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CALCIUM TRANSPORT IN FISH GILLS AND INTESTINE

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Summary

In calcium-transporting epithelia, calcium can move transcellularly (when it passes inwards, from mucosa to serosa) and paracellularly (when it moves in both an inward and outward direction). An epithelium is considered to be 'tight' when the transcellular route dominates and leaky when there is additional significant paracellular transport. The branchial epithelium of the gills of freshwater fish is a good model for tight epithelia, whereas the gills of seawater fish and the intestine present a model for leaky epithelia. Generally, the regulation of transcellular inward calcium transport determines whether net absorption occurs and the regulation of paracellular calcium transport is pivotal to secretion in calcium-transporting epithelia. In its simplest form, transcellular transport requires movement of Ca^{2+} across the apical membrane, through the cytosol and across the basolateral membrane. At the same time, cellular calcium homeostasis must be maintained and, to this end, calcium is buffered in the cytosol by calcium-binding proteins and sequestered in the endoplasmic reticulum and mitochondria. Movement of calcium from the exterior of the cell to the cytosol is passive, down an electrochemical gradient, and appears to be regulated through channel or carrier proteins. The apical membrane contains a hormone-regulated carrier mechanism for Ca^{2+} entry. Movement from the cytosol to the exterior requires energy-consuming extrusion mechanisms, involving Ca^{2+}-ATPase and/or Na^{+}/Ca^{2+} exchange. The roles of such mechanisms in calcium transport phenomena in fish gills and intestine will be addressed.

Calcium transport in intact fish

The extant bony fishes represent the largest class of vertebrates with an estimated 20000 species, and fish are considered to be an evolutionary success. They have developed sophisticated osmo- and ionoregulatory mechanisms, allowing them to inhabit and to thrive in virtually every aquatic niche, be it an ice-cold sea or a tropical soda lake. Recently, it has become clear that fish have also developed mechanisms for calcium transport to realize a calcium homeostasis that appears to be comparable with that of higher vertebrates. The calcium transport mechanisms underlying the homeostasis have made fish essentially independent of their aquatic environment, which may be strongly hypocalcic, with micromolar levels of calcium in soft water, or hypercalcic, with some $10 mmol\,l^{-1}$ calcium in sea water. An essential difference between terrestrial vertebrates and aquatic fish is that the latter have a specialized organ for calcium uptake, the gills. This situation provides a continuous constraint on calcium regulatory mechanisms, the

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ion composition of the water being directly or indirectly (*via* hormonal regulation) the determinant factor regulating these mechanisms.

Unlike terrestrial vertebrates, fish show significant variations in plasma calcium levels, not only between species but also within species, e.g. as occurs when a fish is confronted with water containing different levels of calcium (Urasa and Wendelaar Bonga, 1987). Fish are able to survive the extreme hypercalcaemia (up to $10\,mmol\,l^{-1}$ total calcium, $4.5\,mmol\,l^{-1}$ Ca^{2+}; Hanssen *et al.* 1989) that develops when their source of hypocalcaemic hormone, the corpuscles of Stannius, has been removed. Such observations indicate that fish can tolerate fluctuations in plasma calcium concentration to an extent not encountered in terrestrial vertebrates. Fish cells must have very strict control over Ca^{2+} entry.

Bones and scales, crucial elements in vertebrate life, derive their sturdiness from deposited calcium phosphate minerals. Most fish grow continuously and, to do this, accumulate calcium in their body throughout their life. This is realized through a continuous uptake of Ca^{2+} *via* their gills (Herrmann-Erlee and Flik, 1989; Flik *et al.* 1993*a*,*b*).

The gills are the most important site of contact between fish and water, covering a surface area 10–60 times larger than the area of skin covering the body surface (Parry, 1966). The large surface area of the gills reflects their role in the gas- and ion-exchange processes that are determined by the environment and the lifestyle of the fish. Such a large area of integument in contact with the water requires strict control over calcium movements across this epithelium.

