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Effects of Zn^{2+} on Ca^{2+} uptake by mitochondria and endoplasmic reticulum in permeabilized tilapia gill cells

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Abbreviations: BSA, bovine serum albumin; EGTA, ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; HEEDTA, N-(2-hydroxyethyl) ethylene diamine-N,N',N'-triacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid; NTA, nitrilotriacetic acid

Abstract

An intracellular ATP-dependent Ca^{2+} pumping mechanism, distinct from mitochondrial Ca^{2+} accumulation, was identified within tilapia gill cells. Cell suspensions treated with 0.003% saponin, which selectively permeabilizes the plasma membrane, were used to characterize the Ca^{2+} sequestering mechanisms as endoplasmic reticulum and mitochondria and to determine the effect of Zn^{2+} on their Ca^{2+} storing activity. Of the Ca^{2+} taken up by the endoplasmic reticulum, 80% was released by IP_3 (10 µmol l^{-1}). The Ca^{2+} pump of the endoplasmic reticulum was 2.5 times less sensitive to Zn^{2+} (IC_{50} = 0.05 nmol l^{-1}) than was the mitochondrial uptake mechanism (IC_{50} = 0.20 nmol l^{-1}). The results indicate that Ca^{2+} is stored predominantly within the endoplasmic reticulum at 0.1 µmol l^{-1} and that this storing capacity is seriously attenuated by nanomolar concentrations Zn^{2+}.

Introduction

Branchial epithelial cells are involved in both ion and gas exchange but are still similar to other cells in that they maintain resting levels of intracellular Ca^{2+} of about 100 nmol l^{-1} (95 ± 15 nmol l^{-1}; Li *et al.* 1995). In cells in general a wide variety of actions are regulated by fluctuations in free Ca^{2+}. Neurohumoral substances can induce rapid, transient increases in cytosolic Ca^{2+} either by stimulating Ca^{2+} influx *via* receptor operated Ca^{2+} channels in the cell membrane and/or by releasing Ca^{2+} from intracellular stores through inositol-1,4,5-triphosphate (IP_3) activated channels (Rasmussen *et al.* 1986; Berridge 1993).

In addition to serving a role as second messenger, Ca^{2+} is also transferred transepithelially during branchial calcium absorption associated with the calcium homeostasis of the freshwater fish (reviewed by Fenwick 1989). It is well established that this uptake occurs *via* the ionocytes – mitochondria rich cells – in the gills and that the uptake occurs transcellularly (Perry and Flik 1988; McCormick *et al.* 1992; Marshall *et al.* 1995). This means that during transepithelial transport the transported calcium could affect the cytosolic Ca^{2+} concentration. The current model for active Ca^{2+} absorption involves passive entry across the apical membrane, diffusion through the cytosol, and ATP- and Na^+-driven efflux across the basolateral plasma membrane (Flik *et al.* 1993; Verbost *et al.* 1994b). And little is known concerning the state of Ca^{2+} on its route through the cytosol. Active Ca^{2+} sequestration by intracellular organelles and

buffering by calcium binding proteins occurs in rat intestinal epithelium (Wasserman and Fullmer 1982; Rubinoff and Nellans 1985; Van Corven *et al* 1987; Feher *et al* 1992) and this mechanism may be generally involved in the intracellular handling of Ca^{2+} during transit.

The current model for mammalian epithelial cells suggests that the Ca^{2+} pumps in the plasma membrane and endoplasmic reticulum (ER) are of equal importance for maintaining low intracellular Ca^{2+} (Van Os *et al.* 1988). On the other hand, the mitochondria appear to perform little if any role in maintaining low intracellular Ca^{2+} concentration (Nicholls and Akerman 1982; Van Os *et al.* 1988) because they do not have a sufficiently high affinity for Ca^{2+}. The mitochondria do have a large capacity to store Ca^{2+}, but this can result in activation of various mitochondrial enzymes essential for the regulation of ATP production (Denton and McCormack 1985).

