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Calcium Regulation in Fish

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Calcium is important for growth, reproduction, and many other physiological processes such as muscle contraction, nerve signal transduction, and control of membrane permeability and cellular metabolism. These processes are dependent on an effective control of the ionic calcium levels of the extracellular fluid (ECF). Although fishes seem to be slightly more resistant to changes of ionic calcium of the ECF than the terrestrial vertebrates, deviations from the normal physiological concentration ("setpoint") have essentially the same effects in all vertebrates: hypocalcemia leads to increased excitability of cellular membranes and may result in tetanic seizures, whereas hypercalcemia reduces membrane excitability with lethargy and cardiovascular dysfunction as consequences. Precise control of the setpoint of ionic calcium is the rule in fish as well as in other vertebrates.

The maintenance of constant calcium levels in the ECF is effected in fish by the continuous exchange of calcium between the animals and the environment, mainly via the gills. In full-grown animals influx and efflux of calcium are in equilibrium, whereas during periods of growth and reproduction net calcium accumulation occurs. During such periods large amounts of calcium are transported from the sites of entry through the blood and other compartments of the ECF to bones or ovaria. This represents a special challenge for the mechanisms that control the calcium concentration of the ECF.

There are several important differences between fish and the terrestrial vertebrates with respect to the organs involved in the maintenance of the calcium balance of the body, and in the endocrine mechanisms controlling these organs. Firstly, whereas

in terrestrial vertebrates calcium is obtained exclusively from the food via the gut, in fish the gills are in general the main route of uptake of calcium with the gut in a secondary role. The ambient water seems sufficient as a calcium source in fish. Our unpublished studies on the African cichlid *Oreochromis mossambicus* have shown that calcium-deficient food does not have any effect on the calcium balance of tilapia, which confirmed similar observations on goldfish by Ichii and Mugiya (1983). The uptake of calcium in fish then is an essentially continuous process, in contrast to the calcium uptake in terrestrial vertebrates, which is episodic and restricted by the calcium content of the food. The second important difference between fish and terrestrial vertebrates is the role played by the skeleton. In the absence of a continuous access to external calcium sources, terrestrial animals have developed the bone tissue as a calcium store which is used for the minute-to-minute control of the calcium level of the body fluids. We will discuss below that a calcium exchange capacity of the skeleton in fish is not required because the control of calcium homeostasis in fish is mainly effected at the level of the gills, in particular by regulation of the calcium influx from the water. Thirdly, and not surprisingly, given the above-mentioned characteristics of calcium metabolism in fish, there are essential differences in the hormonal mechanisms regulating the calcium balance between fish and the terrestrial vertebrates. In this paper we will concentrate on these regulatory aspects.

A. Terrestrial Vertebrates

In the terrestrial vertebrates two hormones are specifically involved in the control of calcium metabolism: parathyroid hormone (PTH) and calcitonin. PTH is the dominant hormonal factor. It stimulates the intestinal uptake and reduces the renal excretion of calcium, processes mediated by a vitamin-D3-metabolite 1,25-dihydroxycholecalciferol. PTH further mobilizes calcium from the skeleton. When the ionic calcium concentration of the ECF falls below the physiological setpoint (about 1.24 mM Ca^{2+}) the

PTH-cells respond within seconds with the enhanced secretion of PTH (Brown *et al.* 1987). The hormone stimulates the osteoclasts, and this leads to a rapid rise of the extracellular Ca^{2+} level. Calcium concentrations above the setpoint inhibit PTH secretion. The rapid response of the PTH cells is mediated by the special Ca^{2+}-sensing capacity of these cells (Jones and Fitzpatrick 1990).

