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Minireview

Parathyroid hormone-related protein in teleost fish

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Abstract

A brief description is given of the discovery of PTHrP and the roles of the peptide in mammalian physiology. Next, the occurrence of PTHrP in the earliest vertebrates, sharks, skates and fishes, is reviewed and the calciotropic functions of PTHrP are addressed more specifically in fishes. Parathyroid hormone-related protein (PTHrP) is a hypercalcemic hormone in teleostean fishes, but also has para- and autocrine functions. After the isolation and identification of fish PTHrP and PTHrP receptors and the subsequent development of recombinant protein and a real-time quantitative PCR, a calciotropic role of PTHrP in fish physiology could be assessed. PTHrP influences calcium physiology via regulation of calcium mobilisation from internal sources (bone and scales) and via calcium uptake from the environment (water and diet). Continuous variations in the need for calcium and in the availability of environmental calcium require fast calciotropes to guarantee calcium balance, in which PTHrP is pivotal for the fish. PTHrP is essential in fish bone physiology, *e.g.* in mineralisation and calcium reabsorption from the scales. Moreover, PTHrP plays a role in vitellogenesis, cortisol production, regulation of renal Mrp2 activity and melatonin synthesis. The plethora of functions of PTHrP in fish concern endocrine, paracrine and autocrine (and possibly intracrine) functions; calciotropic actions of PTHrP at the organismal and cellular level are prominent in fish. The strong conservation of the *pthrp* gene in the vertebrate lineage and the N-terminal similarity of the coded proteins relates to the important role of PTHrP in calcium physiology that is of paramount importance to all physiological processes. Recent and ongoing studies will contribute to our rapidly expanding knowledge of the original physiological functions of PTHrP in teleost fish.

Keywords: PTHrP; Calcium physiology; PTH1R; CaSR; Endocrine interaction

1. PTHrP, its discovery

Parathyroid hormone-related protein (PTHrP) was discovered in 1987 in humans as the circulating peptide responsible for the syndrome of humoral hypercalcemia of malignancy (HHM; Moseley et al., 1987). PTHrP and parathyroid hormone (PTH), the main hypercalcemic hormone in vertebrates, evolved from a common, ancestral gene. Within a species, the two proteins share a high, about 70% N-terminal amino acid (aa) homology and bind with similar affinity to a shared PTH/PTHrP receptor (Jüppner et al., 1991). PTH is secreted from the parathyroid glands; however, *pthrp* is expressed in and PTHrP secreted by a variety of tissues, including many epithelia (de Papp and Stewart, 1993). In humans, there are three different *pthrp* mRNA transcripts, encoding for isoforms of 139, 141 and 173 aa long (Mangin et al., 1989). These isoforms have different physiological functions; have three bioactive subdomains (N-terminal, mid segment and C-terminal sequences) and mostly act in an intra-, auto- or paracrine fashion. Besides the pathological effects of overproduction of PTHrP in HHM, the main functions of PTHrP in 'normal physiology' are regulation of transepithelial calcium transport, regulation of smooth muscle tonus (relaxation of stomach, urinary bladder and arterial segments) and tissue growth and differentiation, as well as cell proliferation (de Papp and Stewart, 1993; Philbrick et al., 1996; Martin et al., 1997). The aa sequence of PTHrP is strongly conserved among mammalian species, which indicates its importance in mammalian physiology; the presence of PTHrP in fishes (and elasmobranch species) suggests that

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this signal protein has played an important role in vertebrate physiology throughout evolution. Very recently, an expert review was published on PTHrP in fish (Guerreiro et al., DOI: 10.1152/ajpregu.00480.2006); this topical review is somewhat more speculative and focuses on new functions and unexpected relationships with other endocrine factors.

2. PTHrP in the earliest vertebrates

PTHrP has been detected in tissues and plasma of the lamprey (*Geotria australis*; Trivett et al., 2005), in sharks and rays as well as in a lungfish (*Neoceratodus forsteri*; Danks et al., 1998), establishing that this protein is at the roots of vertebrate evolution. Elasmobranch species have a cartilaginous skeleton that contains calcium minerals, albeit less compared to that of bony fishes, which have a skeleton with apatite as the main mineral, just as in mammals. PTHrP immunocrossreactivity was found in tissues of the dogfish (a shark!), *Scyliorhinus canicula* (Ingleton et al., 1995) and in the red stingray (*Dasyatis akajei*), a PTH/PTHrP receptor (now PTH1R) was demonstrated (Akino et al., 1998). Trivett and colleagues (2002) detected PTHrP in tissues of representative species from different elasmobranch families, using antisera against human (1–14)PTHrP, (1–16)PTHrP and (67–84)PTHrP. In general, the distribution of PTHrP in elasmobranch species is similar to the distribution found in mammals, pointing to the conservation of PTHrP and PTHrP production sites in evolution. Basal, circulating PTHrP levels in elasmobranch species (Ingleton et al., 1995; Akino et al., 1998; Trivett et al., 1999, 2002) are consistently higher than the PTHrP levels found in healthy humans and comparable to the levels found in patients with HHM. Caution in the outcome of the studies on elasmobranches is needed (because in all instances heterologous antisera were used) till specific data with homologous probes make the observations unequivocal.

