Parathyroid hormone-related protein-stanniocalcin antagonism in regulation of bicarbonate secretion and calcium precipitation in a marine fish intestine

Juan Fuentes, Deborah M. Power and Adelino V. M. Canário

Am J Physiol Regul Integr Comp Physiol 299:R150-R158, 2010. First published 21 April 2010;
doi: 10.1152/ajpregu.00378.2009

Am J Physiol Regul Integr Comp Physiol 299: R150–R158, 2010.
First published April 21, 2010; doi:10.1152/ajpregu.00378.2009.

Parathyroid hormone-related protein-stanniocalcin antagonism in regulation of bicarbonate secretion and calcium precipitation in a marine fish intestine

Juan Fuentes, Deborah M. Power, and Adelino V. M. Canário

Centro de Ciências do Mar, CIMAR-Laboratório Associado, Campus de Gambelas, Faro, Portugal

Submitted 6 July 2009; accepted in final form 19 April 2010

Fuentes J, Power DM, Canário AV. Parathyroid hormone-related protein-stanniocalcin antagonism in regulation of bicarbonate secretion and calcium precipitation in a marine fish intestine. *Am J Physiol Regul Integr Comp Physiol* 299: R150–R158, 2010. First published April 21, 2010; doi:10.1152/ajpregu.00378.2009.—Bicarbonate secretion in the intestine (duodenum) of marine fish has been suggested to play a major role in regulation of calcium availability for uptake. However, while the end process may lead to carbonate precipitation, regulation of transport of calcium and/or bicarbonate may actually result in fine-tuning of calcium availability for transport. To test this hypothesis, sea bream (*Sparus auratus*) duodenal preparations were mounted in Ussing-type chambers and the effect of parathyroid hormone-related protein (PTHrP) and stanniocalcin 1 (STC 1) on the control of intestinal bicarbonate secretion and calcium transport was analyzed. As expected, PTHrP increased net calcium uptake, as a result of an increase of calcium uptake without changes in calcium efflux. In contrast, purified sea bream STC 1 caused a minor decrease of calcium uptake and a two- to threefold increase in calcium efflux. As a result, STC 1 was able to invert the calcium flux from net calcium uptake to net calcium loss, which is in keeping with its known actions as a hypocalcemic factor. Furthermore, both PTHrP and STC 1 regulate intestinal bicarbonate secretion. PTHrP increased calcium uptake and simultaneously reduced the single factor that induces calcium precipitation, bicarbonate secretion. In contrast, STC 1, while reversing the calcium net flux to make it secretory, promoted intestinal bicarbonate secretion, both actions directed to decrease the calcium gradient across the epithelium and promote immobilization in the form of bicarbonate in the intestinal lumen. Together our results provide robust evidence to support an antagonistic action of PTHrP and STC 1 in the fine control of movements of both calcium and bicarbonate in the intestine of seawater fish.

ion regulation; sea water; sea bream

MARINE TELEOSTS live in an environmental osmolality of ~1,000 mosmol/kgH_2O while keeping their internal milieu at ~350 mosmol/kgH_2O. As part of the regulated osmoregulatory process, high rates of water ingestion have been described for marine fish (8). The fluid imbibed is processed along the intestinal canal, with a primary step of net ion assimilation in the esophagus that allows water absorption in the intestine by a process driven by NaCl via a Na^+-K^+-$2Cl^-$ cotransporter (24). In addition to water ingestion and absorption, the formation of carbonate aggregates in the intestine has been proposed to be central to the preservation of body fluid homeostasis in marine fish (21). Thus calcium reaches the intestine at concentrations ranging from 7 to 15 mM (9, 39), which upon precipitation in the form of $CaCO_3$ driven by HCO_3^- secretion lowers intestinal fluid osmolality between 15 and 25 mosmol/kgH_2O and favors water absorption (13).

Address for reprint requests and other correspondence: J. Fuentes, Centro de Ciências do Mar, CIMAR-Laboratório Associado, Campus de Gambelas, 8005-139 Faro, Portugal (e-mail: jfuentes@ualg.pt).

