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Stimulation of Cortisol Release by the N Terminus of Teleost Parathyroid Hormone-Related Protein in Interrenal Cells *in Vitro*

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The mode of action of PTHrP in the regulation of sea bream (*Sparus auratus*) interrenal cortisol production was studied *in vitro* using a dynamic superfusion system. Piscine (1–34)PTHrP (10^{-6}–10^{-11} M) stimulated cortisol production in a dose-dependent manner. The ED_{50} of (1–34)PTHrP was 2.8 times higher than that of (1–39)ACTH, and maximum increase in cortisol production in response to 10^{-8} M of (1–34)PTHrP was approximately 7-fold lower than for 10^{-8} M of (1–39)ACTH. In contrast to (1–34)PTHrP, piscine (10–20)PTHrP, (79–93)PTHrP, and (100–125)PTHrP (10^{-9}–10^{-7} M) did not stimulate cortisol production. The effect of piscine (1–34)PTHrP on cortisol production was abolished by N-terminal peptides in which the first amino acid (Ser) was absent and by simultaneous addition of inhibitors of the adenylyl cyclase-protein kinase A and phospholipase C-protein kinase C intracellular pathways but not by each separately. The PTHrP-induced signal transduction was further investigated by measurements of cAMP production and [H^3]myo-inositol incorporation in an interrenal cell suspension. Piscine (1–34)PTHrP increased cAMP and total inositol phosphate accumulation, which is indicative that the mechanism of action of PTHrP in interrenal tissue involves the activation of both the adenylyl cyclase-cAMP and phospholipase C-inositol phosphate signaling pathways. These results, together with the expression of mRNA for PTHrP and for PTH receptor (PTHR) type 1 and PTHR type 3 receptors in sea bream interrenal tissue, suggest a specific paracrine or autocrine steroidogenic action of PTHrP mediated by the PTHRs. (*Endocrinology* 146: 71–76, 2005)

PTHrP IS A protein that shares sequence similarity to the N terminus of PTH, a product of the parathyroid gland and a key hormone in calcium metabolism in mammals. PTHrP is a paracrine factor that is produced in a range of different tissues and has a wide spectrum of biological activity. Posttranslational processing of PTHrP, which contains multibasic endoproteolytic sites (multi-K/R), has been shown to give rise to amino-terminal, mid-region, and carboxy-terminal mature secretory forms of PTHrP (1). The processing pathways and activities of the various peptides arising from PTHrP are still not fully characterized in mammals and remain the subject of considerable study.

PTHrP has been identified in most vertebrate groups, including teleost fish in which a PTHrP-like protein was identified in tissue and plasma, and subsequently, the cDNA and gene were isolated from sea bream (*Sparus auratus*) and the puffer fish (*Fugu rubripes*), respectively (2, 3). Moreover, a PTH-like factor has also recently been identified in fish (4), which shares 59% identity with PTHrP isolated from puffer fish, sea bream, and flounder (*Platichthys flesus*). Multiple sequence alignment of the amino acid sequence of PTHrP from fish and mammals indicates that they share 52% identity in the N-terminal region [(1–34)PTHrP] (2), raising the question of whether there is conservation of function.

PTHrP brings about its biological activity by binding to the G protein-coupled, PTH receptor (PTHR), and stimulates the adenylyl cyclase (AC)-cAMP and phospholipase C (PLC)-inositol phosphate (IP) signaling pathways (5). Of both the PTHRs cloned in human, only PTHR type 1 (PTH1R) responds to PTHrP and strongly activates the AC-protein kinase A (PKA) signaling pathway and, to a lesser extent, the PLC-protein kinase C (PKC)-intracellular Ca^{2+} signaling pathway (6). Recently, three different teleost PTHRs [(PTH1R, PTHR type 2 (PTH2R), and PTHR type 3(PTH3R)] were identified in zebrafish (*Danio rerio*), and receptor activation was characterized *in vitro* (7, 8). However, PTHRs have still not been isolated from any other teleost, and the tissue distribution and binding characteristics of the native receptors to fish PTHrP remains to be characterized.

In mammals, PTHrP has recently been reported in various tissues of the hypothalamus-pituitary-adrenal axis (9). The interaction between hypothalamic CRH and pituitary ACTH regulates cortisol production (10). Although a range of other hormones and peptides also modulate this process, PTHrP was recently shown to stimulate cortisol release from dispersed human adrenocortical cells (11). Moreover, in mammals, glucocorticoids regulate the expression of the gene encoding PTHrP (12). The teleost fish interrenal gland is homologous to the mammalian adrenal gland, and in both, the basic structure is made up of cords. However, whereas

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Abbreviations: AC, Adenylyl cyclase; CIP, corticotropin-inhibiting peptide; IMBX, 3-isobutyl-1-methylxanthine; IP, inositol phosphate; PIP, phosphoinositide phosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PTH1R, PTH receptor type 1; PTH2R, PTH receptor type 2; PTH3R, PTH receptor type 3; PTHR, PTH receptor.

