 1 (ed. P. K. T. Pang & M. B. Schreibman), pp. 439–464. New York: Academic Press.

WILLIAMS, D. R. & GIESY, J. P. JR (1978). Relative importance of food and water sources to cadmium uptake by *Gambusia affinis*. *Envir. Res.* **16**, 326–332.