Fish have two sites for the uptake of calcium: their gills (containing an abundance of ion-transporting cells or ‘chloride cells’, also known as ionocytes) and their intestine. The branchial epithelium may be considered to be a specialized skin area. In the euryhaline teleosts *Oreochromis mossambicus* (McCormick *et al.* 1992) and *Oncorhynchus mykiss* (Marshall *et al.* 1992), parts of the skin covering the inner operculum have also been shown to transport Ca^{2+}. Using an Ussing chamber, it has been demonstrated that this transport is probably mediated by the ionocytes found in this part of the skin under these conditions. The skin has been shown to contain numerous ionocytes at typical densities of around $200\,mm^{-2}$. Extrapolation of the positive correlation between skin ionocyte numbers and inward calcium transport rates suggests that skin devoid of ionocytes is essentially impermeable to Ca^{2+} (Marshall *et al.* 1992). The contribution of extrabranchial calcium transport *via* the skin is considered to be small, and this is reflected in the absolute numbers of ionocytes in gills and skin (Marshall *et al.* 1992; Perry *et al.* 1993). Care should be taken with the extrapolation of data on calcium transport *via* extrabranchial sites, as Marshall *et al.* (1992) have shown that calcium transport *via* the skin is not affected by La^{3+}, a powerful blocker of branchial calcium transport. It has also been shown that trout stanniocalcin had no effect on calcium transport in *Fundulus heteroclitus* opercular membranes (W. S. Marshall, personal communication), although this hormone has been shown to inhibit the movement of calcium over the apical membrane of branchial ionocytes of a variety of species, including trout (Lafeber *et al.* 1988), eel and tilapia (Verbost *et al.* 1993*a*,*c*). It may be that a homologous hormone is required to show an effect in *Fundulus heteroclitus.* These

observations suggest that differences exist in the way apical membrane calcium transport is controlled in ionocytes in gills and in skin.

Ca^{2+} can be taken up directly from the water *via* the gills, but also through the intestine by drinking water. Food may be another source of Ca^{2+} through intestinal absorption. The role of the intestine in the calcium metabolism of fish is not very clear and has only been sparsely studied. The primary function of the intestine must be the uptake of nutrients, which may depend on the uptake of ions (e.g. Na^+-coupled sugar uptake). The intestine may become active in the uptake of calcium from food (a freshwater fish confronted with osmotic water influx should not drink) in times of extra need for calcium, such as during gonadal maturation or when ambient calcium levels are very low (Berg, 1968; Ichii and Mugiya, 1983). This has been shown for the Atlantic cod, *Gadus morhua*, in which intestinal calcium absorption increases drastically in prespawning fish (Sundell and Björnsson, 1988), which require vast amounts of calcium for the development of the gonads. Very little is known about the relative contribution of the gills and the intestine to calcium uptake in seawater fish, with the exception of data from the stenohaline Atlantic cod. Sundell and Björnsson (1988) and Björnsson and Nilsson (1985) estimated that intestinal absorption of calcium in seawater fish made up around 30% of the total calcium intake.

For two species (eel and tilapia) studied in our laboratory, it has been shown that net branchial Ca^{2+} influx in sea water was not lower, but comparable or slightly higher, than in fresh water (G. Flik and P. M. Verbost, unpublished observations). Seawater fish do drink and sea water is high in calcium (approximately $10 mmol\,l^{-1}$). The water is drunk to compensate for osmotic water efflux but at the same time presents a significant calcium load. Considering the branchial influx that suffices for growth and homeostasis, one would predict that intestinal uptake is not required. All studies on intestinal calcium transport in fish indicate that in the proximal parts of the intestinal tract active transport underlies net uptake of calcium, in freshwater as well as in seawater species (Flik *et al.* 1990*b*; Schoenmakers *et al.* 1993).

Measurements have been made of branchial and intestinal calcium uptake for the stenohaline Atlantic cod, *Gadus morhua*. The estimated contribution of the intestine to total calcium uptake comes to 30% (Sundell and Björnsson, 1988). In a recent study on tilapia (Schoenmakers *et al.* 1993), evidence was provided that this species reduces calcium absorption to a minimum when in sea water, indicating that species differences in intestinal calcium handling may exist.