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in mammals, a central medulla of chromaffin tissue is surrounded by a zoned adrenal cortex, in fish, adrenocortical cells are intermingled with chromaffin cells (13). The peripheral zona glomerulosa of the adrenal gland produces aldosterone, whereas a zona fasciculate produces glucocorticoids, including cortisol. Aldosterone is not commonly detected in teleost fish (13). The presence of PTHrP in the brain, pituitary gland, and interrenal tissue of the sea bream (2, 14) raises questions about its potential involvement in control of cortisol production in fish. In the present study, the capacity of N-terminal peptides of piscine PTHrP to stimulate cortisol release from sea bream interrenal tissue is established. To characterize the corticotropic activity of PTHrP and the amino acid residues important for receptor activation in this tissue, several N-terminal truncated forms of piscine PTHrP are used. The PTHR types present in piscine interrenal tissue and the intracellular signaling mechanisms activated after PTHR binding are established.

Materials and Methods

Chemicals

Piscine (1–34)PTHrP, (2–34)PTHrP, (3–34)PTHrP, (7–34)PTHrP, (10–20)PTHrP, (79–93)PTHrP, and (100–125)PTHrP were synthesized by Genemed Synthesis, Inc (San Francisco, CA). Human (1–39)ACTH, corticotropin-inhibiting peptide (CIP), phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine, IMBX), PLC inhibitor (U-73122), BSA, MEM, collagenase/dispase 2-phenoxyethanol, and other laboratory reagents were purchased from Sigma-Aldrich (Madrid, Spain). SQ-22536, an AC inhibitor, was obtained from Calbiochem (La Jolla, CA). The RIA kit for cAMP and [H^3]myo-inositol (specific activity, 629 GBq/mmol) were purchased from Amersham Biosciences (Little Chalfont, UK), and an in-house assay was used to measure cortisol.

Fish

Sexually immature gilthead sea bream (*S. auratus*), weighing 80–120 g, were maintained at the Ramalhete experimental station of the University of Algarve (Faro, Portugal) under constant environmental conditions; the photoperiod was 14 h of daylight and 10 h of dark, and the water temperature was 22 ± 1 C. Fish were kept in 5 m^3 seawater tanks in an open circuit, which received a continuous flow (250 $cm^3{\cdot}h^{-1}$) of well-aerated seawater. Tanks were stocked at a density of 4 $kg{\cdot}m^{-3}$, and fish were fed twice daily at 0900 h and 1800 h with 1% body weight commercial dry pellets (PROVIMI, Lisbon, Portugal). Food was withheld from fish for 24 h before sampling.

In vitro superfusion

All fish belonging to the same sampling group were rapidly captured and anesthetized with an overdose of 2-phenoxyethanol (1:1000 vol/vol). Intact head kidney was quickly dissected from each fish placed in superfusion chambers and superfused with buffered HEPES Ringer solution (HEPES, 15 mM; pH 7.38) containing NaCl (171 mM), KCl (2 mM), $CaCl_2/2H_2O$ (2 mM), 0.25% (wt/vol) glucose, and 0.03% BSA (wt/vol). This medium was pumped through the superfusion chambers at a rate of 75 $\mu l{\cdot}min^{-1}$ by means of a multichannel peristaltic pump (Masterflex; Cole-Palmer, Vernon Hills, IL) and fractions of 1.5 ml were collected in ice. The whole system was maintained at a constant temperature of 18 C. Previous studies with this system indicated that cortisol reaches stable basal levels after 3 h of superfusion (14). Therefore, when a steady basal state was reached, tissue was stimulated for 20 min with the different treatments applied. To characterize the dose-response curves of cortisol production for different regions of the PTHrP molecule, the head kidney was perfused with the following peptides: *Fugu* and flounder (1–34)PTHrP from 10^{-6}–10^{-12} M; sea bream (10–20)PTHrP from 10^{-7}–10^{-9} M; sea bream (79–93)PTHrP from 10^{-7}–10^{-9} M; and sea bream (100–125)PTHrP from 10^{-7}–10^{-9} M. The overall cortisol synthetic capacity of the head kidney was determined by perfusing 10^{-8} M human (1–39)ACTH. The effect of N-terminal truncated forms of sea bream PTHrP on cortisol production by the head kidney was determined by comparing the production rates when perfusing with 10^{-8} M *Fugu* (1–34)PTHrP, 10^{-8} M *Fugu* (2–34)PTHrP, 10^{-8} M *Fugu* (3–34)PTHrP, and 10^{-8} M *Fugu* (7–34)PTHrP. To establish whether the cortisol stimulatory activity of PTHrP was occurring through its binding to the ACTH receptor or through binding to a PTHR, 10^{-8} M *Fugu* (1–34)PTHrP was perfused alone or in the presence of 10^{-6} M of CIP, *Fugu* (2–34)PTHrP, *Fugu* (3–34)PTHrP, and *Fugu* (7–34)PTHrP. In a final group of experiments aimed at establishing the principal signaling pathway used by PTHrP in the head kidney, *Fugu* (1–34)PTHrP (10^{-8} M) was used to perfuse a head kidney preparation alone or in the presence of a PLC inhibitor (10^{-4} M SQ-22536) and/or an AC inhibitor (10^{-5} M U-73122).

