Piscine PTHrP regulation of calcium and phosphate transport in winter flounder renal proximal tubule primary cultures

Pedro M. Guerreiro, Adelino V. M. Canario, Deborah M. Power, and J. Larry Renfro

1Centre of Marine Sciences, University of Algarve, Faro, Portugal; 2Department of Physiology and Neurobiology, University of Connecticut, Storrs, Connecticut; and 3Mount Desert Island Biological Laboratory, Salisbury Cove, Maine

Submitted 19 August 2009; accepted in final form 18 May 2010

Guerreiro PM, Canario AVM, Power DM, Renfro JL. Piscine PTHrP regulation of calcium and phosphate transport in winter flounder renal proximal tubule primary cultures. Am J Physiol Regul Integr Comp Physiol 299: R603–R611, 2010. First published May 19, 2010; doi:10.1152/ajpregu.00509.2009.—Multiple factors control calcium (Ca$^{2+}$) and inorganic phosphate (Pi) transport in the fish nephron, and the recently discovered members of the piscine parathyroid hormone-like protein family are likely participants in such regulatory mechanisms. The effects of an NH$_2$-terminal peptide (amino acids 1–34) of Takifugu rubripes parathyroid hormone-related protein, (1–34)PTHrP, on Ca$^{2+}$ and Pi transport were investigated in winter flounder (Pseudopleuronectes americanus) proximal tubule cells in primary culture (PTCcs). RT-PCR performed on RNA extracted from PTCcs and from intact kidney tissue indicated that expression of PTHrP and types 1 and 3 PTH/PTHrP receptors occurred both in vivo and in vitro and that circulating levels of PTHrP measured by specific radioimmunoassay averaged 2.5 ± 0.13 ng/ml. PTC monolayers were mounted in Ussing chambers, and under neutral electrochemical conditions, addition of 10 nM (1–34)PTHrP to the basolateral side induced a slight increase in Ca$^{2+}$ transport rate from luminal to peritubular side, significantly stimulating net Ca$^{2+}$ reabsorption. (1–34)PTHrP also significantly increased the Pi, secretory flux, and slightly reduced Pi reabsorption, evoking a significant increase in Pi net secretion. This stimulatory effect was partially inhibited by bisindolylmaleimide, an inhibitor of protein kinase C. Incubation of ex vivo flounder renal tubules with (1–34)PTHrP resulted in apparent reduction of Na$^+$-Pi, cotransporter type II (NaPi-II) protein in tubule membranes. PTHrP seems therefore to participate in the modulation of Ca$^{2+}$ and Pi homeostasis by fish kidney.

parathyroid hormone-related protein; renal transport; proximal tubule cell; Pseudopleuronectes americanus

Parathyroid Hormones are well known as major endocrine controls of calcium (Ca$^{2+}$) and inorganic phosphate (Pi) homeostasis in terrestrial vertebrates. The discovery of several parathyroid hormone (PTH)-like peptides in the fishes (8, 13, 23) has led to studies indicating that these hormones have physiological functions in the aquatic environment as well (27, 54). PTH-related protein (PTHrP) was the first of these peptides to be unequivocally identified and characterized in fish (12, 14, 20, 27, 31, 39). This peptide is involved in a multitude of processes in normal mammalian physiology, including the regulation of smooth muscle tone, tissue proliferation and organ development, and the modulation of transepithelial Ca$^{2+}$ and Pi transport (10, 38, 58). These functions are explained by the fact that PTH and PTHrP share high sequence similarity at the NH$_2$ terminus and, as a consequence, are able to bind to a common G protein-coupled receptor (37). Thus both are active in bone turnover and renal transport, promoting Ca$^{2+}$ mobilization and conservation, respectively, and reducing Pi reabsorption in the kidney tubules of mammals (10) while stimulating net secretion in reptiles and birds (9, 44).

In light of the actions of these peptides on Ca$^{2+}$ and Pi homeostasis in terrestrial vertebrates, it is likely that their presence in fish may reflect a similar regulatory role. Teleosts, either freshwater or marine, face a constant Ca$^{2+}$ influx from the environment, and compensation includes active excretion by several routes, including the urine (6, 25). Pi, however, is normally scarce in the aquatic environment, the only adequate source being the food, and therefore fish must balance the sporadic dietary intake with metabolic utilization and renal excretion lest Ca$^{2+}$ activity is perturbed (33, 41).