Calcitonin has long been considered the hormonal antagonist of PTH, because of its reportedly hypocalcemic action. Although hypocalcemic effects have been shown after injection of the hormone in young and growing mammals, birds and reptiles, consistent hypocalcemia could not be demonstrated in adult animals (Copp and Kline 1989). Neither chronically elevated calcitonin levels, nor removal of the calcitonin-producing C-cells by thyroidectomy (in mammals) or ultimobranchialectomy (in birds and amphibians) are inducing persistent imbalance of the extracellular Ca^{2+} levels (see Wendelaar Bonga and Pang 1991). This sharply contrasts with the effects of parathyroidectomy, which include hypocalcemia, tetany and death. One role of calcitonin is the reduction of hypercalcemia that occurs after intestinal absorption of calcium following a meal (VanderWiel and Talmage 1981). It promotes the deposition of ingested calcium into the bone. It further protects the skeleton against excessive demineralization during periods of increased calcium demand, such as pregnancy and lactation in mammals (Stevenson *et al.* 1979). In conclusion, in the terrestrial vertebrates the exchange of calcium with the environment is mainly regulated by PTH, that also controls the Ca^{2+} concentration of the ECF. The bone plays an essential role as a calcium buffer. Compared to PTH, calcitonin only has a minor function in the homeostatic control of extracellular Ca^{2+}.

B. Fish

Our knowledge of the calcium metabolism and the endocrine control in fish is mainly based on freshwater teleosts and therefore we will mainly deal with this group. Teleost fish maintain plasma total and ionic calcium within narrow limits, but these limits are to some extent dependent on the growth rate and on the cal-

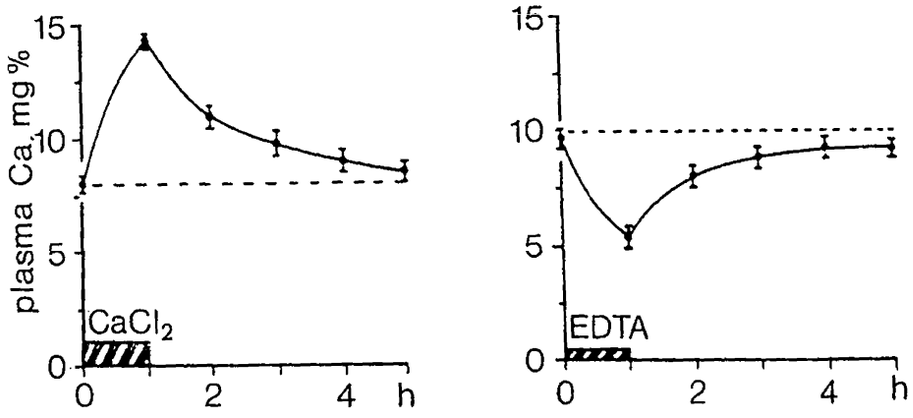

Fig. 1. Recovery from induced hyper- or hypocalcemia in rainbow trout (Copp *et al.* 1985).

cium concentration of the ambient water (Urasa and Wendelaar Bonga 1987). When plasma calcium levels of teleost fish are changed experimentally, normal levels are rapidly restored. In rainbow trout (Copp *et al.* 1985) and American eels (Fenwick and Brasseur 1991) it took 3 to 6 h before a twofold increase of plasma total calcium had decreased to control values (Fig. 1). Restoration of a 50% drop in plasma calcium induced by EGTA infusion in rainbow trout lasted a similar period (Copp *et al.* 1985; Fig. 1). These data show that teleost fish can successfully restore changes in the calcium concentration of the extracellular fluid. The response may be similar to or even more rapid than in the higher vertebrates. The changes in calcium levels in the terrestrial animals are compensated by modulating the exchange of calcium between the bone and the ECF. How is this compensation effected in fish?

B. 1. Bone

In amphibians and fishes, the calcium-exchange capacity of the bones has been frequently considered as limited. This does certainly not imply that calcium mobilization from bone does not occur. There are several reports for teleost fish with cellular as well as acellular bone indicating that the calcium concentration of bone varies in response to environmental or physiological changes (Fleming *et al.* 1973). In tilapia, a species with

acellular bone, reduction of the water calcium concentration or of the water pH - both stimulate calcium efflux across the gills - causes a transient decrease of the calcium concentration of the bone (Wendelaar Bonga and Dederen 1985). Induction of scale regeneration by removal of mature scales is accompanied by a transient reduction of the calcium concentration of the remaining scales (Weiss and Watabe 1978). Forced gonadal maturation by injection of estrogens may lead to calcium mobilization from the bone and/or the scales (Mugiya 1982). Thus, calcium can be mobilized from skeleton and scales in fish. The contribution of calcium mobilization from the bone to the calcium homeostasis of the ECF is unclear, however. Except for estrogens no hormone is known that stimulates bone calcium release in fish. The function of bone as a readily accessible and hormonally controlled calcium store seems to have been evolved after the water-to-land transition of the vertebrates, in conjunction with the appearance of PTH and a system of bone cells with calcium-releasing activity that rapidly responds to this hormone (Pang *et al.* 1980; Wendelaar Bonga and Pang 1991).