The cortisol released in the head kidney fractions was determined by RIA and expressed as nanogram of cortisol per gram of head kidney per hour or as percentage of basal release, considering as 100% basal the release in the 30 min preceding the application of a stimulus.

Interrenal cell suspension

The preparation of an interrenal cell suspension for the measurements of cAMP and [H^3]myo-inositol incorporation was carried out as previously described (15). In brief, the head kidney of individual fish was removed and placed in a culture dish containing complete medium (MEM supplemented with 5 $g{\cdot}liter^{-1}$ BSA; 2.2 $g{\cdot}liter^{-1}$ $NaHCO_3$; 2 $g{\cdot}liter^{-1}$ NaCl, pH 7.4; 348 mosmol). The tissue was washed with complete medium, resuspended in fresh complete medium containing 2 $mg{\cdot}ml^{-1}$ collagenase/dispase, and incubated for 15 min with gentle agitation. The solution was then filtered with a 60-μm mesh cloth, and the filtrate was centrifuged at 1500 rpm for 5 min. The pellet was resuspended in complete medium, and the cells were counted using a hemocytometer. Cell viability estimated by tryptan blue at the end of the isolation procedure was 92 ± 4.6%.

Measurement of cAMP production

To measure cAMP, isolated interrenal cells were plated into 24-well sterile culture plates (Costar, Corning Incorporated, Corning, NY) at 2×10^6 $cells{\cdot}well^{-1}$ in 1 ml complete medium. After 30 min of preincubation in complete medium containing 1 mM IMBX, cells were incubated for an additional 30-min period in complete medium-IMBX in the presence or absence of 10^{-8} M *Fugu* (1–34)PTHrP or 10^{-8} M *Fugu* (7–34)PTHrP. Culture plates were then centrifuged, the medium was aspirated, and cells were resuspended in 0.01 M PBS with 4 mM EDTA. Subsequently, the cells were sonicated, and aliquots were taken for protein determination. Thereafter, samples were boiled and centrifuged at 5000 rpm for 5 min at 4 C. The supernatant was removed and stored at −20 C. cAMP concentration was determined by RIA using a kit (TRK 432; Amersham Biotrak). Results are reported as picomoles of cAMP per milligram of total protein. Total protein was determined by the Lowry method (16).

Measurement of [H^3]myo-inositol incorporation

[H^3]myo-inositol incorporation was determined following the method described by Ramirez *et al.* (17) with small modifications. Interrenal cells were plated in 24-well plates at 2×10^6 $cells{\cdot}well^{-1}$ in 1 ml complete medium. After 1 h of preincubation, cells were incubated for 30 min in complete medium containing [H^3]myo-inositol (37 $MBq{\cdot}ml^{-1}$; specific activity, 629 $MBq{\cdot}nmol^{-1}$) in the presence or absence of 10^{-8} M of *Fugu* (1–34)PTHrP or 10^{-8} M *Fugu* (7–34)PTHrP. The reaction was stopped by centrifugation at 2500 rpm at 4 C and by suspending the pellet in 500 μl ice-cold 10% trichloroacetic acid. The contents were transferred from 24-well culture plates into 1.5-ml microtubes and sonicated. An aliquot was taken for total protein determination, and the remainder was centrifuged at 12,000 rpm for 15 min at 4 C. The supernatant, containing IPs, was removed and measured in a scintillation counter (LS6100; Beckman Coulter, Fullerton, CA). Chloroform-methanol (2:1, vol/vol) was then added to the pellet to extract the phosphoinositide phosphates (PIPs). After centrifugation at 12,000 rpm for 15 min at 4 C, this supernatant was also removed and counted. Counts obtained for IPs and PIPs were pooled, and the results reported corresponded to the total amount of [H^3]myo-inositol incorporated in each sample (phos-

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phoinositides plus PIPs) and are expressed as counts per minute per milligram protein.