Our understanding of the endocrine factors and the transport mechanisms that regulate renal Ca$^{2+}$ and Pi balance in fish is incomplete. Salmon stanniocalcin (STC), an antihypercalcemic factor in fish, has no apparent effect on renal Ca$^{2+}$ transport but significantly stimulates Pi net reabsorption in a concentration-related manner from the luminal side to the peritubular side of polarized flounder renal proximal tubule primary cultures (36). Surprisingly, bovine (1–84)PTH produces a similar effect (36), and growth hormone, prolactin, and somatolactin all stimulate Pi reabsorption (35) likely via protein kinase A (PKA) activation. Fish, like most nonmammalian vertebrates (5), are capable of net renal Pi secretion, a phenomenon apparently regulated by protein kinase C (PKC)-mediated-pathways (41). However, the primary messenger stimulating renal Pi secretion in the fishes is unknown, and the roles of other classic hormones such as calcitonin and vitamin D in fish kidney Ca$^{2+}$ and Pi physiology are unclear (1, 21, 25, 26, 33, 36).

Calciotropic effects of synthetic Takifugu rubripes NH$_2$-terminal (1–34)PTHrP (39) were demonstrated in sea bream (Sparus auratus) larvae. Exposure to PTHrP produces a dose-dependent increase in whole body Ca$^{2+}$ uptake, a consequence of both increased influx and reduced efflux (26), but the mechanism of action and sites at which PTHrP acts to bring about these changes remain uncertain. Whereas in those in vivo experiments, increased uptake was mostly due to extraintestinal absorption (26), recent studies have shown that (1–34)PTHrP also stimulates Ca$^{2+}$ uptake across intestinal preparations in vitro (22), indicating that both gills and intestine are involved in the calciotropic action of this peptide in fish. However, whether the observed reduction in whole body Ca$^{2+}$ loss was due to increased renal reabsorption or decreased filtration and secretion in the nephron remains to be determined.
The objective of the present study was to investigate the effect of (1–34)PTHrP on renal transepithelial transport of Ca$^{2+}$ and P$_i$ by primary monolayer cultures of winter flounder (Pseudopleuronectes americanus) renal proximal tubule cells (pPTCs) mounted in Ussing chambers. In this in vitro system, the peptide stimulated renal P$_i$ secretion, reduced P$_i$ reabsorption, and promoted Ca$^{2+}$ reabsorption.

**MATERIALS AND METHODS**

**Animals.** Winter flounder, *P. americanus* (Walbaum, 1792), Pleuronectidae, were collected by otter trawl in Long Island Sound, offshore of Niantic, Connecticut, or in the Narragansett Bay, Rhode Island. Animals (250–400 g) were maintained in the laboratory in Living Stream Units (Toledo) filled with artificial seawater (Utikem) at 12°C. This animal research adhered to the American Physiological Society’s “Guiding Principles in the Care and Use of Animals” and the protocols approved by the University of Connecticut Institutional Animal Care and Use Committee (protocol no. A04-107).

**Solutions and chemicals.** Modified medium 199 with Earle’s salts (M199; Sigma Chemical, St. Louis, MO) was supplemented with (in mM) 30.0 NaCl, 4.2 NaHCO$_3$, 1.0 L-glutamine, 25.0 HEPES, 14.75 NaOH (pH 7.5), 347 mosmol/kg H$_2$O), and 20 mg/l tetracycline. Modified M199 was supplemented with 10% flounder serum to form the final plating medium. The Ca$^{2+}$- and Mg$^{2+}$-free solutions (CMF) used to remove extrarenal tissues contained the following (in mM): 150.0 NaCl, 4.0 KCl, 2.0 CaCl$_2$, 1.0 MgSO$_4$, 0.4 NaH$_2$PO$_4$, 4.2 NaHCO$_3$, 25.0 HEPES, 5.5 glucose, 1.0 l-glutamine, 1.0 t-glutamine, and 10% flounder serum to form the