While in higher vertebrates duodenal HCO_3^- secretion is considered one of the most important defense mechanisms against acid injury in the duodenum, the intestine of fish is able to produce a highly alkaline microenvironment that appears even in unfed fish (39), indicating that this process is dissociated or independent from digestion. This high alkalinity is the result of HCO_3^- secretion and has the capacity to precipitate divalent cations (35, 37–39). Intracellular hydration of CO_2 in the intestine epithelial cell plays an important role in the generation of HCO_3^- for apical secretion (14, 15). However, if bicarbonate is routed via the transcellular pathway, the organization of bicarbonate secretion (BCS) entails a dual component of bicarbonate, one basolateral for internalization in the epithelial cell and the second to accomplish the actual secretion to the lumen of the intestine (21).

Regardless of how it is generated, secreted bicarbonate immobilizes calcium in the form of calcium carbonate aggregates. As such, in addition to the proposed idea of water availability and enhancement of intestinal water absorption, BCS has also been suggested to participate in calcium metabolism (39). Accordingly, to prevent excessive calcium load, BCS would inhibit calcium uptake in the intestine by decreasing or annulling luminal calcium availability by precipitation. A lack of calcium absorption as a consequence has been proposed (37). However, this is not the case, and net intestinal calcium absorption has been shown to take place in the intestine of seawater fish by regulated processes (9, 30).

In keeping with a calcium and bicarbonate cross-regulation, the endocrine control of one or the other may play a crucial role in substrate availability for carbonate aggregate formation. A model for calcium regulation that incorporates a single endocrine factor, stanniocalcin (STC), has been proposed in fish. It is justified on the basis of the nearly endless calcium pool available in seawater, the absence of parathyroid glands in fish, and the presence of fish-specific corpuscles of Stannius, which produce STC, a hypocalcemic or antihypercalcemic factor (3, 28, 34, 36). However, this model is thrown into doubt by *1*) the recent identification of parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP) in fish tissues and *2*) their demonstrated role in calcium regulation as hypercalcemic factors (2, 4, 9–11, 19, 25). In addition to its hypercalcemic nature, PTHrP was shown to regulate drinking rates in one of our previous studies (19) in sea bream larvae in seawater, a role in keeping with a potential regulation of imbibed fluid processing.

Despite its physiological relevance (17, 38) and potential importance for other models of biomineralization, the process of BCS in seawater fish intestine has not been fully characterized at the mechanistic level (21), and no information is available at the regulatory endocrine level. We hypothesize that calcium and bicarbonate transport combine into a functional system and that the same endocrine factors that control

calcium movements also regulate directly or indirectly BCS in the intestine. The present study was designed to characterize calcium and bicarbonate transport in the intestinal epithelium in vitro and their regulation by PTHrP (hypercalcemic factor) and STC (hypocalcemic factor).

MATERIALS AND METHODS

Peptides and Chemicals

The PTHrP(1–34) (2) from puffer fish was synthesized by Geneemed Synthesis (San Francisco, CA). All chemicals were of the highest grade and were obtained from Sigma-Aldrich (Madrid, Spain) unless stated otherwise.

Animals

Sea bream (*Sparus auratus*) were obtained as fry or juveniles from the stock of Ramalhete Experimental Marine Station (University of Algarve) and kept for at least 1 mo before experimentation/death. Fish were held in 600-liter seawater tanks (density <8 kg fish/tank), with flowing seawater (salinity 38‰; water temperature 18–24°C), under a natural photoperiod and were fed twice a day (9 AM and 6 PM) with 1% body weight commercial dry pellets (Provimi). All fish were fasted for 48 h before experimental manipulations.

For tissue collection fish were anesthetized with 2-phenoxyethanol (1 ml/l water, Sigma-Aldrich) and killed by decapitation. All animal manipulations were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. All animal protocols were performed under license of Group-1 from the Direcção-Geral de Veterinária, Ministerio da Agricultura, do Dessevolvimento Rural e das Pescas.