RT-PCR of PTHrP and PTHRs

Sea bream head kidneys were collected and immediately frozen in liquid nitrogen. Total RNA was extracted with TRI reagent (Sigma-Aldrich, St. Louis, MO), and the final concentration was measured using a spectrophotometer (GeneQuant; Amersham Biosciences, Lisbon, Portugal). The quality of extracted total RNA was determined by agarose gel (1.5%) electrophoresis. cDNA synthesis was carried out by adding the following to each reaction tube: 1.25 μl oligo dT (1 $\mu g \cdot \mu l^{-1}$; Amersham Biosciences); 2 μl of 5× RT buffer (Life Technologies, Inc.-BRL, Barcelona, Spain); 0.25 μl of MMLV Reverse transcriptase (200 $U \cdot \mu l^{-1}$; Life Technologies, Inc.-BRL); 0.5 μl deoxynucleotide triphosphate (10 mM; Amersham Biosciences), and 1 μl dithiothreitol (0.1 M). The reaction was allowed to proceed for 3 h at 37 C and was then stopped by heating at 65 C for 5 min and stored at −20 C until use. For the RT-PCRs, 1 μl of cDNA was added to a reaction mixture (50 μl) containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 1.25 U *Taq* DNA polymerase (Promega, Madison, WI), and 10 pmol each of forward and reverse primers for the gene of interest. The sea bream PTHrP specific sense primer (5′-GAATTCAGGAGGTCAGTGAGCCAC-3′) and antisense primer (5′-AACAGACCGTGCCCGCCTCCTCTTCTTGTC-3′) yielded a 359-bp product. The sea bream PTH1R specific sense primer (5′-GCGGCAGTAACACATACAGC-3′) and antisense primer (5′-TTTCTCTTTCCTCCTCCACC-3′) yielded a 290-bp product. The sea bream PTH3R specific sense primer (5′-ACATCCACATTCACTTCTTCAC-3′) and antisense primer (5′-GATGAGGGCCACAGGTAGT-3′) yielded a 274-bp product. As a positive control, plasmids containing cloned PTH1R and PTH3R substituted cDNA in the PCR. Elongation factor 1α was used as a control to establish the relative quantity of cDNA used in each reaction (18). PCR products were visualized by ethidium bromide staining after electrophoresis on 1.5% agarose gels. The bands were excised, purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), cloned into pGem-T-easy plasmid, and used to transform competent *Escherichia coli*. Plasmid DNA was prepared from recombinant *E. coli* using the alkaline lysis method (19) and sequenced (Macrogen Inc., Seoul, South Korea). DNA sequence analyses were performed using BLAST (20). GenBank accession numbers for sea bream PTHrP, PTH1R, and PTH3R are AF197904, AJ619024, and AY547261, respectively.

Biochemical analysis

Cortisol was determined in head kidney perfusate using a cortisol RIA. The cross-reactivity of this antiserum in relation to cortisol was 54% for 11-desoxycortisol, 10% for cortisone, 16% for 17,21-dihydroxy-5-pregnan-3,11,20-trione, 5% for 11β,17,21-trihydroxy-5-pregnan-3,20-dione, 0.05% for 11-hydroxytestosterone, and less than 0.001% for testosterone. Head kidney perfusates (15 μl) were diluted in phosphate buffer (300 μl) containing 0.5 $g \cdot l^{-1}$ gelatin (pH 7.6) and denatured at 70 C for 30 min.

Data analysis

Results are presented as mean ± SEM of six separate head kidney perfusions. Data analysis was carried out using one-way ANOVA followed by the Student-Newman-Keuls test. When nonhomogeneous variances were found, the Mann-Whitney and Kruskal-Wallis test were applied. The level for accepted statistical significance was $P < 0.05$. Significant differences in the figures are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$). Standard curve analysis was carried out with the Pharmacology Module of Sigma Plot, version 8.0 (SPSS Inc., Chicago, IL), by fitting of a four-parameter logistic function.

Results

Of all the piscine PTHrP peptides tested, only the intact *Fugu* and flounder N-terminal fragment (1–34)PTHrP stimulated, in a dose-dependent fashion, the *in vitro* release of cortisol from isolated sea bream interrenal glands (Fig. 1).