**Primary culture of flounder renal proximal tubule epithelium.** The method used was first described by Dickman and Renfro (15) and later modified by Gupta and Renfro (28). Briefly, kidneys were perfused with modified M199 and dissected, and tubules were teased apart. Hematopoietic and lymphoid tissues were removed by brief incubation of tubules in CMF containing 0.2% trypsin at 22°C. Epithelial cells were released from tubule fragments by 3 days of trypsinization (5°C) in modified M199 (without supplement) supplemented with 0.05% trypsin. Dissociated cells were washed, suspended in complete culture medium, and plated on native (nondenatured) rat-tail collagen. Collagen gels were released after 4 days. After 12 days, epithelial monolayers had contracted from the initial 35-mm diameter to 17 mm and assumed the morphological structure, glucose transport capacity, and transepithelial resistivity consistent with fully differentiated trans-epithelial transport capabilities (43). Analysis of gene expression by RT-PCR in intact kidney and pPTCs. A kidney fragment or a single pPTC preparation containing ~10$^5$ cells was homogenized in TRI Reagent (Sigma) by following the manufacturer’s protocol. The integrity (A$_{260}$/A$_{280}$) and quantity of total RNA were assessed using a GeneQuant spectrophotometer (Pharmacia) and by agarose electrophoresis. Complementary DNA as synthesized from 5 μg of total RNA using 2 μl of poly(T) oligonucleotide (1 μg/μl; Promega); 8 μl of 5× RT buffer (GIBCO), 0.2 μl of RNase inhibitor (39 U/μl; Promega), 0.2 μl of MMLV reverse transcriptase (200 U/μl; GIBCO), 1 μl of dNTPs (10 mM; Promega), and 2 μl of DTT (0.1 M). PCRs were performed in a thermocycler (RoboCycler; Stratagene) using primers (see Table 2) designed based on sea bream, European flounder (*Platichthys flesus*), and puffer fish published sequences for PTHR, PTH/PTHrP receptor type 1 (PTH1R), and PTH/PTHrP receptor type 3 (PTH3R) and synthesized by MWG Biotech (Ebersberg, Germany) and Metabion International (Martinsried, Germany). The cycling conditions were 94°C for 3 min, followed by 25–35 cycles of 94°C for 1 min, 55–60°C for 1 min, 72°C for 1 min, and finally 72°C for 5 min. A 10-μl volume of each reaction product was then run on a Tris-borate-EDTA 1.5% agarose gel stained with ethidium bromide.

**Determination of transepithelial Ca$^{2+}$ and P, fluxes.** PPTCs were mounted in Ussing chambers with an aperture size of 0.332 cm$^2$ and fluid (FS) volume of 1.2 ml/hemichamber. Temperature was maintained at a constant 20°C, and fluid inside the chambers was constantly and vigorously stirred with small magnetic stir bars turned by external stir plates and insulated with humidified 99% O$_2$–1% CO$_2$. Transepithelial potential difference (TPD) was determined with Ag–AgCl electrodes connected to the luminal and peritubular (interstitial) compartments with 3 M KCl–2% agar bridges. Electrical properties were determined with a pair of computer-controlled, high-impedance automatic dual-voltage clamps (DVC 1000; World Precision Instruments, Sarasota, FL). Electrode asymmetry was corrected at the beginning and end of each experiment. Short-circuiting electrodes were connected to the luminal and peritubular solutions with 3 M

---

**Table 1. Multisequence alignment and similarity of the NH$_2$-terminal (1–34)PTHrP among the pufferfish (used in this study), two Pleuronectiforms, and the sea bream**

<table>
<thead>
<tr>
<th>Species</th>
<th>Mature NH$_2$-Terminal Amino Acid Sequence</th>
<th>Accession No.</th>
<th>%Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiger puffer fish</td>
<td>SVSHAQLMHDKGRSLQEFRRMVLHELLEEVITA</td>
<td>CAB94712</td>
<td>91</td>
</tr>
<tr>
<td>European flounder</td>
<td>SVSHAQLMHDKGRSLQEFRRMVLHELLEEVITA</td>
<td>CAG27316</td>
<td>91</td>
</tr>
<tr>
<td>European plaice</td>
<td>SVSHAQLMHDKGRSLQEFRRMVLHELLEEVITA</td>
<td>ABW97685</td>
<td>91</td>
</tr>
<tr>
<td>Gilthead sea bream</td>
<td>SVSHAQLMHDKGRSLQEFRRMVLHELLEEVITA</td>
<td>AAF79073</td>
<td>91</td>
</tr>
</tbody>
</table>

Sequences show amino acids 1–34 of the parathyroid hormone-related protein (PTHrP); shaded letters indicate the sites of (conservative) amino acid substitutions. Data for %similarity include 2 values: top value is the percentage of amino acid identity, and bottom value is the percentage of amino acid conservation.