Any surplus calcium taken up by fish is excreted, largely *via* extrabranchial routes, i.e. the renal system or the intestinal tract (Hickman, 1968; Hickman and Trump, 1969; Björnsson and Nilsson, 1985). It has been shown that freshwater eels produce hypotonic urine containing 1.5–$2 mmol\,l^{-1}$ calcium at a considerable rate ($40\,\mu mol\,day^{-1}\,kg^{-1}$ fish), so the renal route is a significant pathway for calcium secretion (Hickman and Trump, 1969; Björnsson and Nilsson, 1985) while the kidneys of seawater fish are renowned for their secretion of divalent ions (Ca^{2+}, Mg^{2+}, SO_4^{2-}) and are considered to be essential to keep calcium flows balanced. The contribution of the complete intestinal tract to calcium excretion has been analyzed only in cod (Sundell and Björnsson, 1988). These studies indicate that the intestinal excretion ($1.22\,\mu mol\,kg^{-1}\,h^{-1}$) represents 20 %

of the total calcium excretion (renal and extrarenal, 4.2 and 2.0 μmol kg^{-1} h^{-1}, respectively) and 50% of the extrarenal excretion. The very few physiological studies that have been carried out further indicate that the urinary bladder may modify the calcium content of the urine significantly, and this means that the bladder epithelium should also be regarded as a calcium transport site.

The endocrine control of plasma calcium levels in fish is very different from that in higher vertebrates. Prolactin (Flik *et al.* 1986, 1989*a*; G. Flik, F. Rentier-Delrue and S. E. Wendelaar Bonga, in preparation) and somatolactin (Kaneko and Hirano, 1993) from the pituitary gland and the steroid cortisol (Flik and Perry, 1989) produced by the interrenal cells are calciotropic hormones with hypercalcaemic effects in fish. Stanniocalcin, a unique fish hormone produced by the corpuscles of Stannius, is the predominant hypocalcaemic hormone in fish (Wendelaar Bonga and Pang, 1986). Calcitonin, a hormone with hypocalcaemic effects in higher vertebrates, first isolated from salmon ultimobranchial bodies, has no clear calcitropic effects in fish and its role in fish physiology remains enigmatic. The involvement of hormones in calcium regulation in fish has been reviewed extensively (Wendelaar Bonga and Pang, 1986, 1989).

Calcium-transporting cells

Ideally, the study of calcium transporters at the molecular level in a fish calcium-transporting epithelium should start with the specification of the cell type where the calcium transport takes place. Cellular physiological considerations indicate that each cell will contain calcium extrusion mechanisms to keep resting cytosolic Ca^{2+} concentrations low (around 100 nmol l^{-1}). Since every cell is in contact with extracellular fluid (typical Ca^{2+} levels around 1.5 mmol l^{-1} in bodily fluids, 1–2 mmol l^{-1} in fresh water, 10 mmol l^{-1} in sea water and 10–30 mmol l^{-1} in intestinal luminal fluids), every cell will also be to some extent permeable to Ca^{2+}. Cells specialized for Ca^{2+} transport should contain more Ca^{2+} extrusion mechanisms and function under some endocrine control. On this basis, we present a review of calcium transporters in fish gills and intestine.

Correlations between numbers of cells and transport rates provide substantial, albeit still circumstantial, evidence for inward calcium transport mediated by the ionocytes in the skin. This is supported by electrophysiological studies. By extrapolation, it is generally assumed that calcium transport is also mediated by these cells in the gills. Indeed, as will be discussed below, there is an abundance of biochemical, morphological and physiological evidence for this hypothesis.

The intestinal epithelium of the few species (tilapia, eel, cod, trout) studied with respect to calcium transport phenomena may be considered as a homogeneous source of enterocytes with 10% or fewer mucocytes and endocrine cells. In some species (e.g. the tilapia and goldfish), it has been shown that the mucosa can be relatively easily stripped of its underlying layers of submucosa and muscles. Such epithelia yield good preparations for electrophysiological analysis, using Ussing chambers, and have allowed the assessment of transcellular Ca^{2+} transport as a basis for calcium absorption studies (Flik *et al.* 1990*b*; Schoenmakers *et al.* 1993).