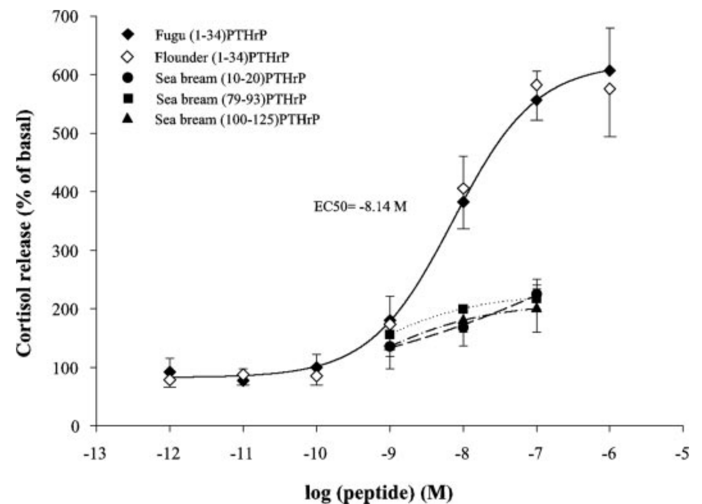

FIG. 1. Effect of increasing doses of *Fugu* (1–34)PTHrP, flounder (1–37)PTHrP, and sea bream (10–20)PTHrP, (74–93)PTHrP, and (100–125)PTHrP on the *in vitro* release of cortisol from superfused sea bream head kidney. Data are given as percentage of basal release and represent the mean ± SEM of six independent determinations.

The baseline secretion established for control tissue was 7.41 nM. The time lag between application of the piscine (1–34)PTHrP stimulus and the increase in cortisol production by the interrenal tissue was similar to that observed when ACTH was used to stimulate tissue (both occurred within 20 min). This is indicative that piscine (1–34)PTHrP has a rapid stimulatory action on cortisol production by interrenal tissue in fish. Truncated forms of *Fugu* (1–34)PTHrP, in which the first amino acid (2–34), first two amino acids (3–34), or first six amino acids (7–34) were removed, failed to stimulate cortisol release from head kidney (Fig. 2A). These results indicate that amino-terminal deletions of teleost PTHrP affect the ability of these truncated forms to stimulate *in vitro* cortisol release from interrenal tissue.

The way in which PTHrP brings about its stimulatory action on cortisol production by interrenal tissue was assessed in a series of experiments with specific inhibitors. Piscine (1–34)PTHrP (10^{-8} M)-stimulated cortisol production from interrenal tissue was unaffected by 10^{-6} M CIP. In contrast, CIP effectively blocked 90% of ACTH corticotropic activity (data not shown). This suggests that the PTHrP effect is not mediated by its binding to an ACTH receptor. However, the stimulatory effect of (1–34)PTHrP could be removed by co-perfusion with 10^{-6} M of any of the truncated peptides, including (7–34)PTHrP, which is known to act as an antagonist by binding to the PTHR. This is indicative that PTHrP binds to a PTHR in the head kidney. Supporting the existence of such receptor(s) is the demonstration by RT-PCR that sea bream interrenal tissue expresses PTHrP and two PTHRs, PTH1R and PTH3R (Fig. 3). Overall, these results strongly suggest that PTHrP most likely brings about its effect on cortisol production by binding to specific PTHRs.

To establish which of the receptors and/or signaling pathways is mediating the PTHrP-stimulated cortisol production, the effect of AC (10^{-4} M SQ-22536) and PLC (10^{-5} M U-73122) inhibitors was investigated. When either of the inhibitors was added to sea bream interrenal tissue *in vitro* in the

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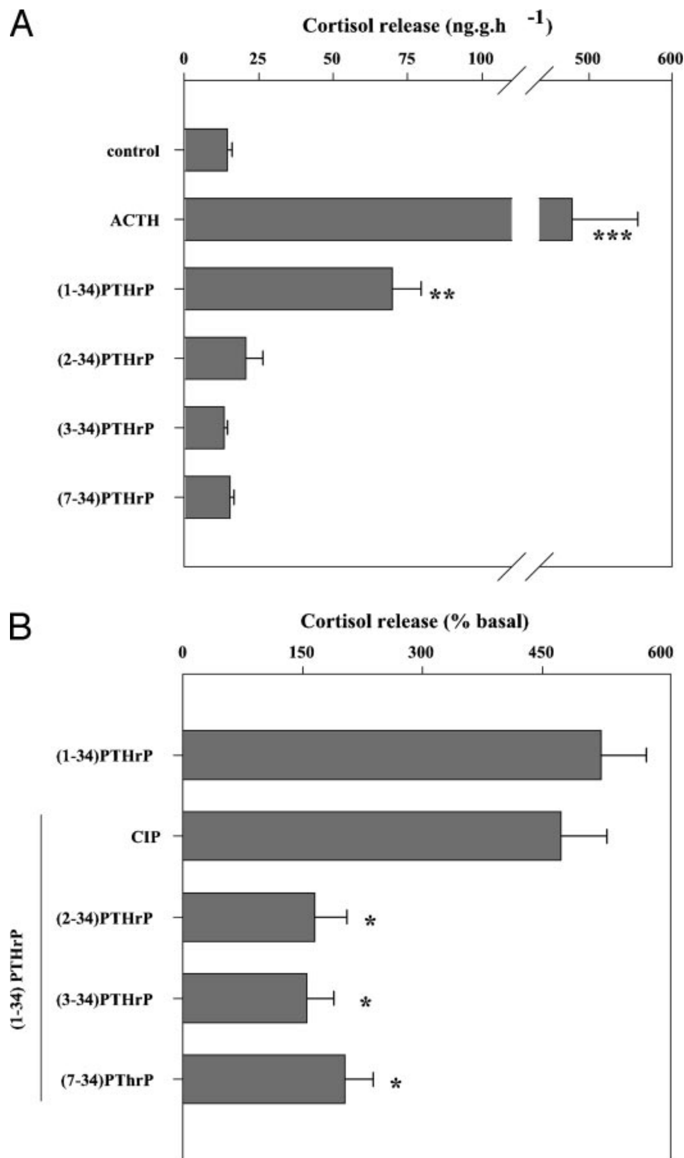