Purification of STC

In vitro culture of corpuscles of Stannius. Corpuscles of Stannius were collected from 60 (0.8- to 1-kg body wt) seawater-adapted sea bream (anesthetized with 1 ml/l 2-phenoxyethanol) and placed in several wells of a sterile 48-well plate with 0.2 ml each of modified Cortland's saline culture medium (in mmol/l: 160 NaCl, 2.55 KCl, 1.56 $CaCl_2$, 0.93 $MgSO_4$, 17.85 $NaHCO_3$, 2.97 NaH_2PO_4, and 5.55 glucose, pH 7.8 when equilibrated with a 99% O_2-1% CO_2 gas mixture). The saline was supplemented with 10 μl/ml of vitamins (MEM 100x Vitamins; Sigma-Aldrich), 20 μl/ml essential amino acids (MEM 50x; Sigma-Aldrich), 10 μl/ml nonessential amino acids (MEM 100x; Sigma-Aldrich), 10 μl/ml antibiotics (penicillin 10,000 IU/ml, streptomycin 10,000 μg/ml; GIBCO), and 20 μl/ml L-glutamine (200 mM; Sigma-Aldrich). Plates were introduced into a chamber with a controlled gas atmosphere of 99% O_2-1% CO_2 and gently shaken throughout their incubation at 22°C for 24 h. After the culture, the corpuscles of Stannius and culture media were stored separately at −20°C for further processing.

SDS-PAGE and Western blot. Culture medium or homogenates of three or four corpuscles were fractionated by SDS-PAGE (15% polyacrylamide) under reducing conditions at 100 V and blotted onto enhanced chemiluminescence (ECL) membranes (Hybond ECL, Amersham Biosciences). The transfer was carried out in a vertical tank transfer system (Mighty Small Hoefer, Amersham Pharmacia) for 60 min at 100 mA.

The membrane was incubated with blocking solution [10% (wt/vol) milk; 0.1% (vol/vol) Tween 20 in Tris-buffered saline (TBS)] overnight at 4°C. Subsequently, the membrane was incubated for 1.5 h at room temperature with constant agitation with the primary antisera (anti-trout STC, kindly provided by Prof. G. Flik, Radboud University, Nijmegen, The Netherlands) diluted 1:1,000 in TBS. Excess antisera was removed, and the membranes were washed three times for 10 min each in TBS-Tween 20 (TBST) before addition of the secondary antibody (anti-rabbit IgG-peroxidase conjugate 1:2,500; GE Healthcare). Excess secondary antisera was removed and membranes were washed three times for 10 min each in TBST and developed in 3,3′-diaminobenzidine (DAB; 0.2 mg/ml in 100 mM Tris, pH 7.5) and H_2O_2 (0.003%) color enhanced with nickel (0.40 mg/ml $NiCl_2$).

Mass spectrophotometry of proteins. The putative band for STC identified by positive Western blot was excised from the gel, and protein identification was conducted by the Centro de Genómica y Proteómica (Facultad de Farmacia, Universidad Complutense Madrid, Madrid, Spain). The proteins were subjected in gel to trypsin digestion, and the molecular weights of the peptides and amino acid sequences were analyzed by matrix-assisted laser desorption ionization (MALDI) tandem time of flight (TOF) mass spectrophotometer, with the postsource decay (PSD) technique. Unambiguously identified peptides were further examined by tandem mass spectrometry (MS/MS) fragmentation. The resulting amino acid sequences were identified by both peptide mass fingerprinting and MS/MS fragmentation. Database searches using the peptide mass fingerprint integrated with MS/MS were performed with the MASCOT program (http://www.matrixscience.com) and BLASTP 2.2.15.

Protein separation with continuous-elution electrophoresis. After confirmation of identity, culture media from several fish (15 ml) were pooled and concentrated with Ultrafree-15 centrifugal filter devices with a molecular mass cutoff of 5 kDa (Millipore, Bedford, MA) and run on SDS-PAGE (12% polyacrylamide gels). Purification and analytical separation of proteins from concentrated culture medium was carried out by preparative SDS-PAGE under denaturing conditions with continuous-elution electrophoresis using a model 491 Prep Cell (Bio-Rad). The resolving gel was composed of 15% polyacrylamide in 0.375 M Tris·HCl, pH 8.8. The stacking gel was 4% polyacrylamide in 0.125 M Tris·HCl, pH 6.8. The dimensions (height) of the resolving gel and the stacking gel were ~54 mm and 28 mm, respectively. Electrophoresis was carried out with 0.025 M Tris·HCl, 0.19 M glycine, 0.1% SDS, pH 8.3 as the running buffer, and samples (3 ml of concentrated culture medium) were mixed with 1.5 ml of sample buffer (100 mM Tris·HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol), heated at 100°C for 5 min, centrifuged briefly, and run at a constant current of 20 mA over ~18 h, maintaining a constant temperature (18°C). Proteins fractionated on the polyacrylamide gel were allowed to migrate off the end of the gel into the elution chamber and collected as 2.5-ml fractions at 8°C. Fraction collection was initiated when the ion front (bromophenol blue) had migrated out of the gel. Every 10th fraction was analyzed by SDS-PAGE (15% polyacrylamide gels) and stained with silver nitrate in order to determine which fractions contained the proteins of interest (identified on the basis of size). Additional Western blots were performed to identify first the range and second the individual fractions reacting with the anti-STC antibody. Fractions containing the putative hormone, i.e., giving a single clear band, were pooled. Concentration of pooled fractions and substitution of buffer was achieved by means of Ultrafree-15 centrifugal filter devices with ultrafiltration membranes (Millipore) against 20 mM Tris·HCl, pH 7.5 by several steps of centrifugation at 1,900 *g* and 4°C for 40 min.

