The parathyroid hormone family of peptides: structure, tissue distribution, regulation, and potential functional roles in calcium and phosphate balance in fish

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Guerreiro PM, Renfro JL, Power DM, Canario AVM. The parathyroid hormone family of peptides: structure, tissue distribution, regulation, and potential functional roles in calcium and phosphate balance in fish. Am J Physiol Regul Integr Comp Physiol 292: R679–R696, 2007. First published October 5, 2006; doi:10.1152/ajpregu.00480.2006.—Parathyroid hormone (PTH) and PTH-related protein (PTHrP) are two factors that share amino acid sequence homology and act via a common receptor. In tetrapods, PTH is the main endocrine factor acting in bone and kidney to regulate calcium and phosphate. PTHrP is an essential paracrine developmental factor present in many tissues and is involved in the regulation of ossification, mammary gland development, muscle relaxation, and other functions. Fish apparently lack an equivalent of the parathyroid gland and were long thought to be devoid of PTH. Only in recent years has the existence of PTH-like peptides and their receptors in fish been firmly established. Two forms of PTH, two of PTHrP, and a protein with intermediate characteristics designated PTH-L are encoded by separate genes in teleost fish. Three receptors encoded by separate genes in fish mediate PTH/PTHrP actions, whereas only two receptors have so far been found in terrestrial vertebrates. PTHrP has been more intensively studied than PTH, from lampreys to advanced teleosts. It is expressed in many tissues and is present in high concentration in fish blood. Administration of this peptide alters calcium metabolism and has marked effects on associated gene expression and enzyme activity in vivo and in vitro. This review provides a comprehensive overview of the physiological roles, distribution, and molecular relationships of the piscine PTH-like peptides.

parathyroid hormone-related protein; calcium regulation; phosphate regulation; molecular evolution

FISH, WHETHER LIVING in seawater or freshwater, are surrounded by a readily available source of ionic calcium (Ca2+), and, intuitively, the existence of a hypercalcemic factor that promotes Ca2+ mobilization or renal Ca2+ retention seems unnecessary. This view appeared to be consistent with the specific role of stanniocalcin as an endocrine factor with antihypercalcemic action (137). Nevertheless, several studies performed using heterologous antisera have indicated the presence of parathyroid hormone (PTH)-like immunoreactivity in fish pituitary glands, brain, and plasma (37, 54, 66, 96, 99). Since the discovery of PTH-related peptide (PTHrP) and its hypercalcemic effect in mammals (84), immunoreactivity for this protein also has been found in plasma and tissues of agnatha, elasmobranchs, and teleosts (3, 19, 22, 23, 63, 131). Definitive confirmation of the existence of PTH-like proteins in fish came from the isolation of cDNAs for functional PTH receptors in zebrafish, Danio rerio (118, 119), and the cloning and characterization of the cDNA and gene for piscine PTHrP in the marine sea bream, Sparus auratus, and pufferfish, Takifugu rubripes (33, 108). More recently, the cloning of the corresponding cDNAs has established that two PTH and two PTHrP genes are expressed in zebrafish and in the pufferfishes T. rubripes and Tetraodon fluviatilis (13, 20, 42) and that a fifth gene encodes a PTH-like peptide (13). This review integrates the information available on PTH-like peptides in teleosts and elasmobranchs, focusing mainly on their structural characteristics, tissue distribution, physiological functions, mode of action, and potential regulatory mechanisms.

PTH AND PTHrP

Calcium and phosphate are present in the circulation in both bound and ionized forms, with the latter being the most physiologically relevant and therefore strictly controlled. In tetrapods, the endocrine factors controlling Ca2+ and ionic phosphate (Pi) are well characterized and include PTH, calcitonin, and 1,25-dihydroxyvitamin D. PTH is perhaps the most important endocrine regulator of Ca2+ and Pi concentration in extracellular fluid. It is synthesized as a preprohormone, processed, and secreted as a linear peptide of 84 amino acids by cells of the parathyroid glands (85, 86). If the concentration of Ca2+ in blood drops below normal levels, PTH is secreted to restore normocalcemia, and its effect is counteracted by calcitonin. PTH accomplishes its task by 1) inducing the differentiation and activation of osteoclasts that mobilize Ca2+ and Pi from bone, 2) stimulating the production of 1,25-dihydroxyvitamin D or calciferol, which enhances the absorption of Ca2+
from the small intestine, and 3) stimulating tubular reabsorption of Ca\(^{2+}\) in the kidney, reducing Ca\(^{2+}\) loss in urine. In parallel, PTH acts in the renal tubule to decrease its P\(_i\) reabsorptive capacity. Thus, whereas P\(_i\) absorption from the intestine is efficient and minimally regulated, the concentration of P\(_i\) in the blood is reduced due to a PTH-stimulated loss in urine (85, 87).

PTHrP was discovered in association with certain types of cancer that caused elevated blood Ca\(^{2+}\) levels (a syndrome called humoral hypercalcemia of malignancy, or HHM) in affected patients. The hypercalcemia, caused by the uncontrolled secretion of PTHrP, is induced via an endocrine-like pathway and is a consequence of increased Ca\(^{2+}\) resorption from bone and suppression of urinary Ca\(^{2+}\) loss, similar to what is seen with hyperparathyroidism (105, 110). PTHrP and PTH share some sequence and conformational homologies between their 1–34 NH\(_2\) termini, which explains their ability to bind with equal affinity to a single common PTH/PTHrP receptor, PTH receptor type 1 (PTH1R; Ref. 40).

PTHrP is a multifunctional protein, produced in a range of different tissues, with broad physiological actions in mammals mediated via intracellular, paracrine, and endocrine pathways. Mammalian PTHrP is encoded by a single gene, but several variants are generated by alternative splicing of the primary transcript and through the use of alternative posttranslational cleavage sites. Posttranslational processing of mammalian PTHrP gives rise to at least three mature fragments: NH\(_2\)-terminal (1–36)PTHrP, structurally related to PTH; a midregion (38–94)PTHrP; and a COOH-terminal (107–139)PTHrP (62, 105). The processing pathways and activities of the various peptides arising from PTHrP still are not fully characterized in mammals and remain the subject of considerable study. Recent comprehensive descriptions of known and emerging actions of PTHrP are given by Clemens et al. (18) and Gensure et al. (41). The critical role of PTHrP for normal life is emphasized by the PTHrP or PTH1R knockout mice, which die at birth or in utero (67, 75).

**PTH AND PTHrP GENE AND PROTEIN STRUCTURE**

The mammalian and avian PTH gene comprises two introns that divide the gene into three exons encoding, respectively, 1) the 5’ untranslated region (UTR), 2) the signal peptide, and 3) the mature peptide plus the 3’ UTR. The homology among species is high in the region that codes for the prepro-PTH, and substantial homology is also retained in the gene flanking regions, introns, and mRNA UTRs (60). The structure of the recently identified pufferfish (20) and zebrafish (42) PTH genes is similar to that described for tetrapods, with three exons separated by two introns (42). In both zebrafish (z)PTH1 and zPTH2 genes, the first exon contains the 5’ UTR, the second exon is almost identical in size in both genes and comprises the remainder of the 5’ UTR and part of the preprosequences. The third exon initiates with the remainder of the presequence and continues with the mature peptide of just over 200 nt and the 3’ UTR. In zPTH1 and in its pufferfish homolog, (p)PTH1A, the last exon is much larger than in zPTH2 and the corresponding pPTH1B homolog due to the existence of a 3’ UTR that is three times longer (13, 42).