FIG. 2. A, Effect of ACTH (10^{-8} M) and intact or truncated forms of (1–34)PTHrP (10^{-8} M) on *in vitro* cortisol secretion from superfused sea bream head kidneys. B, Effect of CIP (10^{-6} M) and truncated forms of (1–34)PTHrP (10^{-6} M) on (1–34)PTHrP (10^{-8} M)-stimulated *in vitro* cortisol secretion from superfused sea bream head kidneys. Head kidneys were treated with peptides for 20 min. Data represent the mean ± SEM of six independent experiments. *Asterisks* indicate significant difference from control basal release (A) and PTHrP only (B).

presence of piscine (1–34)PTHrP (10^{-8} M), no significant inhibition of cortisol release was obtained. However, with the simultaneous addition of both inhibitors, 10^{-4} M SQ-22536 and 10^{-5} M U-73122, the stimulation of cortisol release from sea bream interrenal tissue was totally abolished (Fig. 4). This is indicative that both the AC and PLC pathways can be used to bring about PTHrP corticosteroidogenic response. cAMP release and [H^3]myo-inositol incorporation by dispersed interrenal cells was stimulated 2.5- and 1.8-fold, respectively, by piscine (1–34)PTHrP (10^{-8} M) (Fig. 5). The truncated forms of (2–34)PTHrP and (3–34)PTHrP failed to stimulate cAMP release and [H^3]myo-inositol incorporation (data not shown),

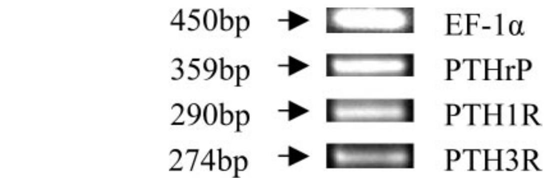

FIG. 3. Amplified products of specific RT-PCR for sea bream PTHrP, sea bream PTH1R, and sea bream PTH3R using cDNA prepared from sea bream head kidney mRNA. RT-PCR products were fractionated on a 1.5% agarose gel and stained with ethidium bromide. EF-1α, Elongation factor 1α.

whereas (7–34)PTHrP significantly increased ($P < 0.05$) [H^3]myo-inositol incorporation but not cAMP release (Fig. 5). This indicates that the first seven amino acids of the N terminus of piscine PTHrP are important for optimal AC-cAMP signaling pathway activation, but the PTHrP activation of the PLC-IP signaling pathway is more complex.

Discussion

The results from the present study demonstrate that PTHrP has corticosteroidogenic activity in fish and the ligand determinants of receptor signaling in interrenal cells. Piscine (1–34)PTHrP stimulated cortisol release in a concentration-dependent manner in interrenal tissue of sea bream. The ED_{50} of (1–34)PTHrP was 2.8 times higher than that of (1–39)ACTH (14), and the percent increase of cortisol production in response to 10^{-8} M of (1–34)PTHrP was approximately 7-fold lower than for 10^{-8} M of (1–39)ACTH. In contrast to (1–34)PTHrP, piscine (10–20)-PTHrP, (79–93)PTHrP, and (100–125)PTHrP failed to stimulate corticosteroidogenesis at 10^{-9}–10^{-7} M. The effect of piscine (1–34)PTHrP was abolished by N-terminal peptides in which the first amino acid (Ser) was absent and by inhibitors of the AC and PLC intracellular pathways. These results, together with the expression of mRNA for PTHrP and for PTH1R and PTH3R receptors in sea bream interrenal tissue, suggest that this is a specific effect mediated by PTHRs, and as observed in mammals (1, 21), this is indicative of a paracrine or autocrine effect of PTHrP modulating corticosteroid production in this tissue (22).