Cell-mediated calcium transport involves the passage of Ca^{2+} over apical and basolateral plasma membranes. Transport of Ca^{2+} into the cell is passive, requiring no input of energy, given the prevailing electrochemical conditions (millimolar concentrations of calcium outside the cell and submicromolar concentrations inside and a transmembrane potential of 50–70mV, inside negative). Transport of Ca^{2+} out of the cytosol requires the operation of energy-consuming mechanisms to overcome the electrochemical gradient for Ca^{2+}.

Movement of calcium across apical membranes

Gills

The mechanisms of calcium transport across ionocyte apical membranes are only partly understood. The presence of non-voltage-gated Ca^{2+} channels in ionocytes is indicated by the finding that blockers of L-type channels (Perry and Flik, 1988) have no effect on branchial Ca^{2+} influx, whereas Co^{2+} or La^{3+} inhibits the transcellular influx of Ca^{2+} without entering the ionocyte (Perry and Flik, 1988; Verbost *et al.* 1989). Also, in isolated gill cells loaded with the fluorescent Ca^{2+} probe Fluo-3, depolarization with 60mmol l^{-1} KCl had no effect on the intracellular Ca^{2+} level (P. M. Verbost, personal observation), indicating the absence of voltage-gated Ca^{2+} channels.

The major hypocalcaemic hormone in fish, stanniocalcin, is a likely candidate for the rapid control of transcellular Ca^{2+} influx (Lafeber *et al.* 1988). In eels, stanniectomy, the removal of the corpuscles of Stannius where the hormone is produced, results in a loss of inhibitory control over Ca^{2+} influx from the water and shifts the animal's net branchial calcium uptake to a positive value, provoking a severe hypercalcaemia. Conversely, injection of stanniocalcin rapidly decreases Ca^{2+} uptake in stanniectomized eels (Verbost *et al.* 1993*c*). The resting potential for Ca^{2+} (calculated according to the Nernst equation and applying the water and plasma Ca^{2+} activities) is always more negative than the transepithelial potential (TEP) measured in fresh water (which varies from around 0 to −16mV at neutral pH; Maetz and Bornancin, 1975; McWilliams and Potts, 1978; Perry and Wood, 1985; Perry and Flik, 1988). Thus, in the gill epithelium, the electrochemical gradient for Ca^{2+} (driving the passive flux) is directed outwards. We assume that the enhanced Ca^{2+} influx seen in eels deprived of their corpuscles of Stannius also occurs transcellularly. This seems a reasonable assumption because the rise in plasma calcium concentration after this operation will increase the chemical and electrical gradients further, preventing passive paracellular Ca^{2+} influx. Moreover, the TEP will become less negative after stanniectomy instead of more negative. Stanniocalcin thus inhibits branchial Ca^{2+} influx from water to blood. In a trout isolated head preparation, stanniocalcin has been shown to inhibit Ca^{2+} influx within 15min (Lafeber *et al.* 1988). There are several lines of evidence indicating that stanniocalcin is a fast-acting hormone reducing Ca^{2+} entry through fish gills by controlling Ca^{2+} channels or carriers in the apical membrane. Injection of stanniocalcin inhibits the entry of Ca^{2+} into the gill cells *via* a La^{3+}-sensitive pathway (Verbost *et al.* 1989). The operation of the Ca^{2+} pumps in the basolateral plasma membrane appears to be independent of stanniocalcin, *in vitro* as well as *in vivo* (Verbost *et al.* 1993*c*), and this excludes the possibility that the hormone

inhibits calcium transport by targeting calcium transporters in the basolateral plasma membrane. Stanniocalcin reduced the branchial epithelial cyclic AMP content in trout and tilapia (Flik, 1990; Verbost *et al.* 1993*c*; G. Flik and P. M. Verbost, personal observation), suggesting that the hormone acts through a second-messenger pathway that conveys the endocrine signal from the basolateral membrane to a second-messenger-dependent Ca^{2+} channel or carrier in the apical membrane.