3. PTHrP in teleosts

In the late 1970s, the research on PTH-related factors in teleosts started. The development of specific antisera for immunohistochemical studies and quantitative immunoassays and later the development of molecular biological techniques have clearly advanced the evidence for the presence of PTHrP proper in teleosts. The first reports in the literature on a PTH-like substance in teleostean species gave evidence for a hypercalcemic factor in the fish pituitary gland that was shown to be immunologically related to mammalian PTH (Parsons et al., 1978). Next, in extracts of corpuscles of Stannius from European eel (*Anguilla anguilla*), a PTH-like substance was demonstrated by immunocytochemistry; surprisingly, this substance exerted hypocalcemic actions in fish, a phenomenon that remains enigmatic (Milet et al., 1982; Lopez et al., 1984). However, it was confirmed by the demonstration of comparable hypocalcemic effects of bovine (1–34)PTH and corpuscles of Stannius extracts (Wendelaar-Bonga et al., 1986) injected in fish adapted to low calcium water. Differences in tissue sources (different species), extraction procedures and bioassays used must be at the basis of the discrepancies in these studies. The corpuscles of Stannius are not homogeneous in cell make up (at least two cell types; Wendelaar Bonga et al., 1989) and products produced, and it may thus be that differential effects of PTHrP and stanniocalcin in an extract (and dependent on the relative abundance of these products) have caused these apparent discrepancies. Harvey et al. (1987) detected an immunoreactive PTH-like substance in the circulation and in several tissues of trout (*Salmo gairdneri*) and goldfish (*Carassius auratus*). Kaneko and Pang (1987) demonstrated the presence of a PTH-like substance in brain of goldfish.

True immunoreactive PTHrP in teleosts was probably first shown in the pituitary gland of Coho salmon (*Oncorhynchus kisutch*) by Fraser and colleagues (1991), who suggested a role for the protein in calcium regulation and by Danks and co-workers (1993), who demonstrated PTHrP in the pituitary gland and plasma of sea bream (*Sparus auratus*) with an antiserum against human N-terminal (1–16)PTHrP. Molecular biology unequivocally demonstrated the presence of PTHrP in fish. The cloning of a *pthrp* cDNA from fugu (*Fugu rubripes*) revealed a 2.25 kb cDNA gene product encoding for a 126 aa peptide (Power et al., 2000). Flanagan and co-workers (2000) cloned a 1.8 kb cDNA from sea bream, encoding a 125 aa gene that showed 85% overall homology with the fugu *pthrp* gene. Rotllant and Du (2004, Accession No. AY608915.1) and Rubin et al. (2005, Accession No. DQ022615.1 and Accession No. DQ022616.1) reported the presence of the zebrafish *(Danio rerio) pthrp* gene which establishes its wide occurrence in fishes. As fugu and zebrafish diverted 300 million years ago (Hedges, 2002), we may conclude that the gene is very well conserved indeed.

The piscine PTHrP shares an overall 36% aa homology with its tetrapod counterpart. However, this homology varies greatly per gene region: indeed, the N-terminus shares around 62% homology with mammalian PTHrP. The piscine PTHrP lacks the 'mammalian' C-terminal domain responsible for osteoclast inhibitory activity, which suggests that this function arose after the water-land transition of vertebrates. Also, the piscine *pthrp* gene, encoding for a protein with insertions between positions 38 and 54 (fugu) or 38 and 65 (sea bream) that is absent in the mammalian *pthrp* gene and this opens the possibility of a unique function for this segment of the peptide in fish physiology (Power et al., 2000).

PTHrP was quantified in plasma by radioimmunoassay using heterologous antisera raised against human peptide (Danks et al., 1993; Devlin et al., 1996) and later with homologous antisera (Rotllant et al., 2003; Abbink et al., 2004, 2006). As holds for elasmobranch species, PTHrP levels in teleostean plasma are consistently and significantly

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higher than these values for humans (basal circulating levels of around 0.5–2.5 pmol l^{-1} in humans and 0.1–0.6 nmol l^{-1} in fish). The difference in levels of circulating PTHrP in elasmobranches and fishes compared to mammals may indicate that PTHrP has lost its endocrine function in normal physiology of the latter and has assumed more local and paracrine functions.