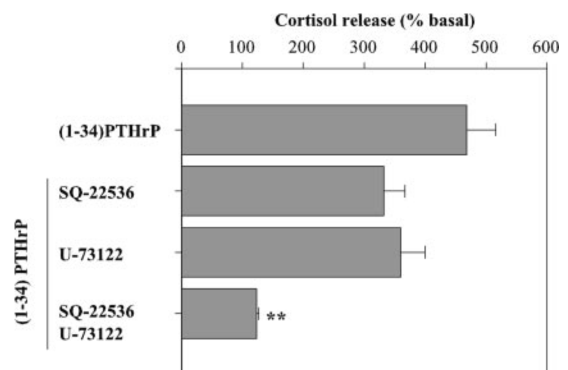

FIG. 4. Effect of AC inhibitor SQ-22536 (10^{-4} M) and PLC inhibitor U-73122 (10^{-5} M) on (1–34)PTHrP (10^{-8} M)-stimulated cortisol secretion from sea bream head kidneys. Head kidneys were treated with peptides and/or inhibitors for 20 min. Data represent the mean ± SEM of six independent experiments. *Asterisks* indicate significant difference from (1–34)PTHrP only.

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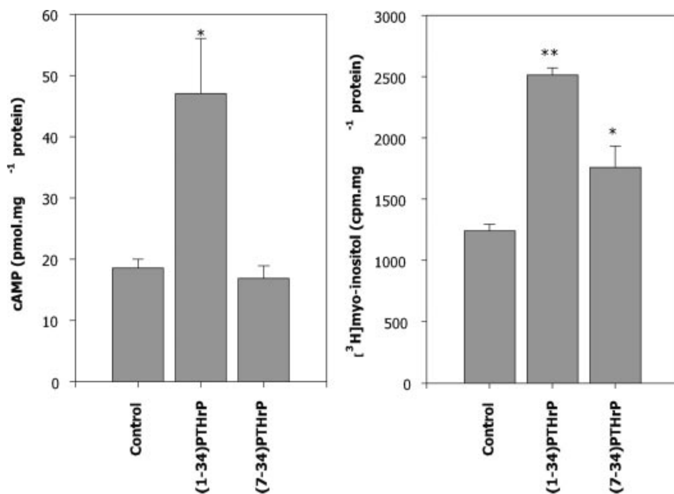

FIG. 5. Effect of (1–34)PTHrP (10^{-8} M) and (7–34)PTHrP (10^{-8} M) on cAMP production (A) and on incorporation of [H^3]myo-inositol (B) by dispersed sea bream interrenal cells. Cells were challenged for 30 min in the presence of IMBX. Data represent the mean ± SEM of two independent determinations in three separate experiments. *Asterisks* indicate significant difference from control.

The possibility that the cortisol stimulatory activity of (1–34)PTHrP in interrenal tissue was a result of direct interaction with the ACTH receptor was eliminated because CIP, at concentrations that effectively abolish ACTH-stimulated cortisol release in interrenal tissue, had no effect on (1–34)PTHrP-induced cortisol production. This demonstrated unequivocally that piscine (1–34)PTHrP stimulates cortisol release via another mechanism and is in agreement with a glucocorticoid secretagogue effect reported for (1–34)PTHrP in human adrenal cells *in vitro* (5). This indicates that the glucocorticoid secretagogue action of the N terminus of PTHrP has been conserved during evolution.

In the present study, amino-terminal deletions in teleost PTHrP, such as removal of the first residue (2–34), first two residues (3–34), or first six residues (7–34), caused a dramatic reduction in the biological potency of (1–34)PTHrP. This suggests that, in fish, the first seven amino acids and, in particular, the first Ser of the N terminus of piscine PTHrP are determinants of the glucocorticoid secretagogue activity. A similar observation has been made in humans, in which the cortisol stimulatory action of human (1–34)PTHrP was abolished by the PTHR antagonist (Leu^{11}, D-Trp^{12})PTHrP (7–34)-amide. The importance of an intact N-terminal region of PTHrP for biological activity appears to be a general phenomenon, and amino acid deletions have been observed to reduce its potency in cAMP second messenger responses of COS-7 cells expressing zebrafish PTHRs (6).