In general, the gene structure of PTHrP across the vertebrates is much more variable than that of PTH (60). Whereas the human gene has eight exons and seven introns and may produce several mature splice variants with combinations from three exons, the avian form has only four exons that translate sequentially into the mature peptide. However, there is high similarity of organization between the pufferfish PTHrP and the PTH genes in the zebrafish and other vertebrates. The pufferfish PTHrPA gene also has three exons separated by two introns, spanning 2.25 kb (108). The organization of exon II has been conserved in all species and encodes the end of the 5’ UTR and the prepro-PTHrPA sequence. Exon III of the pPTHrPA gene encodes a prohormone cleavage site (Arg–Arg), the mature protein (1–126), and the complete 3’ UTR. The cloning and sequencing of the sea bream PTHrP cDNA (33) confirmed there is a high level of nucleotide sequence conservation between the 5’ and 3’ UTRs in both fish (77.5 and 74.8%, respectively). A second PTHrP gene, pPTHrPB, identified in the pufferfish genome (13), shares 67% homology with pPTHrPA, and a further gene has been identified, designated pPTH-L, that is structurally more related to the PTH genes, particularly with regard to size, but encodes a mature protein containing motifs (e.g., MHD) that are more characteristic of the PTHrP genes (Fig. 1). In phylogenetic analysis, this novel gene fails to group with either the PTHrP or PTH clade (Fig. 2).

The teleost PTH and PTHrP amino acid sequences contain a hydrophobic presequence and a putative presequence, indicating that these are secreted peptides. The predicted mature zPTH1 and zPTH2 are 68 and 67 amino acids long, respectively (42), whereas the corresponding pufferfish pPTHA and pPTHB mature peptides are 80 and 62 amino acids, respectively (13, 20). Pufferfish PTHA and pPTHB share ~40% sequence conservation and 53% with the corresponding zebrafish PTHs (Table 1). Among the fish, human, or chicken mature PTHs, the amino acid identity is roughly 20–25%, although the sequence similarity is close to 40%. However, there is high sequence conservation of the first 34 NH2-terminal amino acid residues, and 10 residues are either identical or have undergone conservative substitutions across all PTHs (13). Among the conserved residues in the fish PTHs, several are critical for receptor binding and signal transduction (13, 20, 42).

The pufferfish, sea bream, and European flounder (Platichthys flesus) mature PTHrPAs are 126, 125, and 129 amino acids long, respectively, and share ~90% sequence similarity (Table 1, Fig. 1). In common with what occurs with PTHs, the first 34 amino acids are highly conserved with an average identity of 94 and 97% and similarity of 100% due to two conservative amino acid substitutions. Overall, the total amino acid identity between the piscine and mammalian PTHrP is low at ~36% but is higher (~60%) in the NH2 terminus (see PTH AND PTHrP RECEPTORS) and reaches 50 and 85%, respectively, if only the physicochemical nature of amino acids are considered (Table 1, Fig. 1).

The recently found mature pPTHrPB is 161 amino acids long and shares 63% similarity with pPTHrPA, 67% similarity with zPTHrPB, and 58% similarity or less with tetrapod PTHrPs (13). However, there are regions of high conservation across all the vertebrates, including 22 of the first 34 amino acids, which suggests they interact with the common PTH/PTHrP receptors. Other regions of high conservation are 11 of the 18 amino acids of the Arg-rich RNA binding region (1) and
7 of the 15 amino acids of the nuclear localization sequence (55). Comparison of the COOH-terminal region of PTHrPs reveals it is much shorter in fish than in tetrapods and that PTHrPA is the shortest (13). Finally, only the fish PTHrPs have an insertion of several amino acids starting after residue 37 (usually a conserved Arg; Fig. 1). Such insertion shows less conservation, and its size is longer in pPTHrPBs than in pPTHrPA (13).

All mammalian PTHrPs have regions that are potential sites of posttranslational processing by enzymatic cleavage, amidation, and glycosylation (94), generating several domains with distinct physiological functions. The fish proteins also have such properties in conserved regions [even if some sites are displaced due to the inserted sequence on position 37 (60)], but very little is known about the possible functional roles of the peptides that can arise from the processing of the full-length protein.

Fig. 1. Multisequence alignment of parathyroid hormone (PTH) and PTH-related protein (PTHrP) genes indicating the conservation of the physicochemical characteristics of the amino acid structure. Arrows indicate the beginning of each mature peptide, and the horizontal bar above each sequence shows the length of the 1–34-amino acid NH2 terminus. In both PTH and PTHrP, higher conservation occurs in the NH2 terminus, but only PTHrP shows considerable conservation among species throughout the several regions of the molecule. Note high conservation between PTH and PTHrP within the 1–34-amino acid NH2 terminus, namely, in residues 6–8, 24, 25, 28, and 31 and the conservation of the MHD motif (residues 8–10) in PTHrPs and PTH-L.
PTH AND PTHrP mRNA AND PROTEIN DISTRIBUTION

The initial studies of PTH-like protein localization in fish tissues used either bovine or human antisera to PTH (Table 2). These studies were generally confined to the central neuroendocrine systems: immunoreactivity was demonstrated in plasma and brain and in pituitary extracts of trout (Oncorhynchus mykiss), goldfish (Carassius auratus), salmon (Oncorhynchus kisutch), hake (Urophycis tenuis), and platyfish (Xiphophorus maculatus) (37, 54) and in sections of the hagfish (Eptatretus stouti) and goldfish brain and neurohypophysis (66,96). Similarly, the majority of studies of PTHrP have used human antisera and heterologous gene probes in a wide variety of fish, ranging from the ancient lamprey (Geotria australis) (22, 130, 131) to the more recently evolved teleosts such as the gilthead sea bream (19, 23) and the European flounder (21, 22) and covering several elasmobranch species (3, 22, 63, 131–133). In general, PTHrP protein and (or) mRNA expression are principally associated with the pituitary gland, nerve tissue, and tissues having important roles in ion exchange. In the lamprey, differences in PTHrP localization were noted among individuals at different stages of the life cycle, suggesting that the distributions of PTHrP, and possibly its roles, may change with the stage of development or the environmental conditions (130). In sharks and rays, PTHrP-like immunoreactivity and mRNA were present in several tissues (see Table 2), including the chondrocytes at the edge of the calcified cartilage and within the uncalcified cartilage in the vertebrae, a pattern resembling the PTHrP distribution in early endochondral bone formation in mammals (22, 131–133).