Direct biochemical analysis of Ca^{2+} transport across the apical membrane of ionocytes is hampered by the fact that it has not yet been possible to isolate and characterize an apical membrane fraction of branchial epithelium for biochemical assays. The apical membrane of the ionocytes – and there is general consensus that only these contribute to Ca^{2+} transport in gills (Fenwick, 1989) – forms only a minor part of the total membrane surface facing the water (in the gills of freshwater tilapia the mature chloride cells in contact with the water make up less than 5% of the epithelial cells; Wendelaar Bonga and van der Meij, 1989; Wendelaar Bonga *et al.* 1990). In a comparative study on bullhead (*Ictalurus nebulosus)*, eel (*Anguilla rostrata)* and trout (*Oncorhynchus mykiss)* kept in soft ($0.45 mmol\,l^{-1}$ Ca^{2+}) fresh water, Perry *et al.* (1993) calculated that the fractional areas of the branchial epithelium occupied by the ionocyte apical membranes amounted 1.27, 2.14 and 14.99%, respectively; these values correlate strongly with the branchial calcium influx measured in these fish: 2.3, 4.8 and 16.7 $\mu mol\,h^{-1}\,kg^{-1}$ fish, respectively. It is clear, given the cell physiological conditions (millimolar concentrations of Ca^{2+} outside and submicromolar concentrations inside) that control over the transcellular uptake of Ca^{2+} has to be at the apical membrane.

In sea water, with 8–$10 mmol\,l^{-1}$ Ca^{2+} outside, the need to limit the entry of Ca^{2+} at the apical membrane is even more obvious. The need to reduce the uptake of Ca^{2+} appears to be reflected by the higher levels of stanniocalcin found in seawater species (Flik *et al.* 1989*b*, 1990*a*; Mayer-Gostan *et al.* 1991). In the eel, *Anguilla anguilla*, it has been shown that the metabolic clearance rate and the secretion rate of stanniocalcin were higher in seawater-adapted fish (Hanssen *et al.* 1993). Moreover, stanniectomized eel developed a stronger hypercalcaemia in sea water than in fresh water and the hypercalcaemia was directly correlated with the water calcium content (Flik, 1990).

Intestine

The uptake of Ca^{2+} *via* the intestine is under the control of stanniocalcin. In eel, *Anguilla japonica* (Hirano, 1989), and trout, *Oncorhynchus mykiss* (Takagi *et al.* 1985), the intestinal absorption of Ca^{2+} increases after removal of the corpuscles of Stannius. In the cod, *Gadus morhua*, intestinal absorption of Ca^{2+} is inhibited by stanniocalcin (Sundell *et al.* 1992). The fish intestine provides a useful model since the enterocytes are uniform in size and the apical membrane can be easily isolated by Ca^{2+} or Mg^{2+} precipitation procedures. Thus, the Ca^{2+}-transporting mechanism in the apical membrane can be analyzed directly. In an *in vitro* study on apical membranes from tilapia intestine (Klaren *et al.* 1993), it was concluded that the apical membrane exhibits a low-affinity Ca^{2+}-transporting mechanism with an allosteric regulatory binding site. Whether this mechanism represents a channel or a carrier is still under debate, but recent studies indicate that a carrier protein is the most likely explanation for the results obtained. When

the calcium concentration range used to test the substrate-dependency of the transport was extended above 10mmol l^{-1} to 50mmol l^{-1}, saturation of Ca^{2+} transport was observed. This proves that a carrier or channel was involved, not a simple diffusion process. In equilibrium exchange studies, it was subsequently demonstrated that *trans*-stimulation of calcium transport occurs (tracer uptake into brush-border vesicles was stimulated even when the calcium gradient over the membrane was directed outwards) and this phenomenon is restricted to carrier proteins (P. H. M. Klaren, personal communication). Studies in eel on the effects of stanniectomy and stanniocalcin replacement on brush-border membrane vesicle Ca^{2+} transport are under way to assess the control by stanniocalcin of an apical membrane carrier protein for calcium.