Short-Circuit Current Measurements

The general methods used for Ussing chamber experiments have previously been explained (9), with the exception that the short-circuit current (I_{sc}) values were presented as absolute values, not considering the sign of the current injected. Here the current is presented considering the voltage referenced to the apical side of the preparation.

Sea bream (80–100 g) were killed by decapitation, and the anterior intestine (the region corresponding to the duodenum and comprising a homogeneous region of ~3 cm in length distal to the pyloric caeca) was carefully dissected out and transferred to chilled freshly prepared and gassed (10 mM HCO_3^-, 99:1 O_2-CO_2) basolateral saline (see Table 1 for composition). The intestinal portions were defatted, cleaned with fresh saline, and opened longitudinally to produce a flat sheet. The mucosal crypts were separated to minimize the unstirred boundary layer and

Table 1. *Ionic composition of saline used for Ussing-type chamber experiments in sea bream anterior intestine*

	Basolateral			Apical
Na^+	172	172	172	90
Cl^-	166	166	166	125
K^+	3	3	3	3
Ca^{2+}	1.5	1.5	1.5	7.5
Mg^{2+}	1	1	1	135
SO_4^{2-}	1	1	1	126.5
SO_4^{3-}	2	2	2	1
HCO_3^-	0	5	10	0
HEPES	10	5	0	0
Glucose	5	5	5	
Osmolality	320	320	320	340
pH	7.8	7.8	7.8	7.8–7.9
Gas	O_2	0.3% CO_2	1% CO_2	O_2

Ionic values are expressed in millimoles per liter. Osmolality values (in mosmol/kgH_2O) are measured values.

mounted (with apical and basolateral sides identified) by pinning over the circular aperture (0.5 or 1 cm^2) of an Ussing half-chamber between two Parafilm gaskets to minimize edge damage. The Ussing chamber was assembled, and 3–5 ml of basolateral saline was added to each hemichamber. The saline in the chambers was gassed with a 99:1 O_2-CO_2 mix to provide oxygenation, good mixing by gas lift, and pH control to 7.8. Temperature was maintained between 21 and 22°C throughout the experiments. The preparations were left to stand for at least 60 min or until a steady basal measurement of bioelectrical variables was achieved. Measurement of bioelectrical variables was performed in symmetric conditions to avoid non-zero junction potentials with the saline compositions described in Table 1. I_{sc} ($\mu A/cm^2$) was monitored by clamping of epithelia to 0 mV. All bioelectrical variables were monitored by means of Ag/AgCl electrodes (with tip asymmetry <1 mV) connected to either side of the Ussing chamber with 3-mm-bore agar bridges (3 M KCl in 3% agar). Depending on the clamp amplifier used, epithelial resistance ($\Omega \cdot cm^2$) was automatically calculated from voltage deflections after injection of bipolar 200-ms pulses of 50 μA every minute or manually calculated (Ohm's law) by using the current deflections induced by a 2-s 1-mV pulse every minute. Recording of variables was performed by means of a microcomputer-controlled voltage/current Clamp Electronic with automatic correction for fluid resistance and electrode voltage asymmetry (KMSCI, Aachen, Germany) or a DVC-1000 voltage clamp amplifier (WPI, Sarasota, FL).