Availability of the sequence for the fish PTHrPA gene (31, 108) allowed the production of homologous antisera and fish-specific riboprobes, which confirmed the wide pattern of PTHrP expression in larval and adult teleosts. To date, there are no studies using homologous tools in elasmobranchs and ancient bony fishes. In the sea bream, RT-PCR has been used to detect transcripts in muscle, brain, intestine, skin, kidney, gill filaments, pituitary gland, and operculum, although the overall expression was low (33). Quantitative PCR indicates that expression levels in muscle are roughly 20-fold higher than in skin, brain, kidney, gill, or duodenum, and about eight-, four-, and two-fold higher than in pituitary, heart, and hindgut, respectively (53). In situ hybridization shows signal in epithelial cells of the hindgut, some renal tubule cells, and branchial mitochondria-rich cells. In the pituitary, gene expression is present in the pars intermedia cells of the posterior pituitary surrounding the neurohypophysis, and signal also is visible in some of the epithelial cells of the saccus vasculosus (33). A similar distribution is seen in pufferfish with the exceptions that the nonepithelial supporting cells of smooth and striated muscle, matrix cells of the epidermis, and basement membrane cells of the gill filaments also exhibit abundant gene expression with none detectable in the epithelial cells (108). It has been suggested that this may be related to a possible involvement of PTHrP in the process of expansion and relax-
Table 1. Percentage homology of PTH-like peptides among representative vertebrate species

<table>
<thead>
<tr>
<th></th>
<th>Homo sapiens</th>
<th>G. gallus</th>
<th>D. rerio</th>
<th>T. rubripes</th>
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<tr>
<td>PTHs</td>
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<td>Gallus</td>
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<td>Takifugu</td>
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<td>52 (76)</td>
<td>55 (82)</td>
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<td>Sparus</td>
<td>16 (36)</td>
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<td>Takifugu</td>
<td>47 (73)</td>
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<td>PTHrPs</td>
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<td>73 (82)</td>
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<td>84 (90)</td>
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<td>Danio</td>
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<td>Takifugu</td>
<td>35 (49)</td>
<td>36 (52)</td>
<td>45 (59)</td>
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| Values within each cell indicate protein identity and conserved similarity (in parentheses) for the full-length mature protein (upper value) or the 1–34 amino acid region (lower value). PTH, parathyroid hormone; PTHrP, PTH related protein.

- The distribution of the fish PTHs is still very poorly characterized. The genes were isolated from pufferfish genomic DNA and total zebrafish RNA, and therefore there is no indication of specific tissue source. RT-PCR in pufferfish T. fluviatilis tissues demonstrated the existence of pPTHB mRNA in pituitary, brain, kidney, ultimobranchial gland, and spinal chord but failed to amplify the signal for pPTH or PTH-L (13). Recently, the differential distribution of the two PTH genes and respective peptides in the developing zebrafish embryo using whole mount in situ hybridization and immunohistochemistry were described (57). In zebrafish embryos, at 24 h postfertilization (hpf), zPTH1 mRNA was dispersed in cells along the lateral line in the posterior of the animal and was subsequently present in a discrete zone of cells immediately dorsal to the notochord, and at 4 days postfertilization (dpf), it was observed in the midline of the neural tube. Gene expression of zPTH2 also was detected in two populations of cells running bilaterally on either side of the midline of the animal but not in central nervous tissue. Signal for the zPTH peptides was detected at 3 and 4 dpf in lateral line neuromasts and anterior neuromasts and in small cells dispersed in the anterior of the animal and the developing jaw, where it was located in the first region of the jaw cartilage to become calcified.

- The distribution of zPTHs is coincident in part to that previously observed for PTHrP in sea bream larvae and suggests an involvement of the zebrafish PTH-like peptides in the neural system and in early cartilage and bone development. The widespread distribution of PTHrP immunoreactivity and mRNA presence in ionoregulatory, secretory, and support tissues in different groups of fishes, including the ancient lamprey and elasmobranchs, and the changing pattern during ontogeny and(or) life cycle phases concur with the multifunctional role of this peptide that also may exert its effects in a paracrine manner. These questions can only be answered by...
### Table 2. Distribution of gene and protein expression of PTH-like peptides in tissues of several piscine species

<table>
<thead>
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<th>Phylogenetic Group and Species</th>
<th>Tissue Localization by IHC and In Situ Hybridization</th>
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<td>Petromyzontiformes</td>
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<tr>
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<td>Ceratodontiformes</td>
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<td>Neoceratodus forsteri</td>
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<td><strong>Carassius auratus</strong></td>
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Invited Review

R684 PTH-LIKE PEPTIDES IN FISH
functional studies that include targeting the receptors involved in mediating such roles.

**PTH AND PTHrP RECEPTORS**

PTH receptors are G protein-coupled members of the secretin receptor family that interact with the NH₂-terminal 34 amino acids of both PTH and PTHrP (PTH1R) or PTH only (PTH2R). The fact that the COOH-terminal regions of PTH and PTHrP have distinct functions from those mediated by the known PTH receptors suggests that other receptors may exist for these fragments (42).

Zebrafish and other teleosts possess, in addition to PTH1R and PTH2R, a third receptor, PTH3R, which probably arose from the duplication of PTH1R (118, 119).

Because piscine PTH1R and PTH3R were initially isolated from genomic DNA or from whole zebrafish RNA, little information is available about their expression profiles. In an early report, using a rabbit polyclonal antibody against human PTH1R, Akino et al. (3) identified immunoreactivity in sections and extracts of the choroid plexus and saccus vasculosus of the red stingray. The signal was located in the epithelial cells of both organs, and it was proposed that the ligand/receptor system may participate in regulation of the composition of cerebrospinal fluid. However, to date, there have been no follow-up studies, and nothing else is known about the receptors in elasmobranchs. In sea bream larvae, mRNA expression for the receptor is abundant in the chloride cells of the integument, in the axial muscle cells, and, to a lesser extent, in the eye (107). The highest expression of PTH1R, as determined by quantitative RT-PCR, has been found in the sea bream liver.
and gonad, followed by skin, brain, and pituitary (53). PTH1R also is present in scales (111, 116), whereas PTH3R has been detected in enterocytes (115), and both receptors are expressed in the interrenal glands (114). The PTH2R was initially isolated from catfish *Ictalurus punctatus* liver and kidney and from the zebrafish kidney (118). Whole mount in situ hybridization of zebrafish embryos showed intense transcript expression in the developing CNS at 48 and 72 hpf as well as low level expression in the vascular tissue, similar to its occurrence in mammals (see Ref. 98).

Binding and transactivation studies of zebrafish receptors transiently expressed in COS-7 cells revealed similarities between the fish and mammalian receptors (118, 119). Zebrafish PTH1R binds human (h)PTH and hPTHrP with higher affinity than pPTHrPA, but overall, the three ligands showed similar capability in displacing radiolabeled rat (r)PTH or hPTHrP. zPTH1R is activated (induction of cAMP production) with similar potency by hPTH, hPTHrP, pPTHrPA, and zPTH1, but with lower (≈40-fold) potency by zPTH2 (41, 118). Interestingly, both zPTH1 and zPTH2 elicit an identical response at the hPTH1R receptor, similar in potency to the hPTH and hPTHrP (41), which may result from evolutionary divergence of this receptor type in the marine and terrestrial environments, also suggesting that the two piscine PTHs may have distinct functions in fish. In sea bream scales, pPTHrPA stimulates PTH1R signaling through both the adenylyl cyclase-protein kinase A (AC-PKA) and phospholipase C-protein kinase C (PLC-PKC) signaling cascades. Although the NH₂-terminal truncated forms (2–34, 3–34, and 7–34) had no effect on cAMP release, they stimulated PLC-PKC with equal potency to the intact peptide (116). This may indicate that the first few NH₂-terminal amino acids are not necessary for receptor binding but are important for transactivation of at least the AC-PKA signaling cascade, which seems to be the main pathway in scales (see INVOLVEMENT OF PTH-LIKE PEPTIDES IN FISH Ca²⁺ and P₃ BALANCE AND TRANSPORT). The structure/activity relationship of piscine PTH1R is very similar to that of the human receptor (41, 65). Interestingly, hPTH and bovine (b) PTH, but not hPTHrP, also induce a mild increase in cAMP production in scales, whereas pPTH, pPTHB, and pPTHrPB are without effect. The pPTH-L, however, strongly stimulates cAMP production with a potency equal to that of the intact peptide (13). Sea bream recombinant (1–125)PTHrPA increases the cAMP production levels similar to those caused by (1–34)PTHrPA, confirming that the NH₂ terminus is the region responsible for the actions mediated by PTH1R (4).