---

AJP-Regul Integr Comp Physiol • VOL 299 • AUGUST 2010 • www.ajpregu.org
KCl-2% agar bridges. Transepithelial resistance (TER) was determined by the change in TPD produced by a brief 10-μA pulse controlled by the voltage clamps. TER was corrected for fluid resistance.

Tissues were continuously short-circuited during flux determinations. Preliminary experiments indicated there was no effect of (1–34)PTHrP from the apical side. Thus, in experiments reported in this article, the hormone was added to the peritubular (basolateral) side at the beginning of the 2-h flux measurement to a final concentration of 10 nM. Unidirectional tracer fluxes were initiated by the addition of 1.0 μCi of 45CaCl2 or H332PO4 to the appropriate hemichamber. Duplicate 50-μl samples were taken from the unlabeled side at 30-min intervals over a period of 2 h and replaced with equal volumes of unlabeled solution. The specific activity of the labeled solution was determined at the beginning and end of each experiment. Net flux was calculated as the difference between unidirectional secretory (peritubular to luminal) and reabsorptive (luminal to peritubular) fluxes. Microvillus to luminal fluxes were used to determine changes in amount of NaPi-II protein, since the hormone concentration is roughly 5–10 times higher than that reported in winter flounder whole kidney extracts (Fig. 1A). Whether these transcripts are translated to active proteins was not determined at this point, but their presence suggests that the renal tubule epithelium is a target for PTH-like peptides.

Gene expression studies. Via RT-PCR, PTHrP, PTH1R, and PTH3R transcripts were detected in RNA extracts from cultured fPTCs (Fig. 1A). The fact that these genes are expressed in fPTCs is additional confirmation of tissue differentiation and suitability as a model for studying the effects of this endocrine system, since transcripts for the same two receptors also exist in fPTCs (Fig. 1A). Whether these transcripts are translated to active proteins was not determined at this point, but their presence suggests that the renal tubule epithelium is a target for PTH-like peptides.

**RESULTS**

**PTHrP levels in fish and culture media.** Mean circulating values of PTHrP in winter flounder blood were 2.5 ± 0.13 ng/ml (~1 nM), similar to values reported for the sea bream and lower than those reported for the European flounder (49). PTHrP was not detected in medium collected at different time points from cell culture dishes.

**Gene expression studies.** Via RT-PCR, PTHrP, PTH1R, and PTH3R transcripts were detected in RNA extracts from cultured fPTCs (Fig. 1A). The fact that these genes are expressed in fPTCs is additional confirmation of tissue differentiation and suitability as a model for studying the effects of this endocrine system, since transcripts for the same two receptors also exist in winter flounder whole kidney extracts (Fig. 1B). Whether these transcripts are translated to active proteins was not determined at this point, but their presence suggests that the renal tubule epithelium is a target for PTH-like peptides.

**Effects of (1–34)PTHrP on transepithelial Ca2+ and Pi fluxes.** Figure 2A shows the time course of the effect of PTHrP (10 nM) on unidirectional transepithelial Ca2+ transport (flux in mol/h per surface area) by fPTCs mounted in Ussing chambers and maintained in flounder saline (control situation). The hormone concentration is roughly 5–10 times higher than the measured circulating values reported above. Values in

### Table 2. Primer sequences used for the amplification of PTHrP and PTHrP receptors types 1 and 3 from winter flounder tissues

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Pair</th>
<th>5′ → 3′ Orientation</th>
<th>Accession No.</th>
<th>Band Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PTHrP</strong></td>
<td>Fw: PTHrPFw1</td>
<td>GCT CTA TAG TAA TTC ATC ATT GG</td>
<td>AF197904a</td>
<td>475</td>
</tr>
<tr>
<td></td>
<td>Rv: PTHrPRv1</td>
<td>GCC GCC TAC TCT TCT TCT TCT C</td>
<td>AJ571691b AJ249391c</td>
<td></td>
</tr>
<tr>
<td><strong>PTH1R</strong></td>
<td>Fw: Rec1Fw1</td>
<td>TCA AGG CAA AGC ATA AGG</td>
<td>AJ550897c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rv: Rec1Rv1</td>
<td>ACA GGC GAT GAA AGA GC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PTH3R</strong></td>
<td>Fw: Rec3Fw1</td>
<td>CAC GCC AGG TGG GAA AG</td>
<td>AJ544578c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rv: Rec3Rv1</td>
<td>TGA CCG CCC GAC AGA T</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primer sequences for PTHrP and PTHrP receptors type 1 (PTH1R) and type 3 (PTH3R) were designed based on the nucleotide sequences available for sea bream (S. auratus). European flounder (P. flesus), and puffer fish (T. rubripes).
several species range from ~0.5 to 2.5 nM. The concentration used to obtain the results reported in this article was selected to yield maximal effects and because it was effective in other in vivo and in vitro assays (26). There was very low or no net Ca\(^{2+}\) transport in control flounder saline. Addition of PTHrP to the basolateral side had no effect on secretory flux but caused a significant increase in Ca\(^{2+}\) flux in the reabsorptive direction and altered net transport. Thus active net reabsorption was significantly higher in hormone-treated proximal tubule cells than in paired controls at 30, 60, and 120 min after addition of the hormone.