The purpose of this study was: a) to test if in fish gill cells regulate intracellular Ca^{2+} in the same way as mammalian kidney cells and enterocytes and b) to determine the effects of zinc (Zn) on the Ca^{2+} sequestration by the mitochondrial and non-mitochondrial Ca^{2+} stores. There are two reasons for studying Zn^{2+}. One is to learn about the mechanism of toxicity of this environmental pollutant and, more important for this study, Zn^{2+} can be used as a tool for studying intracellular Ca^{2+} as well as Ca^{2+} transport. Waterborne Zn^{2+} inhibits the branchial uptake of Ca^{2+} (Spry and Wood 1985; Hogstrand *et al.* 1994, 1995a). There is evidence to suggest that the inhibition by Zn^{2+} is caused by the inhibition of basolateral Ca^{2+} transport mechanism after entrance of Zn^{2+} into the cytosol. Concentrations of $Zn^{2+} > 100$ pmol l^{-1} reduced the ATP-dependent Ca^{2+} transport in the basolateral membranes (Hogstrand *et al.* 1995b), the putative driving force uptake of Ca^{2+} (Flik *et al.* 1985a,b). However, nothing is known about the effects of Zn^{2+} on the sequestration of Ca^{2+} by intracellular stores. We set out to determine these effects with the idea that a disturbance of the intracellular Ca^{2+} homeostasis could also partly explain the reduction in Ca^{2+} uptake by Zn^{2+}.

Materials and methods

Holding conditions of fish

Male freshwater tilapia from laboratory stock, weighing around 150 g, were held in 100 l tanks. The aquaria were supplied with running tap water (0.7 mmol l^{-1} Ca, 25°C) under a photoperiod of 12 h of light alternating with 12 h of darkness. Animals were fed Trouvit® fish pellets (Trouw & Co., Putten, The Netherlands) at the rate of 1.5% body weight per day.

Preparation of permeabilized gill cells

Gill cells were isolated as described by Verbost *et al.* (1994a). In brief, the branchial epithelium is scraped from the underlying cartilaginous filaments using the edge of a glass slide. The scrapings were then incubated for 20 min at room temperature in a lysis medium (9 parts 0.17 M NH_4Cl, 1 part 0.17 M Tris/HCl pH 7.4; Yust *et al.* 1976) which produced both blood cell lysis and tissue fractionation. Cells were suspended at the beginning and resuspended at the end of this incubation period by passing them 5 times through a 10 ml pipet (3 mm bore diameter). The lysis was optimal when 2.5 mg tissue scrapings were lysed in 10 ml lysis-medium (around 3.25×10^7 cells per 10 ml). The cells were filtered through 100 µm mesh nylon gauze to remove persistent cell clusters. The resulting suspension was centrifuged at 150 g for 5 min at 4°C in a swing out rotor (BS4402/A rotor, Heraeus). The cells were resuspended in lysis medium (1 mg of protein per ml) containing 135 mM KCl, 1.0 mM $MgCl_2$, 1.2 KH_2PO_4, 10 mM Hepes pH 7.4, and 30 µg ml^{-1} saponin and incubated for 5 min at 37°C. The isolated, permeabilized gill cells were washed twice in the final uptake medium (see below). Saponin treatment yielded 50 to 60% leaky cells. The degree of leakiness was estimated by uptake of trypan blue (0.5%). After saponin treatment cells were pelleted, washed and resuspended to a concentration of 4 mg ml^{-1} in a Ca^{2+} uptake medium containing (in mmol l^{-1}) 120 KCl, 1 $MgCl_2$, 1.2 KH_2PO_4, 5 succinate, 5 pyruvate, 0.5 EGTA, 0.5 HEEDTA, 0.5 NTA, 25 Hepes/KOH (pH 7.1). Cells were used on the day of isolation. The protein content of the cell preparations was

determined with a commercial reagent kit (Bio-Rad) with BSA as a reference. This preparation comprises a mixture of gill epithelial cells with ionocytes (about 10%) and respiratory cells (around 90%).

Isolation of microsomes

Microsomes (the cell membrane fraction mainly consisting of endoplasmic reticulum membranes) were isolated as described by Vercesi *et al.* (1978). First, gill cells were isolated followed by erythrocyte lysis as described above (preparation of permeabilized gill cells). The following steps were all performed at 4°C. The cells were collected by centrifugation (5 min, 220 × g), resuspended in sucrose medium containing (in mmol l^{-1}) 250 sucrose, 0.55 EGTA, 3 Hepes/Tris (pH 7.4) and subsequently homogenized in a douncer with loose fitting pestle (Braun Melsungen) and the homogenate was centrifuged (5 min, 600 × g). The resulting supernatant was centrifuged at 10,000 × g (10 min). The pellet was resuspended in sucrose medium (as above) and centrifuged at 12,000 × g (10 min). This final pellet was resuspended in Ca^{2+} uptake medium (same as given under preparation of permeabilized gill cells) to which mitochondrial inhibitors were added (1 mmol l^{-1} NaN_3 and 5 µg ml^{-1} oligomycin-B). Microsomes were used for the Ca^{2+} uptake studies on the day of isolation.