The mechanism for regulating Ca^{2+} entry across brush-border membranes is still unidentified (Bronner, 1991). The fish intestine may provide a suitable model for the identification of the enigmatic calcium transport mechanism of brush-border membrane, since the hormone stanniocalcin can be used as a tool to control the entry of Ca^{2+} at the apical membrane.

Energized calcium transport

Gills

A complete review of the literature on the definition and the involvement of Ca^{2+}-ATPases in branchial calcium handling was given by Fenwick (1989). After a short summary, we will focus this review on more recent discoveries.

Two categories of ATPases have been demonstrated. The first of these involves a membrane-bound non-specific phosphatase activity with Ca^{2+}-ATP as the preferred substrate. A conformational change in the ATP molecule brought about by the chelation of Ca^{2+} appears to make the substrate accessible for enzymatic breakdown. Confirmatory evidence for this thesis comes from the observation that Mg^{2+} may replace Ca^{2+} with comparable results (Flik *et al.* 1983). In addition to these observations, there is ample evidence to suggest that this activity is not that of a Ca^{2+}-transporting enzyme. First, the hydrolytic activity of the non-specific phosphatase *in vitro* is much higher than the Ca^{2+} transport rates observed in the gills (Flik *et al.* 1985*a*,*b*) and the ATPase activity of the plasma-membrane-bound Ca^{2+} pumps (Flik *et al.* 1984*a*,*b*). Were this phosphatase to be the enzymatic correlate of the plasma membrane calcium pump, this would mean either that ATP is wasted (a stoichiometry of ATP:Ca^{2+} much larger than 1; for most transporters a stoichiometry of 1 is reported) or that high levels of calcium excretion must occur in the fish to compensate for the inward transport. The first possibility is unlikely, the second untrue. Second, the pH optimum of the non-specific phosphatase lies between 8 and 9, a value not compatible with its operation at an intracellular milieu of approximately pH7.2 (Carafoli, 1987). Third, the high levels of Ca^{2+} required to activate the enzyme will never occur in the cell; extrusion mechanisms for Ca^{2+} all have affinities in the submicromolar range (typical values around 100nmol l^{-1}, the resting level of Ca^{2+} in the cell). It is concluded, therefore, that this Ca^{2+}-ATP-consuming phosphatase is not the Ca^{2+} transporter. However, intriguing observations originally made by Fenwick (1976) and by Ma *et al.* (1974) and recently confirmed in more detail by Verbost *et al.*

(1993*b*) suggest that this enzyme activity may be influenced by products of the corpuscles of Stannius. It was shown that the activity of this enzyme in gills increased in stanniectomized eel (Fenwick, 1976) and that this was accompanied by a loss of control over the branchial apical permeability to Ca^{2+}. Moreover, extracts of the corpuscle of Stannius inhibit this enzyme activity (Ma and Copp, 1982; Verbost *et al.* 1993*b*). Verbost and coworkers, have recently shown that the material in the extract inhibiting this enzyme is not stanniocalcin, but a 3kDa glycoprotein that has no inhibitory effect on branchial calcium influx. The physiological importance of this non-specific phosphatase activity remains to be clarified and its function in calcium transport is unclear.

The second category of ATPase present in fish are the Ca^{2+}-pumping ATPases found in the plasma membranes of all vertebrates. In a few species of fish, a calmodulin-dependent, Ca^{2+}- and Mg^{2+}-dependent ATPase with a high affinity for Ca^{2+} (K_m around $100 nmol\,l^{-1}$) and with the capacity to drive Ca^{2+} transport against a gradient has been characterized (see Fenwick, 1989).