Unidirectional and net transepithelial P\(_i\) fluxes by fPTCs were clearly affected by (1–34)PTHrP over a 2-h exposure (Fig. 2B). Although individual cultures sometimes showed slight net reabsorption, average net flux from all control tissues was not significantly different from zero at any time point. (1–34)PTHrP treatment significantly increased active P\(_i\) secretion. This effect was apparent within 30 min and became progressively more pronounced over the 2 h of the experiment. The hormone also induced a reduction in unidirectional reabsorptive P\(_i\) transport that became statistically significant within 60 min and throughout the remainder of the experiment. The net result of the simultaneous increase in secretion and reduction of reabsorption was the induction of a highly significant increase in P\(_i\) net secretion.

Overall, during 2 h of exposure, (1–34)PTHrP (1) increased the amount of reabsorptive Ca\(^{2+}\) transport, inducing the reabsorption of 4.4 ± 1.95 nmol of Ca\(^{2+}\), and 2) increased the secretory transport of P\(_i\), inducing net secretion of 3.2 ± 0.60 nmol of P\(_i\) (shifts from averages of no net transport in controls).
There were no apparent changes in the general condition of the tissues caused by addition of either the peptide or tracer based on the stability of electrophysiological parameters recorded (TPD, TER, and $I_{\text{glu}}$) at the beginning and end of the experiments.

**Effects of PKC inhibitor on PTHrP-modulated $P_i$ fluxes.**

Prior studies (28, 41) indicated that activators of PKC, such as phorbol myristate and diacylglycerol, stimulated net $P_i$ secretion in fPTCs (see Table 3). It was hypothesized that if PTHrP had its effect through PKC activation, then the PKC inhibitor BIM ought to prevent the stimulation. The addition of 1 $\mu$M BIM (at $t = -30$ min) to the FS partially blocked the overall effect of subsequently added (1–34)PTHrP on $P_i$ transport (Fig. 3).

In cell preparations pretreated with BIM, exposure to PTHrP had little effect, and although unidirectional fluxes could not be statistically distinguished, on a paired tissue basis, the combined effect of shifts in the unidirectional fluxes abolished the statistically significant effect of (1–34)PTHrP on the net $P_i$ secretion by fPTCs. Over the 2-h transport period, 0.9 ± 0.37 nmol of $P_i$ were secreted by the BIM + (1–34)PTHrP-treated tissues compared with a total of 3.31 ± 0.63 nmol of $P_i$ by tissues treated with PTHrP alone. The concurrent net reabsorptive transport by the control fPTCs was 0.6 ± 0.92 nmol of $P_i$.

**NaPi-II cotransporter levels in renal tubule cell membranes in response to (1–34)PTHrP.**

The effect of PTHrP on the NaPi-II protein content is shown in Fig. 4, where the immunoreactive signal was shown at ~50 kDa under reducing conditions. This corresponds to the cleaved NH$_2$ terminus region of the protein as previously observed in rat kidney and avian proximal tubule cells with equivalent methods (17). The optical density of the immunoreactive signal was determined by densitometry; in two separate trials, the ratio between signal in lanes loaded with PTHrP-treated samples and control samples was 0.76 and 0.66. This was consistent with the PTHrP-induced reduction in unidirectional $P_i$ reabsorptive flux (see Fig. 2).

**DISCUSSION**

The potential role of the teleost kidney in calcium and phosphate homeostasis may be more predictable based on the present findings. PTHrP was shown to be present in winter flounder plasma, and transcripts of both PTHrP and two PTH/PTHrP receptors were present in the kidney proximal tubule epithelium. fPTCs provided an adequate assay system in which (1–34)PTHrP produced upregulation of net active $Ca^{2+}$ reabsorptive flux, stimulation of active $P_i$ secretion, and inhibition of $P_i$ reabsorption. The peptide’s effects were fully manifest after only 30 min and were maintained throughout the 2-h duration of the experiments.