Ca^{2+} uptake in permeabilized cells and microsomes

ATP-dependent $^{45}Ca^{2+}$ uptake was measured as described by Van Corven *et al.* (1987) and Van de Put *et al.* (1991). The assay was performed at 28°C in the Ca^{2+} uptake medium (described above) but which also contained 10 units ml^{-1} creatine kinase, 10 mmol l^{-1}) creatine phosphate, 1 mmol l^{-1} ATP and 185 KBq ml^{-1} $^{45}Ca^{2+}$. The free Mg^{2+} concentration (0.8 mmol l^{-1}) and the free Ca^{2+} concentration (as indicated) were calculated according to Schoenmakers *et al.* (1992). $^{45}Ca^{2+}$ uptake was started by adding permeabilized cells or microsomes to prewarmed uptake medium. At certain time intervals 100 µl aliquots (40 µg protein) were quenched in 1 ml of ice-cold stop solution containing (in mmol l^{-1}) 150 KCl, 1 $MgCl_2$, 1 EGTA, 20 Hepes/KOH (pH 7.1) and the suspension was filtered immediately (Schleicher and Schüll, GF92). The filters were then washed twice with 2 ml of stop solution, dissolved in scintillation fluid and counted for radioactivity. Total Ca^{2+} uptake was calculated from the radioactivity retained by the filter and is expressed as nmol mg^{-1} protein.

Ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake (Moore 1971), was used (20 µmol l^{-1}). With other mitochondrial inhibitors as oligomycin-B (5 µmol l^{-1}) and sodium azide (5 mmol l^{-1}) very similar results were obtained (results not shown). Thapsigargin (1 µmol l^{-1}) was used to inhibit the Ca^{2+} uptake by the endoplasmic reticulum (Thastrup *et al.* 1990; Hovemadsen and Bers 1993). The inhibitors were added to the permeabilized cells 10 to 20 min before the start of the assay.

Data presentation

Data are expressed as the mean ± SEM. For statistical analysis of the results a repeated measures ANOVA was applied (3 way; Zn*Ca*time). To compare individual means a two way ANOVA on the data from the 2 Ca concentrations was used. Differences were considered to be significant at $p < 0.05$.

Results

Ca^{2+} uptake in permeabilized cells

The time dependence of Ca^{2+} uptake by permeabilized gill cells at 0.1 µmol l^{-1} is shown in Figure 1. Ca^{2+} uptake is stimulated by ATP, reaching a steady state level between 6 and 8 min. The uptake was not affected by a blocker of mitochondrial uptake (ruthenium red or oligomycin-B and sodium azide). Thapsigargin, a specific inhibitor of ER Ca^{2+}-ATPases, inhibited almost all ATP-dependent Ca^{2+} uptake. ATP-independent binding comprises about 30% of the total uptake.

The time dependence of Ca^{2+} uptake at 1.0 µmol l^{-1} Ca^{2+} is shown in Figure 2. For the duration of the experiment (8 min) the uptake was linear with time and was almost completely inhibited by the mitochondrial inhibitor ruthenium red. The uptake in the presence of mitochondrial inhibitors was fur-