In-situ hybridisation (Danks et al., 1998; Trivett et al., 1999; Ingleton et al., 2002) and immunohistochemical studies (Devlin et al., 1996; Trivett et al., 1999; Flanagan et al., 2000) have demonstrated the *pthrp* mRNA and PTHrP protein in gills, operculum, kidney, pituitary gland, brain, saccus vasculosus, muscle, skin, spleen, liver and intestine. This widespread distribution of the *pthrp* gene and protein reflects the wide range of functions that are reported for PTHrP in fish and suggests endocrine, paracrine, autocrine and intracrine functions; at this moment, no reports on intracrine effects in fish tissues are available. The (high) circulating PTHrP-levels in the range of other protein endocrines (nmol l^{-1}) would be in line with a classical endocrine function for (pituitary) PTHrP (Danks et al., 1993) as is its presence in pituitary gland cells (Ingleton et al., 1998; Abbink et al., 2006).

PTHrP was demonstrated in periodic acid Schiff (PAS) positive cells identified as a subpopulation of somatolactin (SL) producing cells (Abbink et al., 2006). Interestingly, in the sea bream pituitary gland, PTHrP is found only in the SL-producing cells that would be similar to the recently described SLα cells in zebrafish (Zhu et al., 2004). Indeed, in an earlier study, Ingleton et al. (1998) reported that in sea bream, PTHrP and SL are both located in PAS positive cells and that some cells do contain both PTHrP and SL. SL is a hormone from the prolactin (*prl*) gene family and is structurally related to both PRL and growth hormone. SL may play a role in regulation of the calcium balance of the fish: changes in SL plasma levels and *sl* pituitary gland mRNA expression at low ambient calcium were observed, albeit only after several days (Kakizawa et al., 1993). This makes short term effects of SL on calcium balance unlikely. However, the presence of PTHrP in SLα cells could indicate that a correlation between somatolactin cell activity and fast hypercalcemic effects reflects a SLα cell activity mediated through PTHrP.

Two other sites in fish may contribute to circulating PTHrP levels, *viz.* the gills and the corpuscles of Stannius (see discussion above). The latter glands can be easily removed in certain fish (*e.g.* stanniectomy of eel), and such an experiment is strongly indicated, but awaits further development of immunoassays to detect PTHrP in such species. The gills were suggested to be evolutionary related to the parathyroid gland vertebrates (Okabe and Graham, 2004), but to us the gills seem a less likely site for circulating PTHrP, as we see lower or unchanged PTHrP levels in fish that have up-regulated levels of *pthrp* mRNA when confronted with limited access to calcium in water and/or diet or when made vitamin D deficient (Abbink et al., 2006, 2007).

PTH1R, the most common receptor in teleosts that binds PTHrP, is a G-protein coupled receptor with similar affinity for PTHrP and PTH (Gensure et al., 2005). Three PTH receptors have been cloned in zebrafish (*D. rerio*), referred to as PTH1R, PTH2R and PTH3R (Rubin and Jüppner, 1999). The PTH2R is activated by human PTH, but not by the human or teleost PTHrP, and, interestingly, this would suggest the presence of PTH in fish (Rubin et al., 1999). Indeed, Danks and colleagues (2003) identified a gene encoding for an 80 aa PTH in fugu and the predicted protein N-terminus of fugu PTH is homologous to the N-terminus of tetrapod PTH. However, the C-termini of fugu PTH and tetrapod PTH show no homology at all. Apparently, there is a lower evolutionary pressure on the fish *pth* gene than on the fish *pthrp* gene, which shows a significant degree of C-terminus homology with human PTHrP. In the zebrafish genome, two *pth* genes were found (*pth1* and *pth2*) that are highly homologous to the human *pth* gene and the proteins derived from the genes have high affinity for the PTH1R (Gensure et al., 2004). Also in pufferfishes (*Takifugu rubripes* and *Tetraodon fluviatilis*) two PTH hormones (PTH_A and PTH_B), two PTHrPs ($PTHrP_A$ and a novel $PTHrP_B$) and a PTH-like ligand (PTH-L) were identified; PTH-L has both PTH and PTHrP characteristics and *pth-l* is proposed to represent the ancestor of the *pth/pthrp* gene (Canario et al., 2006).