Whether the effects observed in this in vitro system translate into changes in intact animals was not tested in this study, but in light of the apparent similarity to mammalian systems, it seems likely that PTHrP may have an important role in actively

---

**Table 3. Known factors influencing transepithelial $P_i$ transport by primary monolayer cultures of winter flounder proximal tubule cells**

<table>
<thead>
<tr>
<th>Ref.</th>
<th>$P_i$ secretion</th>
<th>$P_i$ reabsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>$P_i &gt; 0.5$ mM</td>
<td>$P_i &lt; 0.5$ mM</td>
</tr>
<tr>
<td>28</td>
<td>PKC activation</td>
<td>PKA activation</td>
</tr>
<tr>
<td>28</td>
<td>Diacylglycerol</td>
<td>Forskolin</td>
</tr>
<tr>
<td>29</td>
<td>Metabolic acidosis</td>
<td>Salmon GH and SL</td>
</tr>
<tr>
<td>29</td>
<td>Phospholipase C</td>
<td>Mammalian PRL</td>
</tr>
<tr>
<td>34</td>
<td>$\Delta P_i$</td>
<td>Stanniocalcin</td>
</tr>
<tr>
<td>35</td>
<td>(1–34)PTHrP</td>
<td>Bovine (1–84)PTH</td>
</tr>
</tbody>
</table>

GH, growth hormone; SL, somatolactin; PRL, prolactin. Data are adapted from Renfro (41).

---

**Fig. 3. Effect of bisindolylmaleimide (BIM), a PKC inhibitor, added to both the peritubular and the luminal sides, on (1–34)PTHrP-induced net $P_i$ transport by flounder primary monolayer cultures of proximal tubule cells in Ussing chambers.**

The significant increase in $P_i$ net secretion by 10 nM (1–34)PTHrP was partially abolished by 1 $\mu$M BIM. Tissues were exposed to BIM for 30 min before addition of hormone and radiotracer ($n = 5$). *$P < 0.05$. Negative values indicate reabsorption.
increasing plasma and reducing urine Ca\(^{2+}\) while decreasing plasma P\(_i\) levels and inducing phosphaturia in fish. Such a dual effect supports a predictable hypercalcemic action of PTHrP in increasing the availability of active Ca\(^{2+}\) in plasma. In this context, the present results are consistent with our previous observations using mineralized tissues, where PTHrP significantly induced the mobilization of Ca\(^{2+}\) from scales (40, 48) and reduced the expression of the extracellular matrix Ca\(^{2+}\)-binding protein osteonectin (40, 48).

In rat, human PTHrP caused a reduction of Ca\(^{2+}\) excreted and a rise in urinary P\(_i\), both in vivo and in isolated perfused kidneys (18, 59) while at the same time increasing Ca\(^{2+}\) and decreasing circulating P\(_i\) was observed in PTHrP-infused camels (45). In the present study, the effects of PTHrP on Ca\(^{2+}\) transport were particularly notable, since winter flounder renal tubule is mostly composed of proximal type II segments and lacks the distal tubule segment (16) usually associated with active Ca\(^{2+}\) regulation in terrestrial vertebrates (30). Conversely, most regulated P\(_i\) transport occurs in the proximal tubule I and II regions of the vertebrate neprhon (41). Well-established fish calcitropic factors, such as STC, have generally had weak or nonexistent effects on Ca\(^{2+}\) transport by fPTCs but strong, distinct effects on P\(_i\) transport (Table 3) (36).

Factors known to stimulate P\(_i\) secretion or reabsorption are shown in Table 3. Based on the information gained from the fPTC assay system, renal phosphate handling is influenced by the P\(_i\) concentration of the surrounding medium and factors that activate PKC or PKA. Several primary messengers that activate PKA and stimulate reabsorptive flux have been identified; however, until now there were no primary messengers indentified that activated the secretory process. The present study indicates that the most likely primary messenger stimulating teleost renal P\(_i\) secretion is PTHrP. The induction and increase of P\(_i\) secretion caused by PTHrP in the winter flounder renal system were associated with the activation of the PKC pathway (41) as shown by the fact that when fPTCs were exposed to the PKC inhibitor BIM, subsequent treatment with (1–34)PTHrP failed to stimulate P\(_i\) secretion.