The zPTH3R binds pPTHrPA with considerably higher affinity than hPTHrP (50-fold) and especially hPTH (50-fold). In transfected COS-7 cells, hPTHrP, pPTHrPA, and zPTH1 are equipotent in stimulating cAMP accumulation and more potent than zPTH2 (3-fold) and hPTH (10-fold). Whether zPTH1 and zPTH2 show differential affinity for zPTH1R or zPTH3R has not been determined. Enterocytes appear to express only PTH3R, which has an apparent of Kₐ of 2.59 nM for pufferfish [¹²⁵I]-labeled PTHrP(1–35 Tyr³⁵) (115). This is the same order of magnitude as for zPTH1R and zPTH3R when pufferfish [¹²⁵I]-labeled PTHrP(1–36 Tyr³⁶) was used to displace human [¹²⁵I]-labeled PTHrP(1–36 Tyr³⁶) (119). As observed for PTH1R, specific binding was identical among the intact and NH₂-terminal truncated forms. The sea bream midregion fragments (79–93)PTHrP and (100–125)PTHrP were ineffective. Only the (1–34)PTHrP fragment, but none of the other forms, stimulated cAMP accumulation in isolated enterocytes, an effect blocked by the AC inhibitor SQ-22536. The absence of stimulation of the PLC-PKC pathway by binding of NH₂-terminal peptides to PTH3R is an important difference from PTH1R and may result in diverse and specific physiological functions (119).

The sea bream interrenal tissue expresses both PTH1R and PTH3R, and (1–34)PTHrP stimulates a dose-dependent increase in cortisol release by isolated interrenal cells (114). (1–34)PTHrP also stimulates the production of cAMP and IP₃. Accordingly, upon PTHrP stimulation, AC and PLC inhibitors separately do not cause a significant reduction in cortisol release. However, in conjunction, cortisol release is maintained at unstimulated control levels. This indicates that, in this tissue, the physiological action of (1–34)PTHrP may be mediated by both the AC-PKA and the PLC-PKC pathways, through PTH1R or both PTH1R and PTH3R (114), whereas (7–34)PTHrP elicits only one-half the IP₃ accumulation and has no effect over cAMP. In addition, the NH₂-terminal truncated forms (2–34, 3–34, and 7–34) have no effect on cortisol release and, when used in conjunction with (1–34)PTHrP, significantly reduce its effects, confirming the importance of the initial amino acids for induction of the intracellular response but not for interaction with the receptor(s) binding site.

In common with observations in mammals, PTH2R in fish does not seem to be involved in the better known PTH functions. PTH2R was the first piscine PTH receptor to be identified, and in the initial cAMP accumulation studies in transfected COS-7 cells, the receptor did not respond to either human or piscine PTHrP but only to hPTH, and with much lower efficacy than PTH1R or PTH3R. Recently, it has been demonstrated that the zebrafish TIP39 induces cAMP accumulation in cells transfected with either the zPTH2R or the hPTH2R and that its potency is about one-half to one-third lower than that of the human TIP39 at the same receptors (98). The EC₅₀ of hTIP39 in stimulating the rPTH2R and the zPTH2R is similar to that of hPTH, but the absolute maximal response to hPTH is only 30–40% of that induced by hTIP39 (56). It would be of interest to compare the efficacies of zPTH1 and zPTH2 at the zPTH2R and how they compare to hPTH and to human and fish TIP39.

The importance of specific amino acid sequences in PTH/PTHrP ligands for receptor binding and activation have not been systematically determined in fish. However, based on sequence analysis and the few functional studies that exist, there is strong evidence for conservation of ligand binding and activation sites throughout the vertebrates (13). As with the human peptides, the fish (7–34)PTHrP is a strong antagonist of (1–34)PTHrP in binding and transactivation studies. Furthermore, for maximum activity, the terminal serine needs to be present, and all activity is lost in the 2–34 and 3–34 truncated peptides (114–116). In the studies reported above, the receptors’ affinity and response to the NH₂-terminal or full-length peptides were indistinguishable. It remains to be seen whether fish, like mammals (24), possess receptors that interact with the mid- and carboxyl regions of these peptides.
CONTROL OF PTH AND PTHrP SECRETION AND THE CaSR

Little is known about the mechanisms that regulate the PTH-like peptides in fish. In mammals and birds, the circulating levels of PTH are greatly altered by minute changes in blood Ca\(^{2+}\) concentration: in humans, the plasma levels of PTH can fall from 75 to 15 ng/l if the free Ca\(^{2+}\) concentration increases from 1.1 to 1.3 mM. A change from 1 to 1.5 mM can reduce PTH release from 100% to nearly 0% (11, 85). It is now well established that PTH secretion is modulated by the action of the Ca\(^{2+}\)-sensing receptor (CaSR) located in parathyroid gland cells. This is a G protein-coupled receptor characterized by seven transmembrane domains, a very large extracellular domain, and a cytoplasmic domain (11, 12). Ca\(^{2+}\) interacts with the extracellular domain and transactivates a series of intracellular signals that in turn alter the production and release of PTH by the cell. In hypercalcemic conditions, the CaSR inhibits PTH secretion. In hypocalcemic conditions, the receptor is unoccupied by extracellular Ca\(^{2+}\), resulting in a stimulation of PTH secretion (11, 16). The secretion and eventual release of PTHrP seems to be more complex because of its many paracrine functions, production sites, and target tissues. The stimulus for PTHrP mRNA production is of diverse origin. In contrast to PTH, PTHrP secretion also is stimulated by increasing Ca\(^{2+}\) concentrations in a number of normal and cancerous cell types via the modulation of the CaSR (17, 120–122). This receptor also is important in the modulation of PTHrP secretion for the maintenance of the placental-fetal Ca\(^{2+}\) transport (73). In smooth muscle cells of the stomach, uterus, and blood vessels, the mechanical stretch itself induces the production of the peptide, but other factors may be involved (9, 91, 127, 144). In the renal glomerulus, the increased activity of renin-angiotensin induces release of PTHrP (77, 95, 106). Treatment of cultured amniotic fluid cells with human prolactin, human placental lactogen, or human growth hormone increased PTHrP secretion after 24 h by 43, 109, and 90%, respectively. Insulin-like growth factors I and II, insulin, and epidermal growth factor also increased PTHrP secretion by 53, 46, 68, and 118%, respectively (25). In addition, a number of other growth factors, cytokines, and steroids have been shown to regulate PTHrP production. Dexamethasone, vitamin D, cortisol, estrogens, and androgens have been shown in vitro to inhibit PTHrP production (39, 44, 68, 79, 90, 100, 123, 136).