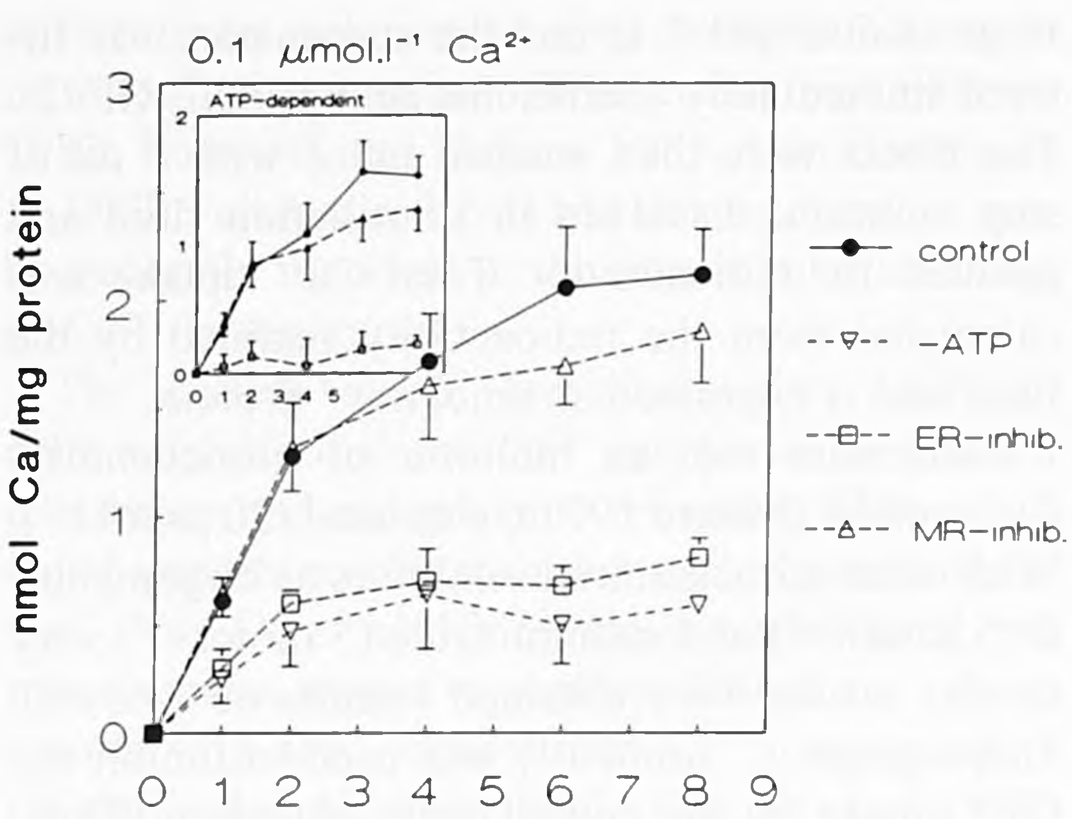

Fig. 1. Time-dependent Ca^{2+} uptake by permeabilized tilapia gill cells at a free Ca^{2+} concentration of 0.1 μmol l^{-1}. Solid circles represent uptake in the presence of ATP (control); this was compared with Ca^{2+} uptake in the presence of thapsigargin (1 μmol l^{-1}) or ruthenium red (20 μmol l^{-1}) and that the absence of ATP (-ATP). The inset shows the ATP-dependent Ca^{2+} uptake (same curves after subtraction of the -ATP values). Mean values of 4 experiments ± SEM are shown.