Our knowledge of the physiological functions of PTHrP in fish is recently rapidly expanding. The availability of recombinant sea bream PTHrP allowed the development of region specific detection and quantification to define post-translational and postsecretory processing of the protein and assess its bioactivities in fish (Anjos et al., 2005). A real-time quantitative PCR was designed by Hang et al. (2005) for sea bream tissues to measure mRNA expression levels for *pthrp*, *pth1r* and the calcium sensing receptor *casr*, which, in humans, has a set point that can detect minor changes (as small as 0.2 mmol l^{-1}) in blood Ca^{2+} and regulates secretion of calcemic endocrines, including PTHrP, to strictly control the plasma Ca^{2+} levels (Chattopadhyay et al., 2000). Quantitative analysis of these messenger RNAs proved to be a powerful tool to study the role of PTHrP in fish calcium handling (Abbink et al., 2006).

4. Functions of PTHrP in fish

The structure of the *pthrp* gene and the presence of the protein and its receptors in a wide array of tissues are under intensive study. A strong focus is on the function of PTHrP in teleosts that relates to calcium physiology. An important line of research addresses how PTHrP influences calcium physiology via mobilisation of calcium from internal bone compartments (skeleton and dermal scales) and via calcium uptake from the environment (water and/or diet; external calcium sources). These two different faculties of calcium mobilisation for calcium balance imply

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the presence of different and possibly independent PTHrP systems for calcium regulation in fish.

4.1. Calcium balance

N-terminal (1–38)PTHrP enhances in a concentration-dependent way the accumulation of calcium in larval sea bream (Guerreiro et al., 2001). The larval undirectional calcium influx (Ca^{2+}-uptake from the water via chloride cells) is stimulated, whereas the epithelial efflux is reduced or unaffected; through these combined effects a strong positive net uptake of calcium ions from the environment into the fast growing larvae occurs, supporting the rapid growth of the skeleton. Little is known about presumed effects on early development of fishes, but clearly this calciotropic action is key in normal skeletal growth. Remarkably, the increase in calcium uptake from the water via the integumental chloride cells is accompanied by a 30% reduction in the drinking rate of the fish, which could be a compensatory mechanism to avoid excessive calcium load from the seawater drunk. Clearly, the findings indicate that PTHrP causes a shift in the way calcium is taken up by the fish and that it orchestrates multiple sites for calcium handling, via gills/skin, intestine and likely kidney as well (see below).

Indeed, a recent study by Fuentes et al. (2006) established a physiological role for PTHrP in intestinal calcium regulation. Increased intestinal calcium uptake was measured after 30 min of exposure to 6 or 30 nmol l^{-1} (1–34)PTHrP *in vitro*, in all three regions of the intestine tested in this study (duodenum, hindgut and rectum). In combination with a remaining (duodenum, hindgut) or decreasing (rectum) intestinal calcium efflux, the overall observation was an up to 4-fold increased net calcium accumulation, depending on the region of the intestinal track. The regional differences that were observed indicate that the calcium transport mechanisms involved vary between the regions, possibly by different active signalling pathways or by the involvement of different receptors (in addition to the PTH1R). The results clearly show that the intestinal tract is (in addition to the gills) a key target for PTHrP-dependent calcemic control.

When PTHrP stimulates calcium uptake from the environment, decreases in environmental calcium availability and an imminent hypocalcemia should provoke enhanced reaction PTHrP-signalling as may predicted for any feedback control mechanism. We assessed the PTHrP system in juvenile sea bream confronted with calcium constraint in water and diet (Abbink et al., 2004, 2006). In gill tissue, expression levels of the *pthrp* and *pth1r* genes were up-regulated on the longer term after calcium constraint; short-term (4 h) constraint did not provoke changes. However, in the pituitary gland, both short and long term exposure to calcium constraint resulted in an unexpected down-regulation of *pthrp* and *pth1r* expression. These observations point to two different and possibly independent responses of PTHrP systems. The pituitary gland PTHrP system is rapidly and persistently down-regulated at the level of mRNA production. Plasma PTHrP levels are not significantly affected, which we take to indicate that the lower calcium turnover under environmental calcium constraint results in lower PTHrP turnover as well. Direct demonstrations of changes in PTHrP turnover await further experimentation. The up-regulation of the branchial PTHrP system during calcium constraint correlates well with a long-term adjustment of uptake mechanisms. The precise role of PTHrP in calcium acquisition remains to be elucidated: branchial and intestinal influxes are very much lower under calcium constraint; yet net calcium accumulation is hardly affected. Clearly then, PTHrP must limit calcium loss via branchial, intestinal and renal pathways (Fig. 1).