Of all the fish PTH-like peptides, only PTHrPA has been identified and quantified in plasma. In sharks, the information concerning PTHrP levels was obtained using heterologous RIAs. In the European dogfish (Scyliorhinus canicula) and the gummy shark (Mustelus antarcticus), the circulating values were ~9 pM (63, 132), whereas in the red stingray, an average concentration of 2.8 pM was detected (3), equivalent to those determined the presence of a hypercalcemic factor in the pituitary gland. Other tissue extracts with significant levels of PTHrP are the esophagus and surrounding tissue, the trunk kidney, and the head kidney, although PTHrP levels in these tissues are ~30–40 times lower than those in pituitary extracts. In elasmobranchs, as in teleosts and mammals, PTHrP mRNA and protein expression occur in structural tissues and transport epithelia (131, 133), but it seems that the pituitary may be an important source of circulating PTHrP with notable hybridization signals observed in the pars distalis and pars intermedia (63, 133).

Circulating levels of PTHrP in fish are influenced by environmental, ionic, and hormonal factors. Juvenile sea bream fed a Ca\(^{2+}\)-deficient diet for 6 wk had significantly increased levels of PTHrP (2). Similarly, fish fed a normal diet but exposed to lower salinity (a change from 10.5 to 0.7 mM Ca\(^{2+}\)) for identical periods also showed elevated PTHrP levels that were 1.5-fold higher than salinity and diet controls. Interestingly, fish deprived of Ca\(^{2+}\) sources, in comparison, maintained normal levels of PTHrP but showed reduced ionic Ca\(^{2+}\) in plasma and lower Ca\(^{2+}\) and P, accumulation, and they experienced growth arrest. Perhaps the maintenance of growth, thus the need for constant Ca\(^{2+}\) uptake, may in part explain the rise in PTHrP levels in hypocalcemic conditions (2).

Ca\(^{2+}\) deprivation also elevates PTHrP in the European flounder, a euryhaline teleost that moves between full seawater and salt water in estuaries. Chronically acclimated seawater and freshwater animals have similar plasma levels of PTHrPA and Ca\(^{2+}\) (142), but when held in deionized water for 14 days, the levels of PTHrP increased significantly. Furthermore, injection of a calcium chelator, EGTA, lowered plasma Ca\(^{2+}\) and significantly elevated plasma PTHrP 6–8 h later (142). Floun-
nder transferred from seawater to freshwater showed elevated PTHrPA levels (compared with fish transferred from seawater to seawater) within 24 h, whereas the change from freshwater to seawater slightly decreased PTHrPA at both 4 and 8 h after transfer (142). Changes in PTHrP immunohistochemical staining intensity also were observed in flounder kidney, gill, and pituitary from animals acclimated to freshwater compared with those in seawater (21). In elasmobranchs, environmental salinity did not seem to alter either circulating PTHrP or signal intensity in tissues (132). However, these measurements were performed after a 7-day adaptation period, and rapid, transient changes may have passed unnoticed. Altogether, the data suggest that Ca\(^{2+}\) availability, in either the diet or the environment, is an important factor regulating PTHrP secretion in fish and that the hormone may be used for rapid compensations in changing environments but also is necessary in long-term hypocalcemic situations.

Elasmobranchs and teleosts express the extracellular CaSR (29–31, 78, 89, 109) and apparently may use it not only to sense their internal milieu Ca\(^{2+}\) but also as a proxy for salinity conditions of their surrounding environment (58, 59, 89). There is, on average, little difference in blood Ca\(^{2+}\) levels between freshwater and seawater fish. However, immediately after transfer from one environment to the other, there may be significant changes in Ca\(^{2+}\) levels that are rapidly restored (71). Whether the integration of information from the environment leads to changes in hormone production and plasma Ca\(^{2+}\) levels per se is not known. The corpuscles of Stannius of the rainbow trout express the mRNA for the CaSR, and the use of calcimimetics, which increase the sensitivity of the CaSR to Ca\(^{2+}\), stimulates the release of stanniocalcin and inhibits the whole body Ca\(^{2+}\) influx in these fish (109). It is likely that a similar mechanism involving the CaSR may modulate the secretion of PTHrP and possibly that of the other PTH-like peptides in an as yet unidentified gland.

There also is strong evidence for the regulation of PTHrP in fish by estradiol. Vitellogenesis is the process through which the calcium/phosphate-rich protein vitellogenin is synthesized in the liver and incorporated into the oocyte. It has long been observed that during vitellogenesis, plasma calcium concentration rises severalfold such that vitellogenesis can be effectively monitored by the change in plasma Ca\(^{2+}\) level (43, 88). Estradiol administration induces vitellogenesis together with a large rise in the protein-bound fraction of circulating calcium, mobilization of Ca\(^{2+}\) from mineralized tissues (103), and increased Ca\(^{2+}\) influx (46), but a direct action of this steroid on the transporting mechanisms has not been demonstrated. PTHrPA levels also rise in response to estradiol and precede the rise in plasma Ca\(^{2+}\), suggesting it could mediate the calcitropic response to estradiol (52). Furthermore, this estradiol-induced hypercalcemia can be blocked by coinplantation of the (7–34)PTHrP antagonist (18, 41), a fact that strongly supports the hypercalcemic actions of PTHrP and its regulation by estradiol. Interestingly, in turn, recombinant (1–125)PTHrP potentiates the estradiol stimulation of vitellogenin production by sea bream hepatocytes in estradiol-primed fish but not in untreated fish (6), thus indicating an involvement of PTHrP in the process of fish vitellogenesis and a close relationship with this steroid.

PTHrP also seems to modulate the hypothalamus-pituitary-interrenal axis and possibly the stress response in fish. (1–

![Fig. 3. Plasma PTHrP (black) and cortisol (gray) relationship during confinement (*P < 0.05; #P < 0.05; significant differences from unstressed animals). During stressful events such as crowding and confinement, cortisol levels significantly rise, whereas PTHrP concentration decreases. Cortisol injection significantly reduces the amount of circulating PTHrP (13), suggesting the existence of a regulation of PTHrP secretion by this glucocorticoid.)
Ca\textsuperscript{2+} and P\textsubscript{i} Balance in Fish

Circulating Ca\textsuperscript{2+} is maintained under strict control, since slight changes may disturb a wide range of physiological mechanisms including neural, muscular, and cardiovascular functions, leading to tetany, lethargy, and, ultimately, death (35, 137). In vertebrates, total calcium is usually between 2 and 3 mM and Ca\textsuperscript{2+} is \( \sim 1.25 \) mM.

In fish, the branchial epithelium and the intestine are the major sites for Ca\textsuperscript{2+} uptake, which enters passively into epithelial cells down a concentration gradient and is then extruded into the blood by means of an active, energy-consuming, and rate-limiting step. Transport across the branchial or intestinal cell basolateral membrane is mediated via a Ca\textsuperscript{2+}-ATPase and( or) a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. In the intestine of seawater fish or during the digestion of calcium-rich food, a passive entry also may be relevant. Excretion of Ca\textsuperscript{2+} may occur passively in the gills and intestine via paracellular routes driven by the outward electrochemical force acting on Ca\textsuperscript{2+} in bony freshwater and seawater (35, 82, 101, 135). Regulated Ca\textsuperscript{2+} excretion occurs in the kidney, and it is mediated by the differential activity of Ca\textsuperscript{2+} pumps in the renal tissue (7). The kidney in freshwater fish produces dilute urine to counteract the osmotic inflow of water, and Ca\textsuperscript{2+} losses are minimized by means of active tubular reabsorption of ultrafiltered Ca\textsuperscript{2+} (8, 124). In seawater bony fish, the kidney has principally a Ca\textsuperscript{2+} excretory function, and tubular reabsorption of Ca\textsuperscript{2+} is reduced (113).