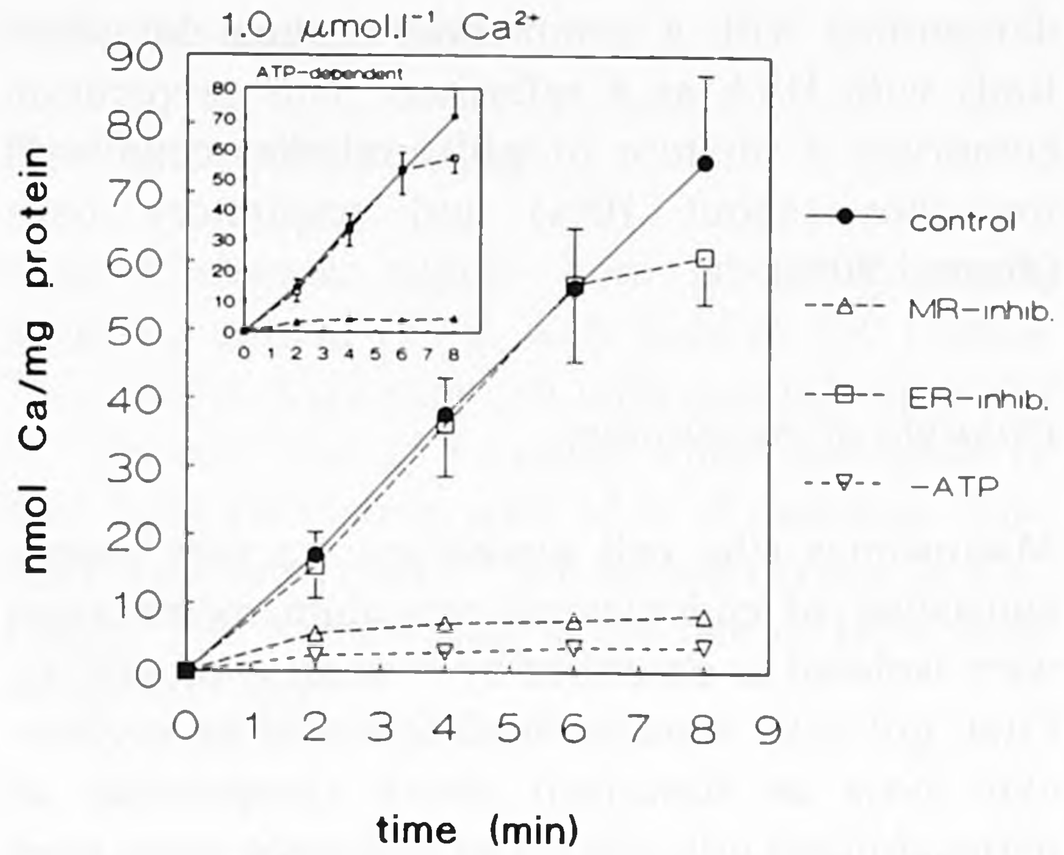

Fig. 2. Time-dependent Ca^{2+} uptake by permeabilized tilapia gill cells at a free Ca^{2+} concentration of 1.0 μmol l^{-1}. Solid circles represent uptake in the presence of ATP (control); this was compared with Ca^{2+} uptake in the presence of thapsigargin (1 μmol l^{-1}) or ruthenium red (20 μmol l^{-1}) and that in the absence of ATP (-ATP). The inset shows the ATP-dependent Ca^{2+} uptake (same curves after subtraction of the -ATP values). Mean values of 5 experiments ± SEM are shown.

ther reduced by the "ER inhibitor" thapsigargin to the level when ATP is omitted (this line is exactly the same as that for the -ATP condition in Fig. 2, and was left out for clarify).

Effects of IP_3 on the uptake of Ca^{2+} in permeabilized cells and microsomes

From the Ca^{2+} taken up ATP-dependently during a 10 min period by permeabilized cells, about 80% is released within 1 min following addition of IP_3 (10 μmol l^{-1}): from 2.85 ± 0.36 to 0.57 ± 0.22 nmol Ca^{2+} per mg protein in permeabilized cells (Fig. 3A). Heparin (10 μmol l^{-1}) completely prevented the IP_3 effect (control); this was done by adding a mixture of IP_3 and heparin. With the microsomes a similar result was obtained. There was an immediate release of Ca^{2+} following addition of IP_3 (64%, from 2.8 ± 0.2 to 1.0 ± 0.1 nmol Ca^{2+} per mg protein microsomes) which could be blocked by heparin (by adding IP_3 and heparin together = control; Fig. 3B).

Effects of Zn^{2+} on Ca^{2+} uptake in permeabilized cells at 0.1 μM Ca^{2+}

There was not significant difference between the ATP-dependent uptake in the presence of 0.1 and 0.5 nmol l^{-1} Zn^{2+} compared to the control (Fig. 4). With 1 nmol l^{-1} Zn^{2+} in the medium, uptake was inhibited significantly at all time points. The uptake was linear with time for up to 2 min and applying the initial ATP-dependent uptake rates from 1 min time points an IC_{50} value of 0.50 nmol l^{-1} Zn^{2+} was calculated.

Effects of Zn^{2+} on Ca^{2+} uptake in permeabilized cells at 1.0 μM Ca^{2+}

The (mitochondrial) uptake in the presence of 0.1 nmol l^{-1} Zn^{2+} was not significantly different from the control (Fig. 5). However, at 0.5 or 1.0 nmol l^{-1} Zn^{2+} uptake was inhibited at all time points (Fig. 5). The uptake was linear with time for the complete time period in this experiment (8 min) and confirms the classic characteristic of the mitochondria as functioning as a calcium sink. From the initial ATP-dependent uptake rates at the 2 min time