The involvement of this Ca^{2+}-ATPase in branchial Ca^{2+} transport is plausible on the basis of its kinetic properties, from a calculation of its capacity (Flik *et al.* 1985*b*) and from its dependence on calcitropic hormones (prolactin, cortisol; Flik *et al.* 1989*a*; Flik and Perry, 1989). In eel, the high-affinity Ca^{2+}-ATPase activity is enhanced (not by effects on its affinity for Ca^{2+}, but by an increased maximum velocity) after transfer of the fish to low-calcium water, in which branchial calcium uptake is stimulated (Mayer-Gostan and Naon, 1992). It appears that more Ca^{2+}-ATPase is active under such conditions in the gills of eel. Calculations of the Ca^{2+}-transporting capacity of the gills based on the maximum velocity of the Ca^{2+}-ATPase indicate that a large (200-fold) overcapacity of this transporter is present in the basolateral plasma membranes compared with the actual transcellular Ca^{2+} flow (Verbost *et al.* 1993*d*). An important conclusion from such calculations is that the minute-to-minute control of calcium movement through the (branchial) ionocytes must occur at the entry step, since calcium homeostasis of the cell seems to be guaranteed by this overcapacity of calcium pumps. When speculating on the advantages such an overcapacity may have for the fish, it should be remembered that the capacity calculations were based on *in vitro* data, and it may well be that *in vivo* only some of the calcium pumps are active. Furthermore, morphological analysis of ionocyte turnover in the gills of tilapia has indicated that only a small percentage of these cells is mature, i.e. in contact with the water (Wendelaar Bonga *et al.* 1990). Cells that are not mature, and not functioning in ion transport, may actually provide the bulk of the enzymes measured in biochemical calcium transport assays.

Prolactin and cortisol are hormones that exert hypercalcaemic actions in freshwater fish and control the number of Ca^{2+} pumps in the branchial epithelium. By doing so, these hormones determine the calcium-transporting capacity of the gills. The calcitropic actions of prolactin and cortisol become noticeable only in the long term, i.e. after approximately 4 days. To the best of our knowledge, no reports have appeared on the direct short-term endocrine control of Ca^{2+}-ATPase or sodium/calcium exchange activity in fish.

Recently, substantial evidence has been presented for another energy-consuming calcium transporter in plasma membranes of branchial epithelium in the form of a

Na^+/Ca^{2+} exchanger (Flik *et al.* 1993*b*; Verbost *et al.* 1993*d*). The role of this Na^+/Ca^{2+} exchanger in branchial calcium transport is, however, not well understood. The exchanger in tilapia gills is half-maximally activated by Ca^{2+} at around $2\,\mu mol\,l^{-1}$ and has a maximum velocity of around $12.5 nmol min^{-1} mg^{-1}$, values roughly 10-fold and threefold higher, respectively, than those of the ATP-driven Ca^{2+} transporter (Flik *et al.* 1993*b*). The low affinity for calcium makes it difficult to assess the contribution of this exchanger to calcium extrusion in the branchial ionocyte and its function in cell calcium homeostasis. To try to understand the function of this transporter in the ionocyte, it was argued that where calcium extrusion is partly mediated by a Na^+-dependent mechanism, such a mechanism might be of greater importance in sea water, where the branchial Na^+ turnover is much higher. However, no differences in enzyme make-up of the basolateral plasma membranes with regard to Na^+/K^+-ATPase, Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange were found when freshwater or seawater tilapia gills were compared (Verbost *et al.* 1993*d*). A speculative explanation for the results would be either that the exchanger provides an emergency mechanism for Ca^{2+} extrusion, when Ca^{2+} concentrations in the cytosol rise to micromolar levels, or that the exchanger is involved in extrusion of very locally elevated levels of Ca^{2+} (e.g. in the direct vicinity of calcium channels in the plasma membrane). Data on the sodium-dependence of calcium transport in fish gills could give a clue to any involvement of the exchanger in calcium transport. Such studies would require an analysis of Ca^{2+} influx in an isolated head preparation, which allows the manipulation of mucosal and serosal media bathing the branchial epithelium, and an analysis of the sodium-dependence of the exchanger in freshwater and seawater fish. Another preparation that may be used in the future to test this hypothesis is the opercular membrane.