P\textsubscript{i} occurs in a variety of inorganic and organic compounds ubiquitously distributed in the tissues as an integral component of nucleic acids, as a substrate for kinase and phosphatase regulation of intracellular signaling, and for energy provision through ATP (5, 87). As in the case of calcium, large reserves of body P\textsubscript{i} are deposited in the mineralized tissues. Total and free P\textsubscript{i} circulating concentrations are very similar to those of calcium and also are very tightly controlled. Fluctuations in P\textsubscript{i} levels are closely associated with alterations in plasma Ca\textsuperscript{2+} concentrations.

Phosphate levels in both freshwater and seawater are generally very low, and animals must rely on dietary P\textsubscript{i} intake and renal excretion to remain in P\textsubscript{i} balance. Entry of P\textsubscript{i} across the fish intestine occurs mostly via a sodium-phosphate cotransporter (NaPi type II) located in the intestinal brush border, although some marginal diffusive uptake can occur. Circulating free phosphate is filtered at the glomerulus, and teleost kidney tubules, in contrast to mammalian tubules, may either reabsorb filtered P\textsubscript{i} or secrete excess blood P\textsubscript{i} into the urine (112). The underlying mechanisms for this are still unclear, but studies have indicated that in fish, the same or different forms of the NaPi-II cotransporter may be located in either the luminal and (or) the basolateral membranes, controlling reabsorption or secretion (72, 140).

**PTH-Like Peptides and Ca\textsuperscript{2+} and P\textsubscript{i} Transport**

The initial physiological studies indicating the possible action of a PTH-like substance in fish were first described in 1978 by Parsons et al. (99), when a factor in pituitary extracts from cod (Gadus morhua) and eel (Anguilla anguilla), which cross-reacted with mammalian PTH antiserum, was able to induce hypercalcemia in rainbow trout. Earlier studies had shown that hypophysectomy reduced plasma Ca\textsuperscript{2+} concentrations (36) and that a carp (Cyprinus carpio) pituitary extract would reestablish plasma Ca\textsuperscript{2+} in hypophysectomized killfish, Fundulus heteroclitus (97).

Since then, only a few studies addressing the physiological actions of PTH-like peptides in fish have been published. Injections of sea bream with high doses of (1–34)hPTHrP caused a reduction in whole body Ca\textsuperscript{2+} uptake (50), an effect similar to that observed in freshwater-adapted tilapia injected with (1–34)bPTH (138). However, the specificity and the biological significance of such observations is unclear, because the identity of the first 34 residues between the piscine PTHrPA and human PTHrP is only 57% (33, 108). Pufferfish (1–34)PTHrPA, which differs by only two amino acid residues

**Fig. 4.** Actions of PTHrP on Ca\textsuperscript{2+} and P\textsubscript{i} balance. A: Whole body Ca\textsuperscript{2+} fluxes in larval sea bream. PTHrP increases Ca\textsuperscript{2+} uptake and reduces Ca\textsuperscript{2+} excretion. [Adapted from Guerreiro et al. (49).] B: P\textsubscript{i} transport in renal proximal tubule cells. PTHrP has no effect on reabsorptive flux but greatly increases P\textsubscript{i} secretion by the renal cells in vitro. *P < 0.05, significantly different from control. Gray-shaded bars indicate the net flux resulting from the subtraction of influx/secretion and efflux/reabsorption. The results suggest that the PTHrP treatment transiently contributes to the retention of Ca\textsuperscript{2+} and loss of P\textsubscript{i}.
from the sea bream peptide, applied in bathing water caused a significant and dose-dependent increase in whole body Ca\(^{2+}\) uptake in sea bream larvae (49). This effect was achieved by both enhanced Ca\(^{2+}\) influx [1.6-fold increase at 10 \(\text{nM}\) (1–34)pPTHrPA] and a reduction in Ca\(^{2+}\) efflux (1.7-fold reduction at 0.1 \(\text{nM}\) and 2.5-fold at 1 and 10 \(\text{nM}\)) over a period of 4 h (Fig. 4A). Identical results were obtained with saltwater-but not with freshwater-acclimated tilapia (Oreochromis mossambicus) larvae, indicating a differential regulation possibly due to variations in endogenous levels in the two environments (48). Surprisingly, when the novel PTH-like peptides were tested for calcitropic effects in sea bream larvae, neither of the parathyroid orthologs, pPTHa and pPTHb, nor pPTHrPB had any effect on whole body Ca\(^{2+}\) uptake. However, pPTH-L elicited a dose-dependent increase in whole body Ca\(^{2+}\) influx rates, showing higher potency than pPTHrPA (13). It appears then that in both fish and tetrapods, two peptides of the parathyroid family have hypercalcemic activity: one is PTHrP, but the other is derived from different lineages in fish (PTH-L) and tetrapods (PTH).

Approximately 40–60% of the Ca\(^{2+}\) uptake by sea bream larvae comes from intestinal uptake in sea water (47, 49), and recent Ca\(^{2+}\) transport studies of sea bream intestine using Ussing chambers have demonstrated for the first time in fish that (1–34)pPTHrPA significantly increases Ca\(^{2+}\) absorption 2- to 3-fold while decreasing the electrical resistance of the intestinal epithelium, effects that vary with the region of the intestine (38). Transient increased intestinal Ca\(^{2+}\) uptake had previously been demonstrated in the perfused chick intestine (145).

Interestingly, in addition to an increase in whole body Ca\(^{2+}\) uptake, one other effect of the peptide was to reduce by 30% the drinking rates of sea bream and tilapia larvae in seawater but not in brackish water, where drinking rates were already low. The reduction in drinking implied that less Ca\(^{2+}\) was available for intestinal absorption and suggested an important action of (1–34)pPTHrPA on branchial Ca\(^{2+}\) uptake mechanisms. This was further supported by the results of a study in seawater tilapia in which the release of 45Ca\(^{2+}\) from isolated gill cells was accelerated upon addition of (1–34)pPTHrPA to the culture medium, an effect that occurs within seconds to a few minutes from the initial exposure (48). Addition of ouabain abolished the effects of (1–34)pPTHrPA on 45Ca\(^{2+}\) release, which suggests the increased Ca\(^{2+}\) transport is facilitated by sodium, probably via a Na\(^+\)/Ca\(^{2+}\) exchanger. A similar effect on Ca\(^{2+}\) release was observed in isolated basolateral membranes of human placenta cells incubated with (1–34)hPTHrP (28). In these cells, Ca\(^{2+}\)-ATPase is considered the main mechanism responsible for the placenta-to-fetus Ca\(^{2+}\) transport process, but studies indicate that the midregion peptide of hPTHrP is the main fragment responsible for the modulation of this action (125).