The physiologically most relevant fraction of calcium in the blood is the ionic fraction, which is most strictly regulated. Calcemic endocrines react swiftly to changes in external calcium availability or increased physiological needs for calcium (rapid growth, vitellogenesis; Björnsson et al., 1999). As indicated above, circulating PTHrP levels remain rather constant or show only mild changes in response to a low water calcium level. Surprisingly, a mild positive correlation between plasma ionic calcium and plasma PTHrP was recently established (Abbink et al., 2006), which we tentatively explain to reflect lower PTHrP turnover at increased plasma calcium levels; also, this would support an endocrine calcium-regulatory function for PTHrP. This is corroborated and strengthened by a similar positive correlation between plasma ionic calcium and pituitary gland *pthrp* mRNA content.

4.2. Skeletal and scale physiology

The skeleton and dermal scales represent significant internal reservoirs of calcium, with in some fish about 99% of the total calcium pool being incorporated mainly in the form of calcium phosphates (Flik et al., 1986). Plasma PTHrP levels increase with increasing body weight of fish and mildly positive and strong correlations between plasma PTHrP and the whole body content of the main minerals in bone (calcium, phosphorus and magnesium) strongly suggest that PTHrP is involved in skeletal calcium physiology (Abbink et al., 2007). A research for a role of the bone compartments in metabolic clearance, production and secretion, as well as distribution space for PTHrP is strongly indicated by these studies. Somehow, plasma PTHrP levels in the fish reflect calcium, phosphorus and magnesium pool sizes in the fish. This could relate to a pool size-dependent enhanced distribution space for PTHrP, or simply a requirement for enhanced PTHrP activity. Little is known about the role of PTHrP in fish mineral handling, but a short resume on PTHrP and phosphorus handling was published by Guerreiro et al. (DOI: 10.1152/ajpregu.00480.2006).

4.3. Calcium reabsorption from scales

Rotllant and co-workers (2005a) established an involvement of PTHrP in calcium reabsorption from scales, which

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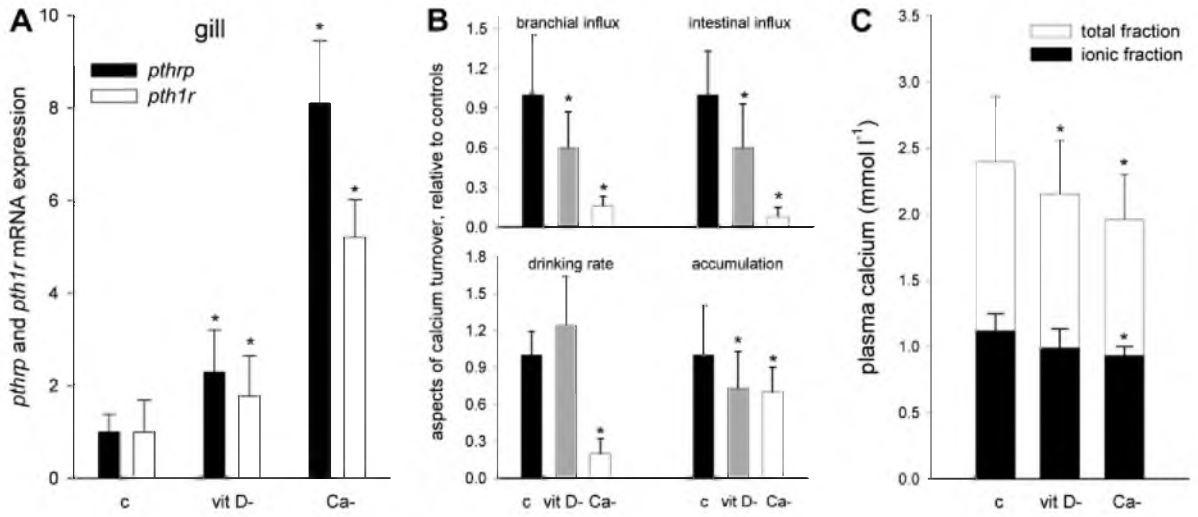

Fig. 1. Juvenile sea bream were confronted with either direct (via a calcium deficient diet and strongly diluted sea water; Ca-group) or indirect (via a vitamin D deficient diet; vit D- group) calcium constraint. The branchial PTHrP system reacts by an up-regulation of branchial *pthrp* and *pth1r* mRNA expression (A). Calcium constraints decrease calcium turnover (B): calcium influxes via gills and intestine, as well as drinking (not in vitamin D deficient fish) decrease. Calcium accumulation is only mildly, but significantly, affected. Plasma calcium balance, best reflected by the important ionic fraction, remains at level to ensure long term survival of the fish (C). Values are given as relative to the control group (A and B) or as mean ± SD ($n = 6$–8). Values in Fig. 1B are given in nmol h^{-1} per g fish (fluxes and accumulation) and in nl h^{-1} per fish (drinking). Asterisks represent significant difference from the control group (group c; $P < 0.05$). Data modified from Abbink et al. (2004, 2006 and 2007; submitted for publication).