B.2. Gills

If the bone does not function as a fast-acting calcium buffer, the most likely mechanism for the minute-to-minute control of the extracellular calcium concentration is the regulation of the calcium-exchange between the fish and its environment. The exchange of calcium is much higher in fish than in terrestrial vertebrates. This is mainly caused by the calcium fluxes across the branchial epithelium. Although this epithelium is very tight, in particular in fresh water, there is still a substantial passive efflux of calcium ions. This loss of calcium ions is compensated by the active uptake of calcium ions through the gills and, to a lesser extent, the intestine. Whereas the branchial calcium efflux most likely follows a paracellular route, the calcium influx is an active, transcellular process. We have investigated this process in detail, and these studies have resulted in a

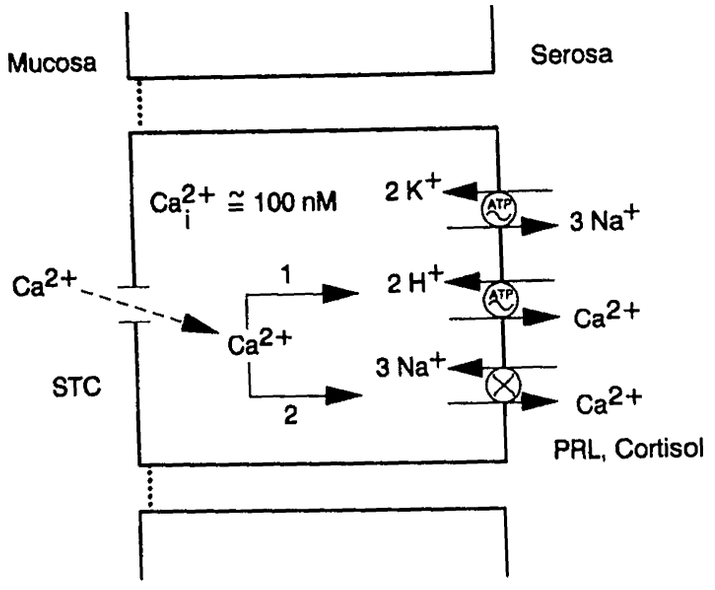

Fig. 2. Transcellular transport of Ca^{2+} in fish gills. Ca^{2+} from the water may enter the cell passively, down an electrochemical gradient. Stanniocalcin (STC) regulates calcium channels in the apical membrane; STC signal transduction requires second (cAMP, DAG, IP_3) and third (Ca^{2+}) messengers. Extrusion of Ca^{2+} from the cell could occur via a Ca^{2+}-ATPase (route 1) or via a Na^+/Ca^{2+} exchanger (route 2). Both extrusion mechanisms are present in branchial epithelium. In gill cells route 1 probably dominates. Hormones with hypercalcemic effects such as prolactin (PRL) in several species and cortisol (in trout) determine the relative abundance of the ATPases and by doing so may determine the calcium transporting capacity of the epithelium (Flik *et al.* 1992).

model that has been verified for a number of teleost species, including trout, tilapia and two eel species (Fig. 2). The calcium-influx is effected by the chloride cells, the cells specialized for ion transport in fish gills. Calcium ions from the water are entering the cytoplasm of the chloride cells via the apical membrane of these cells, probably through voltage-independent calcium channels, and following the electrochemical gradient (Verbost *et al.* 1989). The cytoplasmic ionic calcium concentration is two to three orders of magnitude lower than in the ambient water or in the ECF. This gradient favours the entrance of calcium into the cell. However, special mechanisms are required for the extrusion of calcium ions from the cytoplasm across the basolateral membrane into the ECF. We have identified these mech-

anisms as ATP-driven Ca^{2+}-pumps and Na^{+}/Ca^{2+}-exchangers. Studies on the activity of both mechanisms as a function of the Ca^{2+} concentration have shown that the Ca^{2+}-pump is more important than the Na^{+}/Ca^{2+}-exchanger for the extrusion of calcium from the chloride cells. The reverse is indicated for the intestine (for a review of our studies see Flik *et al.* 1992).