In mammals, PTH and PTHrP are both expressed in the hypothalamus and pituitary gland, whereas PTHRs have been found in all components of the hypothalamus-pituitary-adrenal axis (9). In common with this observation, PTHrP is produced by a variety of endocrine and nonendocrine tissues in fish (2, 3, 22, 23). For example, PTHrP and its mRNA have been demonstrated in the brain, pituitary, and head kidney of sea bream (2, 3, 22). Furthermore, very high to moderate concentrations of immunoreactive PTHrP have been detected, in decreasing order of abundance, in extracts of pituitary, head kidney, esophagus, intestine, gill, skin, and muscle of the sea bream (22). The high pituitary and circulating levels of this hormone measured in the sea bream and flounder may indicate that in teleosts, in contrast to what is observed in mammals, PTHrP, in addition to paracrine and autocrine actions, can also act as an endocrine factor (22, 24).

In mammals, two principal PTHR types have been identified, PTH1R and PTH2R (25, 26). Characterization of ligand-binding affinity indicates that PTH1R binds equally well to human PTHrP and human PTH, whereas PTH2R binds preferentially to bovine tuberoinfundibular peptide (TIP39) (27). PTH1R is the receptor most frequently characterized in nonskeletal tissue, which interacts with amino-terminal fragments of PTHrP (28, 29). There are relatively few studies describing PTHRs in mammalian adrenals. However, those studies indicate that in common with other non-skeletal tissue the receptor expressed in the adrenals is PTH1R (30). The identification in the sea bream interrenal tissue of two receptors, a mammalian PTH1R analog and PTH3R, which has so far only been described in fish, raises questions about the relative importance of these receptors in the responsiveness of the head kidney to PTHrP. Further studies will be required to elucidate the role of these two receptors in the regulation of cortisol production in fish.

In vitro studies of zebrafish PTH1R and PTH3R expressed in COS-7 cells reveal that zebrafish PTH1R was similarly activated by human PTH, human PTHRP, and *Fugu* PTHrP. Conversely, zebrafish PTH3R showed higher affinity for human PTHrP and *Fugu* PTHrP (7, 8).

Until recently, all evidence suggested that PTHRs signal principally via the AC-PKA signaling pathway and secondarily via the PLC-PKC-intracellular Ca^{2+} signaling pathway (6). The previously mentioned zebrafish COS-7 cells transfection studies established that zebrafish PTH1R and PTH2R grouped with isolated mammalian receptors. However, the fish-specific receptor, zebrafish PTH3R, activated the AC-PKA signaling pathway but not the PLC-PKC-intracellular Ca^{2+} signaling pathway (8). In the present study, PTHrP-stimulated cortisol production from the head kidney was only completely blocked when inhibitors to both pathways were used, indicating that most likely PTH1R, which signals via both pathways (AC-PKA and PLC-PKA), is the receptor involved. However, it has not been possible to confirm or determine the relative importance of PTH1R and/or PTH3R in the PTHrP-stimulated cortisol response, and no information about the functional importance of these receptors in fish is available. One observation for which we do not have a good explanation is that, although (7–34)PTHrP was able to inhibit cortisol and cAMP production, it did not appear to be effective in blocking the PLC-PCK pathway. One possibility is that partial blockage of each pathway is enough to block cortisol production, as indicated by the effect of SQ-22536 and U-73122 inhibitors, which only block cortisol when both are used together. The precise ligand regions of (1–34)PTHrP involved in the stimulation of PLC and PKC have not yet been clearly identified. However, it has been shown that (1–31)PTH stimulates AC but not PLC in mouse kidney cells, indicating that the C-terminal amino acids of (1–34)PTHrP are involved in the activation (31). However, replacing Glu^{19} for Arg^{19} in human (1–28)PTH restored PLC signaling and

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ligand-binding affinity, and (1–28)PTH is the shortest human PTH peptide that could fully activate both AC and PLC. Furthermore, PLC activity was reduced by substituting Gly^1 for Ser^1 in human (1–34)PTH and was eliminated entirely by removing either residue 1 or the α-amino group alone. Thus it has been concluded that the N-terminal determinants of AC and PLC activation in human PTH(1–34) overlap but are not identical (32). Our results with piscine PTHrP peptide are consistent with the observations made with PTH in human.

In summary, the present study clearly shows that (1–34)PTHrP behaves as a glucocorticoid secretagogue in interrenal tissue. The stimulatory effect of N-terminal PTHrP is not caused by its binding to ACTH receptors but probably occurs via activation of PTH1R and PTH3R, both of which are expressed by sea bream interrenal tissue. Upon ligand binding, both the AC-PKA and PLC-PKC-intracellular Ca^{2+} signaling pathways are activated in interrenal tissue, leading ultimately to increased cortisol production. The corticosteroidogenic effect of the N-terminal region of PTHrP appears to have been conserved during evolution because it is present in both fish and mammals, although the physiological relevance of this effect remains to be established. It appears that the paracrine action reported for PTHrP and other members of the glucagon family in mammalian adrenals (9) can be extended to the homologous interrenal tissue of the sea bream and possibly other teleosts.

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