was substantiated by the demonstration of the PTH1R in the tissue/cells associated with fish scales. Production of cyclic adenosine monophosphate (cAMP; to evaluate PTHrP-induced signal transduction) and activity of tartrate-resistant acid phosphatase (TRAPC, a marker for osteoclastic activity in mammalian bone; the enzyme activity is also easily demonstrated in tissue associated with fish scales) were enhanced when treated with N-terminal (1–34)PTHrP. These findings would suggest that N-terminal (1–34)PTHrP stimulates an osteoclastic activity in sea bream scales through the PTH1R via a cAMP/AC (adenylate cyclase) intracellular pathway.

4.4. Mineralisation

Calcitriol [1,25$(OH)_2$ vitamin D_3], the active metabolite of vitamin D in vertebrates, plays a key role in bone formation (Haga et al., 2004) and stimulates intestinal calcium absorption (Swarup et al., 1991). Thus, calcitriol will contribute to a positive shift in calcium uptake and accumulation and its effects will be hypercalcemic. Calcitriol receptors were localised in several tissues involved in calcium handling (gill, intestine) in Atlantic cod (*Gadus morhua*; Sundell et al., 1992) and vitamin D metabolites, including calcitriol, have been found in plasma of various fish species (Horvli et al., 1998). Indeed, vitamin D deficiency in juvenile sea bream impedes growth and slows down calcium turnover (*i.e.* calcium influx, efflux and accumulation rates decrease; Abbink et al., 2007). Changes in plasma PTHrP values and mRNA expression levels for the *pthrp* and *pth1r* genes in the pituitary gland and gill mark this first report of a correlation between the hypercalcemic factor PTHrP and calcitriol in fish (the correlation was earlier described in humans by Abe et al., 1998; Tovar Sepulveda and Falzon, 2003) and it was speculated that calcitriol-dependent bone formation is key in this correlation. An interesting parallel exists with the relationship between PTHrP and another steroid that affects calcium handling in fish, *viz.* estrogen. Estrogen is hypercalcemic in fish, but for this effect estrogen depends, at least partly, on a concerted action of PTHrP (Bevelander et al., 2006).

Osteonectin (OSN) plays a key role in bone mineralisation. OSN is described in (mammalian) skeletal biology as a calcium binding glycoprotein that stimulates the mineralisation process following differentiation of the osteoblastic cell lineage (Estevao et al., 2005). High levels of ionic calcium up-regulate *osn* mRNA expression; in higher vertebrates, PTH is known to suppress *osn* gene expression (Nakajima et al., 2002). In agreement with this notion, treatment of sea bream scales in culture with 10 nmol l^{-1} (physiological) or 1000 nmol l^{-1} (pharmacological) (1–34)PTHrP abolishes *osn* mRNA expression; thus PTHrP-regulated bone mineralisation through regulation of the *osn* gene (Redruello et al., 2005) is as old as the fishes (over 450 million years). The dermal scleroblasts that form the scales in teleostean fishes apparently harbour a VDR and PTHrP-receptors as well as osteoclastic and osteoblastic characteristics.

5. Other functions

5.1. Vitellogenesis

Vitellogenesis by the liver is triggered and maintained by estradiol-17β (E_2) and is accompanied by increased plasma calcium and phosphate levels. As described above,

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E_2-treatment increases whole body calcium accumulation and stimulates reabsorption of calcium from dermal scales (Guerreiro et al., 2002). As *pthrp* and *pth1r* are expressed in hepatocytes, a para-/autocrine role for PTHrP in vitellogenesis was indicated, in analogy to the situation in mammals (Cros et al., 1998). A direct involvement of PTHrP in fish vitellogenesis was recently shown: vitellogenin production in E_2-primed cultured sea bream hepatocytes is stimulated by homologous recombinant PTHrP (Bevelander et al., 2006). E_2 stimulates the secretion of PTHrP into the blood stream preceding the rise in plasma calcium levels. This makes PTHrP the candidate of choice for the mediator of the hypercalcemic action of E_2 through mobilisation of Ca^{2+} from either external or internal sources.