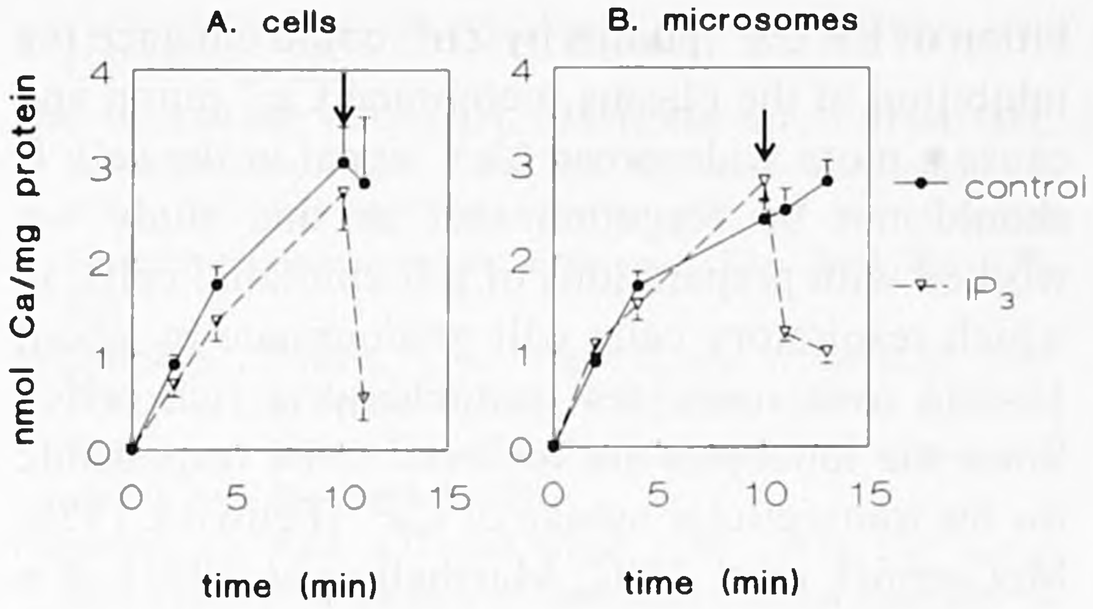

Fig. 3. A. Effects of IP_3 (added at 10 min, indicated by arrow) or a mixture of heparin and IP_3 (control) on the release of ATP-dependently stored Ca^{2+} in permeabilized tilapia gill cells. B. Effects of IP_3 (added at 10 min, indicated by arrow) or a mixture of heparin and IP_3 (control) on the release of ATP-dependently stored Ca^{2+} in microsomes from tilapia gill cells.

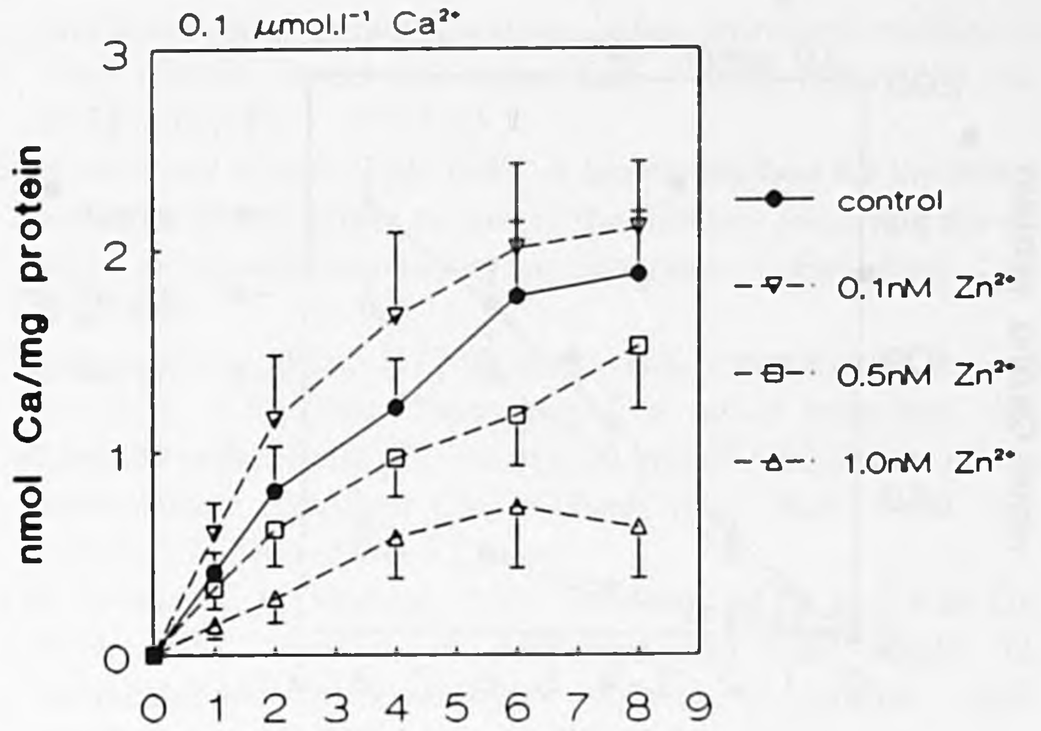

Fig. 4. Effects of Zn^{2+} on the ATP-dependent Ca^{2+} uptake in permeabilized tilapia gill cells at a free Ca^{2+} concentration of 0.1 μmol l^{-1}, representing the uptake by ER. Mean values of 4 experiments ± SEM are shown.

points an IC_{50} value of 0.20 μmol l^{-1} Zn^{2+} was calculated.