PTH1R and PTH3R transcripts were detected both in winter flounder kidney and in cultured fPTCs. PTH3R is thought to be a fish-specific paralog of PTH1R, derived from a gene-duplication event (50). The full range of intracellular pathways triggered by these receptors in fish is still poorly characterized. Sea bream enterocytes and scales express only the PTH1R (47, 48), and incubation of scales with 10\(^{-8}\) M (1–34)PTHrP induces the accumulation of both cAMP and inositol 1,4,5-trisphosphate (IP3) and increases osteoclast-like activity (48). However, in enterocytes, 10\(^{-6}\) M (1–34)PTHrP increases cAMP levels and has no effect on IP3 levels (47). In sea bream interrenal tissue, where both PTH1R and PTH3R are present, (1–34)PTHrP stimulates cortisol release, an effect that could only be significantly reduced if inhibitors for both the PKC and PKA pathways were used together (46), suggesting that both signaling systems are activated and necessary.

Whether both PTH1R and PTH3R participate in the mediation of the (1–34)PTHrP effects on calcium and phosphate transport remains to be determined. The fact is that fish enterocytes, expressing only PTH3R, do not produce IP3 in response to (1–34)PTHrP, and since such a pathway is important for PKC activation and P\(_i\) secretion, PTH1R may be the main receptor type involved. Previous studies using zebrafish receptors expressed in COS-7 cells showed that human PTHrP and human PTH induce significant IP3 accumulation in cells expressing the PTH1R but not in those with the PTH3R (50). These studies also demonstrated that T. rubripes (1–36)PTHrP activates PTH3R with more potency than PTH1R, showing significant cAMP production already at 10\(^{-11}\) M, and whereas the PTH3R interacted preferentially with PTHrP, the PTH1R was similarly stimulated by fish PTHrP, human PTHrP, and human PTH. Clearly, more studies are necessary to characterize the relative importance of these two receptors in fish renal calcium and phosphate transport.

The ion transport mechanisms that respond to PTHrP in fish are still to be characterized. In mammals, PTHrP acts on PTH1R to increase luminal Ca\(^{2+}\) influx via apical Ca\(^{2+}\) channels in the distal tubule and by increasing the activity of the basolateral membrane Na\(^{+}/Ca^{2+}\) exchanger and Ca\(^{2+}\)-ATPase (30). Na\(^+\)-gradient-driven mechanisms for Ca\(^{2+}\) transport in teleost kidneys have not been found (4, 42). Although the mechanism of P\(_i\) secretion in cultured fPTCs and in vivo by proximal tubule cells has not yet been fully determined (41), the exit pathway, cell-to-tubule lumen, is influenced by apical membrane electrical polarity and regulated in part by Ca\(^{2+}\)-activated K\(^{+}\) channels (34). There is Na\(^+\)-driven P\(_i\) uptake by teleost renal cells, and fish are thought to have only a single type of cotransporter, most similar to SLC34A2 (NaPi, type IIb; Refs. 24, 32, 55). Two separate intestinal and renal isoforms have been found (51, 52), but indication that a SLC34A1 (NaPi, type IIA) exists at least in the stickleback (Gasterosteus aculeatus) and the medaka (Oryzias latipes) was recently obtained from molecular databases (51). The presence of fish NaPi-II in the apical membrane has been confirmed by immunocytochemistry signal and is consistent with the uptake of P\(_i\) into the cell (19, 32). However, with the same technique, high abundance of immunoreactive signal for a basolateral NaPi-II in fish has also been demonstrated (19). If this basolateral cotransporter faces inward, that would provide a mechanism favoring secretion and possibly allow the cell to transport P\(_i\) in opposite directions. In fact, most of the basolateral immunoreactivity was found in the proximal segment II, whereas the higher abundance of apical signal occurred in the collecting tubule (19), suggesting that reabsorption of P\(_i\) can also occur distally to the proximal tubule. Nonetheless, it was also indicated that, regardless of whether in a reabsorptive or a secretory state, fPTCs always express NaPi-II-related mRNA (55).

Incubation with (1–34)PTHrP slightly decreased the presence of immunoreactive NaPi-II in the renal cell membranes as shown by Western blot. It is not known if the reduction is significant in relation to ion transport or whether it occurs in the apical or the basolateral membrane or in both. Internalization of apical NaPi-II, a known effect of PTH in mammals (3), would explain the reduction in the reabsorptive capacity of the fPTCs when treated with PTHrP, but a basolateral NaPi-co-transporter (19) might be necessary for the increased secretion observed. Regardless of the possible impact of membrane transporter internalization, the rapid actions of PTHrP in changing the fluxes of both ions indicate that the initial response is due to increased active transport activity.