5.2. Mrp2

Multidrug resistance protein 2 (Mrp2) is a carrier protein that is found among others in luminal membranes of renal proximal tubule cells, where it mediates the active secretion of (endogenous) waste products and xenobiotics (Schaub et al., 1997). In the killifish (*Fundulus heteroclitus*) nephron, Mrp2-mediated fluorescein-methotrexate transport (tubular secretion) is reduced by Ca_i^{2+}-dependent endothelin release via a PKC signalling pathway (Miller et al., 2002). Influx of Ca^{2+} (*e.g.* caused by toxicants) into the tubular cell through L-type calcium channels stimulates release of endothelin which results than in inhibition of Mrp2 activity; also, extracellular high calcium levels reduce Mrp2 mediated transport. These observations prompted the question whether calcemic factors, such as PTHrP, would interfere with this transport pathway. Indeed, PTHrP interferes with the endothelin regulated Mrp2 mediated transport (Wever et al., 2006). The inhibitory effect of recombinant PTHrP on Mrp2 mediated transport is concentration-dependent, with a maximal inhibition of 40% at 20–60 nmol l^{-1}, a concentration that indicates a paracrine action of PTHrP. The endothelin-induced inhibition is additive to the PTHrP-induced effect, indicating that the inhibitions proceed at least partly through separate intracellular pathways. Another interesting observation was that the endogenous PTHrP antagonist stanniocalcin (Verbost et al., 1993), which exerts actions via intracellular calcium and PKC pathways, reverses the combined PTHrP/ET inhibition of Mrp2-transport completely (Wever et al., 2006). Clearly, PTHrP has the nephron as target and studies on PTHrP effects on calcium and phosphate handling by fish kidney are warranted.

5.3. Cortisol

As in mammals (Nussdorfer et al., 2000), in fish the hypothalamus–pituitary–adrenal/interrenal (HPA/HPI) axis expresses *pthrp*, both in the pituitary gland and in the interrenal gland. In isolated and perfused interrenal tissue, sea bream (1–34)PTHrP rapidly stimulates the release of cortisol in a concentration-dependent (range 10^{-6}–10^{-11} mol l^{-1}) way (Rotllant et al., 2005b). The EC_{50} of (1–34)PTHrP was 2.8 times higher than that of (1–39)ACTH and the increase in cortisol production in response to 10^{-8} mol l^{-1} (1–34)PTHrP was about 7-fold lower when compared with 10^{-8} mol l^{-1} (1–39)ACTH.

With the pars intermedia somatolactin cells as a main pituitary source of PTHrP and a regulatory role for pituitary PTHrP in stress-axis activity as suggested above, the search for the organisation of the pituitary vasculature or arrangement of extracellular fluid flows between pars intermedia and (rostral) pars distalis warrants further study.

In-vivo administration of physiological concentrations (1–34)PTHrP rapidly resulted in a dose-dependent inhibition of circulating cortisol (Guerreiro et al., 2006), although this effect was only short-lived (up to 5 h). Increased blood cortisol levels suppress circulating PTHrP levels up to 24-fold, in line with a role for cortisol as a negative feedback regulator of PTHrP production (Guerreiro et al., 2006). The possibility of PTHrP mediated corticotropic effects via (aspecific) activation of ACTH pathways was eliminated since the ACTH blocker corticotropin-inhibiting peptide (CIP) had no effect on (1–34)PTHrP-induced cortisol production. Furthermore, alignment of *acth* and *pthrp* sequences does not give any reason to suspect such interactions at the level of the respective receptors (PTH1R, PTH3R and MC2R). However, the expression of mRNA for *pth1r* and *pth3r* in interrenal cells suggests that the observed effect is mediated directly and specifically via PTHrP receptors, again indicating an auto- or paracrine action of PTHrP.

5.4. Melatonin

Melatonin is a product of tryptophan metabolism mainly in the pineal gland and in the retina. It is synthesised in a rhythmic fashion, with increased synthesis in darkness and inhibited synthesis during the light phase of the light/dark cycle (Falcon, 1999). The production of melatonin is stimulated by increasing plasma Ca^{2+} levels (Begay et al., 1994) and inhibited following hypocalcemia (Meissl et al., 1996). In fish, melatonin is involved in development (Shi et al., 2004) and in the timing of parr-smolt transformation of Atlantic salmon (*Salmo salar*; Porter et al., 1998). In addition, melatonin suppresses osteoclastic-specific TRACP activity, a protective mechanism against excess degradation of the scalar calcified matrix during vitellogenesis (Suzuki and Hattori, 2002). Together, these functional aspects, all (indirectly) related to calcium metabolism, raise questions about a possible regulatory role for PTHrP in melatonin-steered physiology in fish; however, such involvement has yet to be determined directly (Abbink et al., 2007).