B. 3. Endocrine Control of Calcium Metabolism.

a. Prolactin

The absence of parathyroid glands from purely aquatic amphibians and fish has now been well established. This absence has stimulated the search for a hormone with a similar hypercalcemic action. Several studies have indicated that the pituitary gland is the source of such a hormone, with prolactin as the most likely candidate. In the terrestrial vertebrates prolactin has some effects on calcium metabolism, in particular during the female reproductive period: it stimulates intestinal calcium uptake and the transfer of calcium to the offspring via the placenta or the mammary glands (see Wendelaar Bonga and Pang 1991).

In fish the role of prolactin in calcium metabolism seems more important than in the terrestrial animals. Removal of the pituitary gland in freshwater fish usually leads to mild hypocalcemia although data in the literature are not always consistent (Wendelaar Bonga and Pang 1989). Pang *et al.* (1978) have shown that the hypocalcemia of hypophysectomized killifish from low-calcium freshwater can be corrected by injections of pituitary homogenates or mammalian prolactin preparations. The latter produced hypercalcemia in e.g. intact sticklebacks, tilapia, trout, salmon or eel (Wendelaar Bonga and Pang 1989). Recently, using recombinant hormone preparations, we have shown that both forms of tilapia prolactin induce hypercalcemia in intact freshwater tilapia (Swennen *et al.* 1991). The most likely mechanism of action of prolactin is the stimulation of the activity of the ATP-dependent Ca^{2+}-pumps of the chloride cells: prolactins of mam-

malian or teleostean origin, including the recombinant tilapia preparations, increase the Ca^{2+}-ATPase activity and stimulate the Ca^{2+}-influx of the gills (Flik *et al.* 1986, 1989a). It takes several days for these effects to develop, and this contrasts sharply with the immediate responses to PTH in terrestrial animals. It is therefore unlikely that in teleosts prolactin accounts for the rapid restoration of experimentally induced hypocalcemia such as shown in fig. 1, or for the minute-to-minute control of the calcium concentration of the ECF in general. The primary function of prolactin in fish most likely is the control of integumental permeability to water and ions (Hirano 1986; Wendelaar Bonga and Pang 1989). Hypercalcemic actions have been ascribed to some other pituitary hormones of teleosts, but these have not been confirmed (see Wendelaar Bonga and Pang 1989). In trout cortisol was shown to produce hypercalcemia by increasing total body calcium uptake (Flik and Perry 1989). This hormone had no effect on tilapia (our unpublished observations) and it is unlikely to be a hormone with a specific hypercalcemic function.

b. Calcitonin

Calcitonin has been demonstrated in all major groups of fishes with exception of the agnathans. Its function in fish is still in dispute. Administration of mammalian or teleost calcitonin has only occasionally resulted in hypocalcemia. The doses required were high. In many experiments the hormone had no effect on plasma calcium (see Wendelaar Bonga and Pang 1991). An antihypercalcemic action of the hormone was demonstrated by Fenwick and Lam (1988) in mudskippers that were made hypercalcemic by taking them out of the water. On the other hand, removal of the Stannius bodies leads to a marked hypercalcemia, due to elevated total and ionic calcium levels (Hanssen *et al.* 1989) and develop in the presence of the ultimobranchial bodies. These bodies even show signs of hypersecretion of calcitonin in stanniectomized fish (Wendelaar Bonga and Greven 1978). Thus, the antihypercalcemic potency of the hormone is limited, in particular when compared with stanniocalcin, the hormone of the Stannius bodies present in teleostean and holostean fish. Enhanced plasma calcitonin levels

were reported for salmonids during gonadal maturation (Björnsson *et al.* 1989), and enlarged ultimobranchial glands were reported for several female fish during the reproductive period. This is consistent with the elevated plasma calcitonin levels that occur during the female reproductive period of some terrestrial vertebrates, and that have led to the hypothesis that calcitonin protects the skeleton during reproduction (see under A). Calcitonin may have a similar function in fish.