The mechanism of action of PTHrP on Ca\(^{2+}\) transport is largely unknown. To date, only PTHR1 has been identified in branchial tissue, by either RT-PCR or in situ hybridization, and it is not certain whether other PTH receptors are expressed. However, the effect of PTHrPA on intestinal Ca\(^{2+}\) transport appears to be mediated by PTH3R (38, 115). Sea bream enterocytes express PTHrPA and a single class of PTH receptors that biochemical and sequence analysis indicate is a PTH3R. The receptor \(K_d\) of 2.59 nM (115) is of the same order of magnitude as the measured PTHrP circulating levels, suggesting that upregulation of plasma hormone concentrations in response to a stimulus may have a direct action on this receptor. There also is widespread expression of PTHrP and PTH receptor genes in tetrapod intestinal epithelial cells (76). The described actions of the peptide in the mammalian gastrointestinal tract are mostly related to smooth muscle relaxation (18), but as indicated above, rapid stimulation of intestinal Ca\(^{2+}\) transport in avian duodenum also has been reported (145). Whether PTHrP has any relaxing effects on the muscle layers of the gastrointestinal tract in fish is unknown, but its presence in larval and juvenile intestinal smooth muscle cells (60) may indicate a paracrine action possibly activated by vasoactive intestinal peptide and its receptors (9), which are highly expressed in the pufferfish and sea bream duodenum (14, 15).

The kidney is potentially a prime target for PTHrP, since the excretion of Ca\(^{2+}\) and P\(_i\) is controlled at the renal level, and PTHrPA mRNA and protein have been detected in the renal tubules of larval and juvenile sea bream (33, 107). The reduction of Ca\(^{2+}\) efflux induced by pPTHrPA in sea bream larvae suggests that it may induce Ca\(^{2+}\) reabsorption in the kidney tubule as PTHrP and PTH do in the mammalian distal tubule. Furthermore, incubation with piscine PTHrPA enhances the cAMP production by dispersed European flounder renal cells, thus indicating transactivation of one or more types of receptors responsive to PTH-like peptides in this tissue (128, 129). However, although both PTHrPA and PTHR mRNAs are expressed in primary cultures of proximal tubule cells of the winter flounder (45), no secreted protein is measurable in cell culture medium, raising questions about its potential autocrine or paracrine function. Circulating levels in this species are similar to those measured in other fish, consistent with a classic endocrine function.

In Ussing chamber studies, (1–34)pPTHrPA induced a slight increase in Ca\(^{2+}\) transport from the luminal side to the peritubular side of primary monolayer cultures of the winter flounder renal proximal tubule epithelium within 60 min after addition of the hormone to the peritubular side (45). In the control situation, unidirectional secretory and reabsorptive fluxes were equivalent, resulting in a net flux close to zero. Winter flounder renal tubules consist for the most part of cells characteristic of proximal type I and type II. In mammalian renal tubules, active Ca\(^{2+}\) reabsorption occurs mainly across the cells of the distal tubule, which is not present in the winter flounder kidney nephron and thus not represented in the primary cell culture. This may explain the weak stimulatory effect of (1–34)pPTHrPA on Ca\(^{2+}\) transport in these preparations. Notably, exposure of the cell preparations to (1–34)pPTHrPA resulted in a significant increase in the net active P\(_i\) secretory flux across the cell monolayer (Fig. 4B) that lasted for at least 120 min. Whether this translates into an increase in the amount of P\(_i\), excreted in vivo remains to be seen, and the existence of alternative mechanisms involved in Ca\(^{2+}\) and P\(_i\) transport downstream of the proximal tubule (such as differentially oriented NaPi-II in the proximal tubule and in the collecting duct) cannot be ruled out. In addition, other renal processes, such as glomerular filtration rate, may change in parallel with proximal tubule ion transport, as previously reported for mammals (27).
The P$_r$ transport mechanisms altered by (1–34)pPTHrPA to bring about is not known. In this cell system, the activation of the PKC intracellular signaling cascade stimulates an increase of P$_r$ secretion (80). Treatment with bisindolylmaleimide (a PKC inhibitor) 30 min before the addition of the hormone significantly reduced (1–34)pPTHrPA-induced P$_r$ secretion. The effects observed on Ca$^{2+}$ and P$_r$ transport are in agreement with what has previously been described for the mammalian PTH and PTHrP, with the original particularity that, in fish, the P$_r$ secretory flux is enhanced in addition to the reduction of reabsorption typical of mammalian systems. This may occur because in addition to the apical membrane NaPi-II transporter responsible for P$_r$ reabsorption, the fish renal tubule expresses a form of the transporter in the basolateral membrane (26, 139). Incubation of isolated renal tubules with pPTHrPA for 3 h resulted in slightly lower expression of the NaPi-II protein in cell membranes, as evaluated by SDS-PAGE and Western blotting using specific mouse antisera (unpublished data). Again, this is consistent with the known actions of PTH on the renal PTH1R, which is thought to induce internalization of the membrane cotransporter via the production of cAMP (87).

**Ca$^{2+}$ Mobilization From Mineralized Structures**

Although there is still considerable debate on the cellular or acellular nature of fish bone and whether it functions as a Ca$^{2+}$ reservoir, it has been demonstrated that in periods of extra demand, such as during vitellogenesis, fish can mobilize Ca$^{2+}$ and P$_r$ from scales, where scleroclasts and scleroblasts bring about, respectively, the resorption and deposition of Ca$^{2+}$. The role of hormones in these processes is still largely unknown, but at least E$_2$ and calcitonin (102, 103, 126) are involved, respectively, in the activation and suppression of the tartrate resistant acid phosphatase (TRAP), an enzyme used as marker for mobilization activity in fish scale tissue and in mammalian bone.

With the use of an in vitro bioassay of fish scales, the actions of (1–34)pPTHrPA in teleost calcified structures were recently demonstrated (111, 116). Incubation of sea bream scales with (1–34)pPTHrPA resulted in reduced mRNA expression of the extracellular matrix protein osteonectin (OSN), a multifunctional metal-binding glycoprotein with low- and high-affinity Ca$^{2+}$-binding domains, which interacts with the collagen types I, III, V, and IV (111). Expression of OSN in scale tissue incubated for 4 h in 10 or 1,000 nM (1–34)pPTHrPA was reduced more than 20-fold compared with the control tissues. Expression of the genes for collagen I and V also was markedly reduced by exposure to the hormone. OSN expression was used as a marker of the osteoblastic response produced in periods of Ca$^{2+}$ deposition in ossified structures. Reduction of its expression in scleroblasts indicated that Ca$^{2+}$ was not being deposited in the extracellular matrix (64), a fact supported by the observation that the TRAP activity was significantly increased by 10 nM (1–34)pPTHrPA (a potency equivalent to 1,000 nM E$_2$). Together, these results on gene expression and enzyme activity strongly suggest a Ca$^{2+}$-mobilizing action of pPTHrPA in fish scales that is similar to that evoked by mammalian PTH and PTHrP in bone via the PTH1R. Whether PTHrPA acts similarly in fish bone is not yet known. Sea bream bone expresses PTHrPA and PTH1R, which is suggestive of a role in this tissue. The action of PTHrP in scleroclasts is mediated by PTH1R, specifically via the activation of an AC-cAMP signaling cascade, since the PLC-IP3 cascade, also activated by (1–34)pPTHrPA, was equally stimulated by (7–34)pPTHrPA that failed to alter the TRAP activity in sea bream scales (116). Furthermore, the use of SQ-22536 (an AC inhibitor) inhibited the stimulation of TRAP activity by (1–34)pPTHrPA, whereas in a similar experiment using U-73122 (a PLC inhibitor), no reduction of the (1–34)pPTHrPA-induced TRAP activity was observed. The sea bream recombinant (1–125)pPTHrPA is equipotent to pufferfish (1–34)pPTHrPA in stimulating cAMP production (4) and TRAP activity in scales (unpublished observations), indicating that there is no interaction of the mid- or carboxyl region with receptors that use the AC-PKA pathway in this tissue.