Discussion

Our results show that in gill cells the ER is of major importance for Ca^{2+}-sequestration at physiological intracellular levels (0.1 μM). Mitochondria only function as Ca^{2+} buffer at high intracellular Ca^{2+} levels (around 1 μM). This is congruent with the current models for Ca^{2+} buffering that are know for various cells in higher vertebrates (Joseph *et al.* 1983; Kreutter and Rasmussen 1984). Although the complete kinetics of the Ca^{2+} uptake were not determined, the results demonstrate that the affinity for Ca^{2+} of the mitochondria is lower than that of the ER, but that the capacity of the mitochondrial Ca^{2+} uptake is much higher than that of the ER. Thus, the ER will be more important for buffering intracellular Ca^{2+} at normal cell-physiological Ca^{2+} levels, comparable, in this respect, to the function of the Ca^{2+} pump in the plasma membrane.

The maximum Ca^{2+} release from the intracellular, inositol 1,4,5-triphosphate(IP_3)-sensitive Ca^{2+} pool amounts to about 80% of the ATP-dependent, intracellularly stored $^{45}Ca^{2+}$. The remaining 20% of the accumulated $^{45}Ca^{2+}$ is insensitive to IP_3 and appears to be stored in an IP_3-insensitive part of the ER. The remaining activity was not stored by mitochondria as the results clearly show the absence of mitochondrial uptake under these test conditions. The fact that the simultaneous addition of the IP_3 receptor antagonist heparin completely blocked Ca^{2+} release by IP_3 indicates that this release is not an artifact of the addition, but is really mediated by the IP_3 receptor. In microsomes, a rough ER membrane fraction, the same induction of Ca^{2+} release by IP_3 was observed and confirms the ER origin of the Ca^{2+}. Zinc (Zn^{2+}) exerted a strong inhibition of the Ca^{2+}-uptake by both the ER and mitochondria, the mitochondrial uptake being 2.5 times more sensitive than the uptake by ER. But this does not mean that the mitochondria form an earlier target for Zn^{2+} than the ER because the low prevailing Ca^{2+} concentration in the cytosol would dictate negligible mitochondrial Ca^{2+} uptake under these conditions. The situation in which mitochondrial Ca^{2+} uptake exceeds that of the ER will be close to one where the cell is dying. This is perhaps not a very interesting condition from a physiological point of view, although the high sensitivity of the mitochondria to Zn^{2+} may be very detrimental when cytosolic Ca^{2+} is high in the presence of, and perhaps because of the presence of, Zn^{2+}.

It is important to note that the mechanisms of Ca^{2+} uptake are completely different for the two Ca^{2+} stores. ER utilise a Ca^{2+}-pump whereas mitochondria take Ca^{2+} up *via* a uniporter driven by the proton gradient across the inner membrane (Alberts *et al.* 1994). These differences could be the