c. Stanniocalcin

Stanniocalcin is a glycoprotein that has recently been isolated from the Stannius bodies of some teleost species. It is most likely identical with the proteinaceous hypocalcemic factor called hypocalcin by Pang *et al.* (1974), and teleocalcin, purified and partially sequenced by Wagner *et al.* (1986). Stanniocalcin is different from the small glycoprotein isolated from salmon that was originally called teleocalcin (Ma and Copp, 1978), and also different from the glycoprotein called parathyrin of the corpuscles of Stannius isolated from European eels by Milet *et al.* (1980). With the identification (Wagner *et al.* 1986; Lafeber *et al.* 1988) and sequencing (Butkus *et al.* 1987) of stanniocalcin, the confusion about the nature of the major calcium-regulating factor of the Stannius bodies has been solved, and stanniocalcin has now been established as a hormone with unique structural and functional characteristics. It is a dimeric glycoprotein with monomeric constituents with a molecular radius of about 30 kDa (Wagner *et al.* 1986; Flik *et al.* 1989b). The 30 kDa form is dominating in the circulation (Wagner *et al.* 1991).

Injection of stanniocalcin effectively reduces the hypercalcemia that occurs after operative removal of the Stannius bodies. It acts by reducing the branchial influx of calcium, and its effects become noticeable within 15 min after injection (Lafeber *et al.* 1988b). Because it leaves the passive efflux of calcium unaffected, the result is a rapid decrease of the calcium concentration of the blood plasma (Wagner *et al.* 1986; Lafeber *et al.* 1988b). Verbost *et al.* (1989) have shown that stanniocalcin inhibits $^{45}Ca^{2+}$ uptake from the water into the branchial

epithelial cells, and this indicates that it acts by inhibiting the entrance of Ca^{2+} across the apical membranes of the chloride cells. The authors therefore suggested that stanniocalcin is a specific blocker of voltage-independent Ca^{2+}-channels in these membranes. The hormone did not influence the Ca^{2+}-extruding mechanisms in the basolateral membranes of the chloride cells (Flik *et al.*, unpublished). Because stanniocalcin is unlikely to influence the Ca^{2+}-channels of the apical membranes directly, we have postulated that the receptors for stanniocalcin are located in the basolateral membrane, and that the signal is mediated to the channels by second messenger. The hormone indeed modifies the second messenger systems in these cells: it reduces the branchial cAMP content and inhibits adenylate cyclase activity and stimulates the production of 1,2-sn-diacylglycerol in tilapia branchial cells (Flik *et al.* 1992). The latter effect indicates that stanniocalcin acts via the phosphoinositol pathway.

Our present concept of the endocrine control of calcium influx in teleost fish is illustrated in Fig. 2. As discussed above (see under B 2) Ca^{2+} can enter the fish - at least its chloride cells - passively, as long as the Ca^{2+}-channels are open and the electrochemical gradient is favourable. The latter is likely to be the case in most fresh waters, even with very low Ca^{2+} levels. This means that under normal freshwater conditions teleost fish may face hypercalcemia rather than hypocalcemia, even when the water Ca^{2+}-concentration is well below that of the ECF, unless the Ca^{2+} channels are closed by stanniocalcin. This is the essence of our model. It explains why the Ca^{2+} concentration of the blood plasma is rising to extremely high levels when the Stannius bodies are removed. This effect has been consistently observed in freshwater and seawater species and is caused by increased Ca^{2+} influx (So and Fenwick 1979). The total and ionic Ca^{2+} levels produced as a result of the absence of stanniocalcin are much higher than those that can be obtained by injection of hormones with hypercalcemic actions such as prolactin or cortisol. Apparently, in freshwater as well as seawater, the total body uptake of calcium and the control of the Ca^{2+} concentration of the ECF are under inhibitory control by stanniocalcin.

In conclusion then, the endocrine control of the calcium concentration of the ECF is most likely dominated by one hormone only in terrestrial vertebrates and in teleost fish. This contrasts with the earlier concept of two antagonistically acting hormones. In the terrestrial vertebrates it is PTH, which increases the Ca^{2+}-concentration of the ECF, mainly by stimulating the intestinal uptake of calcium and by mobilizing calcium from the skeleton. In teleost fish it is stanniocalcin, which lowers the Ca^{2+}-concentration of the ECF by inhibiting the passive influx of calcium from the ambient water into the branchial chloride cells and, possibly, the epithelial cells of the intestine.

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