6. Concluding remarks

After the isolation and identification of PTHrP and its receptors in teleosts, as well as its definitive detection in various tissues and in plasma, most recent studies have

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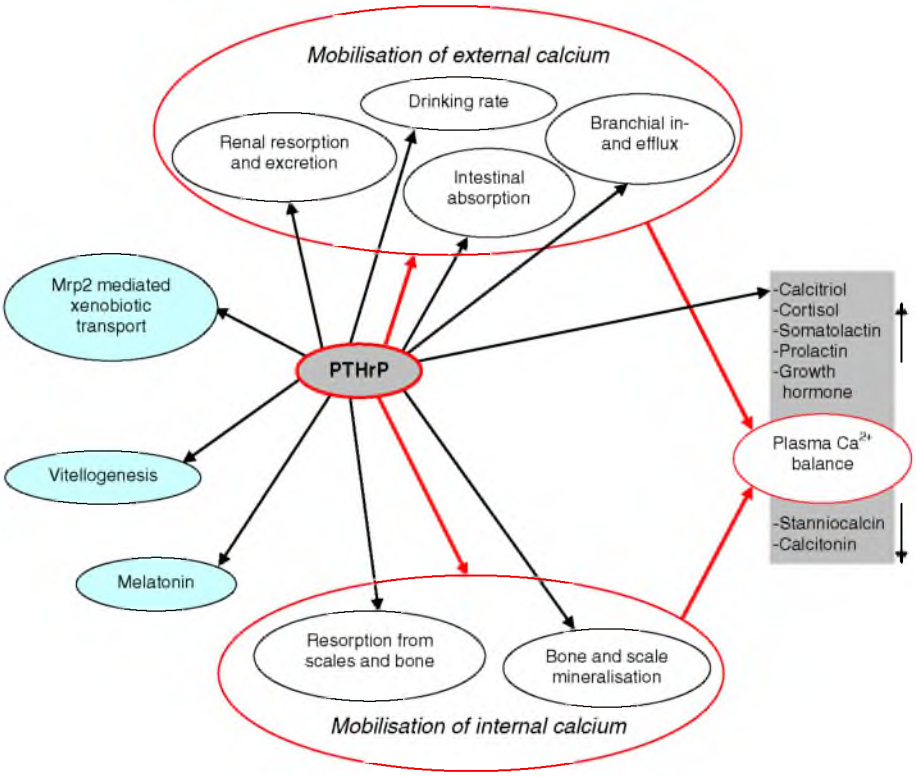

Fig. 2. Illustration of the pleiotropic effects of PTHrP in calcium physiology of fishes as described in this exposé. The figure is not exhaustive, but highlights the regulatory role of PTHrP in fish calcium physiology. Indirect interactions with phenomena in blue circles are likely mediated via alteration in Ca_i^{2+} second messenger pathways; calciotropic calciotropic hormones are highlighted in grey fields.

focussed on the calciotropic role(s) of PTHrP in fish physiology. The strong conservation of the *pthrp* (and *pth*) genes in the vertebrate lineage and the N-terminal similarity of the coded proteins relates to the key role of calcium physiology that is of paramount importance in all physiological processes (Fig. 2). Life originated in a seawater environment and a strictly controlled calcium metabolism (calcium being toxic at elevated levels, inside the cell as well as outside the cell) is key to all life in aqueous and marine environments.

Correlations between plasma ionic calcium and plasma PTHrP and between plasma PTHrP and pituitary gland *pthrp* mRNA expression show that PTHrP, as an endocrine factor, is key in maintenance of fish plasma calcium balance. Variation in the need for calcium (bone mineralisation, vitellogenesis) or the availability of environmental calcium (limited concentrations in water or diet) urge the endocrine system to respond rapidly to regulate the ionic calcium level. PTHrP-involvement in skeletal and scale physiology has been established in bone mineralisation (during growth and development) and in calcium reabsorption from the scales, when processes as vitellogenesis require extra calcium. The very strict control of calcium metabolism (by a plethora of endocrines: PTHrP, PTH, PRL, stanniocalcin, calcitonin, E_2, calcitriol, cortisol etc.) and plasma ionic calcium levels through swift endocrine adjustments make this a rather difficult field of research. However, a new array of paradigms with a key role for fish shows involvement of PTHrP in vitellogenesis, cortisol production, Mrp2 activity and melatonin synthesis (Fig. 2). Such studies will rapidly expand our knowledge on this pleiotropic hormone. They show that the calciotropic actions of PTHrP concern organismal as well as cellular physiological phenomena. Studies on fish give insight in original functions of PTHrP-regulated processes and, once again, show the power of comparative endocrinology.

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