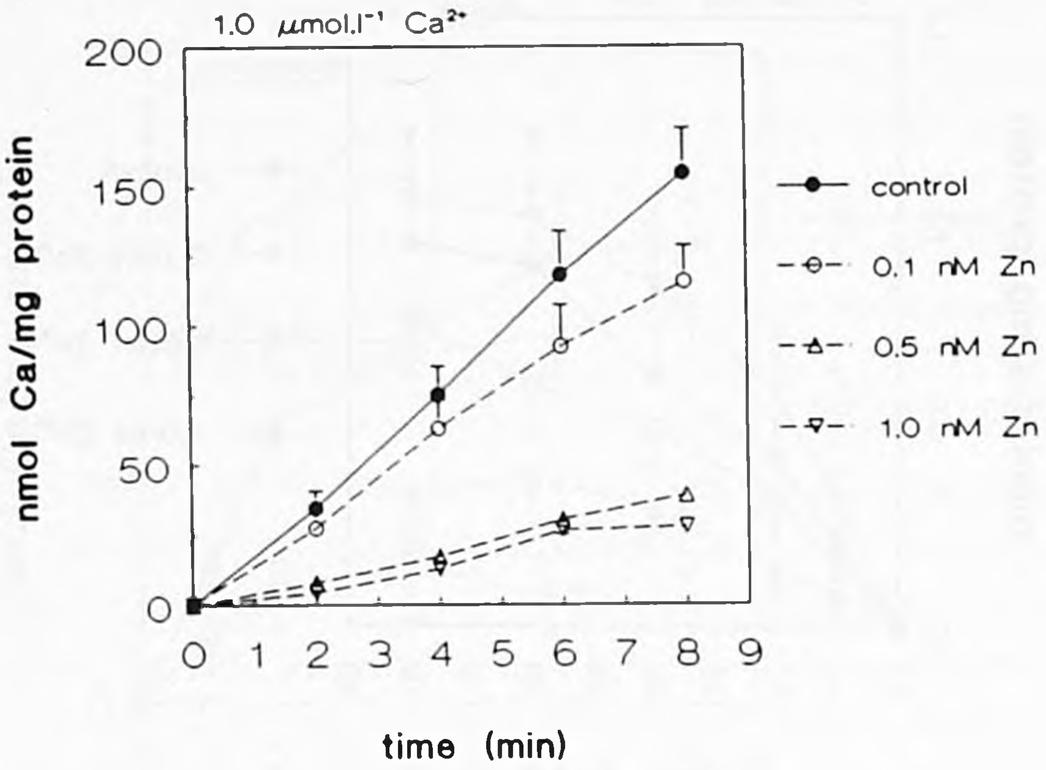

Fig. 5. Effects of Zn^{2+} on the ATP-dependent Ca^{2+} uptake in permeabilized tilapia gill cells at a free Ca^{2+} concentration of 1.0 μmol l^{-1}, representing the uptake by mitochondria. Mean values of 4 experiments ± SEM are shown.

basis for the dissimilar sensitivity to Zn^{2+} of the ER and mitochondria.

How can we fit these data into a complete picture of what will happen when a fish is exposed to water containing Zn^{2+}? At sublethal concentrations in the micromolar range, Zn^{2+} impairs the branchial influx of Ca^{2+} resulting in hypocalcemia (Spry and Wood 1985). Zn^{2+} has 10 times the affinity for Ca^{2+}-uptake sites than Ca^{2+} and clearly outcompetes Ca^{2+} (Spry and Wood 1989; Hogstrand *et al.* 1994, 1995a). Recent studies indicated that Zn^{2+} enters the gill cells (Wicklund *et al.* 1992; Hogstrand *et al.* 1995b) and this has resulted in the hypothesis that Zn^{2+} inhibits transcellular Ca^{2+} uptake by inhibiting the Ca^{2+} pump in the basolateral membrane. A similar mechanism was suggested for the inhibition of Ca^{2+} uptake by Cd^{2+} (Verbos *et al.* 1988, 1989). The effects of Zn^{2+} (free Zn levels in a metal-buffered assay media) on the basolateral Ca^{2+} pump has, to the best of our knowledge, only been studied in trout (Hogstrand *et al.* 1995b). There, it caused a mixed inhibition of the Ca^{2+} transporter. At 0.5 nmol l^{-1} Zn^{2+}, the affinity was reduced 8-fold and the V_{max} was decreased by a factor of 2. The results in this study show that the intracellular sequestration of Ca^{2+} is a putative target of Zn^{2+} entering the cell in addition to the plasma membrane Ca^{2+}-pump. Inhibition of the Ca^{2+} sequestration will result in a rise in cytosolic Ca^{2+} which will in turn lead to a reduction of Ca^{2+} uptake (Marshall *et al.* 1995). In this way, the inhibition of ER Ca^{2+} pumps by Zn^{2+} could enhance the inhibition of the plasma membrane Ca^{2+} pump and cause a more widespread Ca^{2+} signal in the cell. It should not be forgotten that in this study we worked with preparations of gill epithelial cells, in which respiratory cells will predominate by about 10-fold over ionocytes (mitochondria rich cells). Since the ionocytes are believed to be responsible for the transcellular uptake of Ca^{2+} (Fenwick 1989; McCormick *et al.* 1992; Marshall *et al.* 1995), it is not possible to say that we are only dealing with events in calcium transporting cells. This study rather suggests that waterborne Zn^{2+} may lead to an increase in intracellular Ca^{2+} in gill epithelial cells *via* inhibition of Ca^{2+} sequestration by ER (and eventually mitochondria). In ionocytes this could result in reduced transport of Ca^{2+}. For respiratory cells nothing is known at present about the possible effects a rise in cytosolic Ca^{2+} could have on cell function.

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