Intestine

Studies on enterocyte basolateral plasma membrane calcium transporters are limited to one species, the tilapia (Flik *et al.* 1990*b*; Schoenmakers *et al.* 1992, 1993; Schoenmakers and Flik, 1992). However, a wealth of interesting information has recently become available on this species and this will be summarized here.

The transport of calcium in stripped epithelium of tilapia is mostly transcellular and depends on the sodium status of the epithelium. When the sodium gradient across the basolateral plasma membrane is removed, by replacing sodium with *N*-methyl-D-glucamine or by treating of the tissue with the Na^+/K^+-ATPase inhibitor ouabain, no cellular transport of calcium occurs. Analysis of the basolateral membrane of the enterocyte has shown that Ca^{2+}-ATPase activity is very low and that Na^+/Ca^{2+} exchange activity is very high, which may explain at the molecular level the sodium-dependence of the Ca^{2+} flux in this epithelium. In studies with membrane vesicles, the simultaneous operation of the Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger indicates that the extrusion activity of the exchanger exceeds that of the ATPase (Flik *et al.* 1990*b*).

Several studies have demonstrated the operation of the exchanger in the calcium homeostasis of this calcium-transporting cell. Isolated enterocytes loaded with Fura-2 to monitor cytosolic Ca^{2+} concentration and incubated with ouabain to dissipate the Na^+ gradient over the plasma membrane cannot maintain a constant cytosolic Ca^{2+}

concentration when the driving force for Ca^{2+} extrusion *via* the exchanger is absent (Schoenmakers *et al.* 1992).

It has been calculated that the transport of cadmium ions, which follow the calcium transport pathways, occurs at too high a rate for passive diffusion. Ample evidence has been given that Cd^{2+} may enter cells *via* the stanniocalcin-sensitive Ca^{2+} pathway and subsequently block the Ca^{2+}-ATPase in the plasma membranes. The Na^{+}/Ca^{2+} exchanger is less sensitive to Cd^{2+} inhibition and is probably the carrier for cadmium across the basolateral plasma membrane (Schoenmakers *et al.* 1992). It has been demonstrated that the exchanger in its 'Ca^{2+}/Ca^{2+} mode' may exchange Cd^{2+} for Ca^{2+}. These data clearly indicate that the exchanger operates in the enterocyte. By analogy, the cadmium flow may also be explained by movement of cadmium over the basolateral plasma membrane along the Na^{+}/Ca^{2+} exchanger in the gills.

When tilapia are adapted to sea water, they benefit from a reduction of calcium absorption *via* the intestine, because they start drinking sea water containing $10 mmol l^{-1}$ calcium and do not decrease their branchial calcium uptake (which already suffices for growth and homeostasis). It has been shown that calcium absorption *via* the intestine was greatly reduced and that this reduction was correlated with a decrease in the activity of the calcium transporters in the enterocyte plasma membrane. The affinity and the maximum velocity of the ATP-driven calcium pump decreased by almost 30% compared with those of freshwater fish; the affinity of the exchanger also decreased by more than 20% and its maximum velocity by nearly 60% compared with those of freshwater fish. These observations indicate that adaptation to sea water modifies the exchanger and ATPase densities in the enterocyte plasma membrane, in line with a decreased need for calcium transport (Schoenmakers *et al.* 1993).

Conclusions

We hope to have shown that fish gills, opercular skin and intestine provide excellent models for the study of calcium transport phenomena. Serosal and mucosal media can be manipulated *in vivo* as well as *in vitro* in these organs. Defined, hormone-dependent calcium-transport mechanisms appear to be present for active as well as passive transport of Ca^{2+}. The fish intestinal brush-border membrane is unique in its stanniocalcin-dependence and provides the most complete model for Ca^{2+} entry known to us. The prolactin-dependence of the Ca^{2+}-ATPase in the basolateral plasma membranes of the branchial ionocyte and the involvement of the Na^{+}/Ca^{2+} exchanger in calcium extrusion in the enterocyte make fish calcium-transport mechanisms attractive objects to study.

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