**EVOLUTIONARY PERSPECTIVES AND CONCLUDING REMARKS**

The existence of PTH-like peptides and their receptors in fish introduces new concepts and changes several of the previously established views on the evolution of the control of Ca$^{2+}$ balance in vertebrates. It is now clear that the origin of these hypercalcemic factors occurred before the transition to a terrestrial environment and not as an adaptation to a Ca$^{2+}$-depleted milieu as earlier thought (137). Furthermore, it is clear that PTH-like peptides are involved in many physiological processes not necessarily related to Ca$^{2+}$ regulation, as suggested by the ligand specificities of the PTH2R receptor, its relationship to TIP39, and the existence of receptors for the mid- or carboxyl region of PTH and PTHrP (24, 41, 56).

The fact that fish are surrounded by unlimited or readily available amounts of Ca$^{2+}$ and apparently lack parathyroid glands led to the initial arguments for the absence of hypercalcemic hormones, deemed redundant. However, some endocrines, such as cortisol (34) and estradiol (46, 103, 104), have been shown to increase Ca$^{2+}$ uptake, and therefore it seems that hypercalcemic factors are necessary at least in periods of high Ca$^{2+}$ demand. Moreover, fish do not effortlessly extract Ca$^{2+}$ from the environment but depend on energy-driven exchange mechanisms for Ca$^{2+}$ uptake, which must be regulated by endocrine factors.

Thus far, the experimental evidence (described in INVOLVEMENT OF PTH-LIKE PEPETIDES IN FISH Ca$^{2+}$ AND P, BALANCE AND TRANSPORT) indicates that PTHrP may be such a factor, acting on branchial, intestinal, and renal mechanisms. However, despite the good correlation between PTHrP and Ca$^{2+}$ levels in plasma (2) and additional corroborative studies (141, 142), it is still necessary to clearly determine if, and how, low plasma Ca$^{2+}$ stimulates PTH/PTHrP secretion and if, and how, high levels of these peptides increase circulating Ca$^{2+}$ in fish.

Nonetheless, the actions of PTH and PTHrP in Ca$^{2+}$ transport and mobilization have been conserved throughout the vertebrates. The actions of PTHrP on renal cells are in agreement with a hypercalcemic function. If the effects on scale (and possibly bone) resorption are considered, the result would be an increase not only in circulating Ca$^{2+}$ but also in blood P$_r$ levels, since both ions constitute most of the mineralized fraction of these tissues. Increased levels of plasma P$_r$ in parallel with hypercalcemia also have been observed in re-
response to elevated estradiol and vitellogenesis. Retention of PI (hyperphosphatemia) generally causes hypocalcemia in mammals due to excessive precipitation of calcium-phosphate in soft tissues, and there is no reason to doubt the possibility of a similar relationship in fish. The excess PI must therefore be excreted, since attempts to alleviate the hypocalcemia by increasing Ca\(^{2+}\) uptake, from either calcified tissues or the water, via branchial and (or) intestinal routes, will only lead to acceleration of calcium-phosphate deposition in the tissues if enough PI is available. The observations on renal PI transport in proximal tubule primary cultures seem to confirm this dual role in fish, indicating that the initial function of such peptides may have been directed to maintain not only Ca\(^{2+}\) balance but also PI homeostasis. To date, only a few potential phosphate-specific modulators are known (5), and at least one, fibroblast growth factor 23 (FGF23), is present in the zebrafish and pufferfish genomes (69). The interaction between these phosphatotropic factors and the PTH-like peptides as well as with the other calcitropic factors in fish, such as stanniocalcin and calcitonin, is a field unexplored and deserving attention.

A puzzle awaiting clarification is the apparent nonexistence of a parathyroid gland in aquatic vertebrates. Calcitropic factors such as stanniocalcin and calcitonin have their own sources well identified: the corpuscles of Stannius and the ultimobranchial gland, respectively. The relatively high level of circulating PTHrP found in fish blood suggests that a gland is still to be found. Developmentally, the mammalian parathyroid gland is derived from the pharyngeal pouch endoderm, and studies in mice have shown that its formation is under the control of the Gcm-2 gene. It recently was found that Gcm-2 is expressed not only in tetrapods but also in embryonic stages of the zebrafish and the dogfish within the pharyngeal pouches and internal gill buds that derive from them (93). In these studies, zPTH1 and zPTH2 and CaSR also were found to be expressed in the gills of adult fish, which prompted the authors to suggest that the tetrapod parathyroid gland and the gills of fish could be evolutionarily related structures, and that the parathyroid arose as a result of the transformation of the gills during tetrapod evolution (93). These studies showed no evidence of PTH expression in the developing gill, and in adult zebrafish neither zPTH1 nor zPTH2 were detected in any cells likely to correspond to a pharyngeal-derived parathyroid gland equivalent or in the thymus, indicating that another morphological transition may occur between the embryonic and the adult stages (57). It is not as yet known whether the PTHs and the PTHrPs have the same tissue origin. Some tissues are potential main sources, showing higher levels of gene and protein expression, but generally expression appears to be widespread (33, 107, 108).

The coincidence of PTH and PTHrP in the short arm of two evolutionarily related human chromosomes, 11 and 12, respectively, led to the initial suggestion that PTHrP may have derived from PTH (62, 105). However, these two peptides have rather different gene structures in tetrapods, and the same seems true in teleosts, indicative of an early differentiation that took place before, or in the initial stages of, the radiation of fishes. The discovery of a novel PTH peptide in fish, with intermediate primary structural characteristics between that of PTH and PTHrP, suggests that these two peptides may have evolved from a common ancestor and have undergone a process of gene duplication in teleosts. The same event may have originated the fish PTH1R and PTH3R seen in zebrafish and other teleosts. The appearance of two PTH and PTHrP ligands appears to have occurred after the development of distinct PTH, PTHrP, and TIP39 molecules (41). The physiological relevance of duplicated genes in general is a theme of considerable debate, and the persistence of two distinct PTH molecules, PTH1A and PTH2/B, which efficiently activate PTH1R and PTH3R, is intriguing. It also is interesting that only PPTHrPA and PPTH-L, the putative ancestral peptide, but not the PTHs, have calcitropic effects in the fish models tested and may indicate functional divergence has favored their persistence in the genome.

To date, only teleost genes and peptides have been characterized. In light of the recent discoveries, it is likely that at least PTH-L should be present in ancient fishes, such as lampreys, and in the cartilaginous fishes, such as sharks. Our knowledge of PTH-like peptides in elasmobranchs and lampreys is limited to in situ hybridization and immunological studies, either histological or serological. The heterology of the probes and antibodies that have been used in such studies does not exclude the hypothesis that several peptides exist but are being identified as a single signal. To clarify this point and extend our understanding of the evolutionary history of the PTH-like peptides, studies into the genome of such ancient fish (as well as invertebrates) are necessary.

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