The circulating levels of PTHrP measured in winter flounder plasma were very similar to those identified in the sea bream,
and curiously, only half that of its closer relative, the European flounder (49). Although the former two fish species are only partially euryhaline and do not survive in very low salinities, the latter can successfully adapt to freshwater, and it was previously observed in the European flounder that abrupt transfer from seawater to freshwater, or vice versa, would induce a rapid change the PTHrP levels (57). These observations and the relatively high circulating PTHrP in fish suggest an endocrine regulation of the Ca\(^{2+}/P_i\) balance. In mammals, the paracrine actions of PTHrP also change renal function by reducing renal vascular resistance, renal blood flow, and glomerular filtration rate (10). PTHrP may also have a role in kidney maturation and glomerular development, since it causes proliferation of tubular and mesangial cells (10). Whether these actions also occur in fish remains unknown. However, and despite the fact that no immunoreactive protein was found in the conditioned culture medium, the fPTCs produced PTHrP mRNA, also previously detected in sea bream kidney tubules by in situ hybridization (20). This suggests that paracrine/autocrine or intracrine activity may occur in this tissue to mediate this and perhaps other functions not directly related to ion excretion.

Perspectives

Inappropriate increases of plasma P_i (hyperphosphatemia) generally cause hypocalcemia due to excessive precipitation of calcium phosphate in soft tissues, including muscle (53), as well as bone and scales. This is a result of the calcium × phosphate ion product, and thus regulation of Ca\(^{2+}\) and P_i are inextricably linked. Although P_i is a vital nutrient necessary for metabolic control and acid-base balance, in excess it must be removed, since attempts to alleviate hypocalcemia by increasing Ca\(^{2+}\) uptake or mobilization will only lead to acceleration of calcium phosphate deposition in the tissues. Therefore, the fishes, which live in an inexhaustible supply of Ca\(^{2+}\) (seawater concentration is 10 mM) with a continuous inwardly directed Ca\(^{2+}\) gradient, must still mount a regulatory response to P_i surfeit.

Little is known about factors controlling P_i in fish, but is accumulating data on the presence of putative calcio- and phosphatotropic factors in the fishes genomes (27 and references therein). PTHrP belongs to an intricate system of peptides and receptors for which functional information is still scarce (27). In fish, PTHrP’s effects appear to be through activation of both PTH1R and PTH3R. Mammals, however, lack the PTH3R, an indication of differential evolution, and thus exploring these diverse models is relevant to determine whether important differences exist and how Ca\(^{2+}\) and P_i regulation mechanisms evolved among vertebrates. Our hypothesis was that PTHrP is a normal regulator of renal function and that it may be summoned in periods of high need to promote Ca\(^{2+}\) retention. The in vitro model used in the present study strongly supports a role for PTHrP in the rapid renal regulation of plasma Ca\(^{2+}\) and P_i in fishes, substantiating our previous observations that a functional parathyroid-like system evolved before the colonization of land by vertebrates. Such a system, together with the antihypercalcemic peptide STC and calcitriol, equipped fish with mechanisms to thrive in a wide range of Ca\(^{2+}\) concentrations (25) and still keep balance with the low environmental P_i. Clearly, additional studies on the interaction between such systems are justified, and further information should be gathered from in vivo experiments under different Ca\(^{2+}\) and P_i conditions to determine actual excretion levels and hormone secretion and clearance rates. Nonetheless, a multifunctional renal role is already patent. In addition to PTHrP’s involvement in influencing renal mechanisms leading to xenobiotic excretion (56) and in increasing renal calcitriol metabolism (2), our present results indicate its ability to evoke ion transport across the kidney tubule and underline the importance of fish kidney in electrolyte control.

ACKNOWLEDGMENTS

We offer many thanks to Sonda Parker and Elisabete Caldas for skillful technical assistance.

GRANTS

P. M. Guerreiro received Foundation for Science and Technology-Portuguese Ministry of Science Grant SFRH/BD/4646/02. J. L. Renfro was funded by U.S. National Science Foundation Grants IBN-0416954 and IOS-0843253. Travel grants from the Calouste Gulbenkian Foundation, Luso-American Development Foundation, and Journal of Experimental Biology are gratefully acknowledged.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