Intestinal Bicarbonate Secretion

Duodenum preparations were mounted as described above with the following exceptions: *1*) both apical and basolateral sides of the preparation received 3–5 ml of the corresponding saline either apical or basolateral (Table 1) to simulate in vivo conditions; *2*) gas/HCO_3^- and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were modified in the basolateral chamber (see Table 1 for details), while the apical side received 100% O_2; and *3*) the preparations in the Ussing-type chamber were current clamped to 0 $\mu A/cm^2$. For the purpose of BCS measurement, preparations were left to achieve a transepithelial steady-state voltage (generally around 30 min), at which point the saline was replaced in both chambers and left undisturbed for 1.5 h, and then the apical saline was replaced and the hormones incorporated into the basolateral chamber to give a final concentration of 1 μg/ml. The preparation was left undisturbed for an additional period of 1.5 h, and a final collection of apical saline was performed at this point. BCS in response to basolateral hormones was measured with a combination of 10 mM HCO_3^--1% CO_2 in O_2 in the basolateral saline. A single preparation was used for two consecutive measurements, one in the absence (control period) and one in the presence (hormone effect) of hormones. Whole samples of apical saline for either control- or hormone-treated periods were transferred to small titration vessels with a magnetic stirrer and constant gassing (O_2). The samples were gassed for 30 min to remove CO_2 and titrated to pH 3.8 with 10 mM HCl, an additional gassing period of 20 min was applied to remove any remaining CO_2, and the sample was back-titrated to its original pH with 10 mM NaOH. BCS was calculated from the difference in the volume of HCl and NaOH needed for either titration and considering the molarity of the titrant, the time elapsed, and the Ussing chamber opening. BCS is expressed as nanomoles per hour per square centimeter.

Intestinal Calcium Fluxes

The Ussing chamber (0.5-cm^2 opening) was assembled exactly as described for BCS with asymmetric saline (basolateral 10 mM HCO_3^--1% CO_2 in O_2) and gas in the apical and basolateral half-chambers. Preparations were left in saline for 30 min, followed by a further 15 min in new, freshly gassed saline. For the measurement of calcium uptake, ^{45}Ca ($CaCl_2$ 0.2 μCi; NEN Life Sciences Products) was added to the apical (uptake) or basolateral (efflux) sides and left to mix for 15 min after addition of tracer. Saline (100 μl) from the cold half-chamber was collected and replaced by 100 μl of fresh saline (*time 0*), followed by similar procedures at 30-min intervals for the duration of the experiments comprising two consecutive periods of 1.5 h in the absence (control period) or the presence of either STC or PTHrP(1–34) on the basolateral side. Samples (100 μl) were also collected from the ^{45}Ca-labeled saline at *time 0* and at the end of the experiments for calculation of specific activities. All radiotracer experiments were performed under current clamp to 0 $\mu A/cm^2$. The quality of experiments was checked for initial velocity and linearity of flux measurements. At the end of the experimental period, to confirm the physical integrity of the tissue the volume of one of the half-chambers was removed to verify that no fluid moved from the full to the empty half-chamber.

Unidirectional fluxes were calculated according to the following equations:

$$\text{Calcium uptake: } J_{in}^{Ca2+}(\text{nmol} \cdot \text{cm}^{-24} \cdot \text{h}^{-1}) = \Delta[^{45}Ca]_{Bl}(1/SA_{Ap})[\text{volume}_{Bl}]/[(\text{time})(\text{area})] \quad (1)$$

where $\Delta[^{45}Ca]_{Bl}$ represents the increase in radioactivity on the basolateral half-chamber and SA_{Ap} represents the apical side specific activity (cpm/nmol),

$$\text{Calcium efflux: } J_{out}^{Ca2+}(\text{nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}) = \Delta[^{45}Ca]_{Ap}(1/SA_{Bl})[\text{volume}_{Ap}]/[(\text{time})(\text{area})] \quad (2)$$

where $\Delta[^{45}Ca]_{Ap}$ represents the increase in radioactivity on the apical half-chamber and SA_{Bl} represents the basolateral side specific activity (cpm/nmol), and

$$\text{Calcium net flux: } J_{ne}^{Ca2+}(\text{nmol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}) = J_{in}^{Ca2+} - J_{out}^{Ca2+} \quad (3)$$

Statistics

Results are shown as means ± SE unless otherwise stated. After assessment of normality and homogeneity of variances, differences between groups were established by paired Student's *t*-test or one-way analysis of variance (ANOVA) followed by the post hoc Bonferroni test to identify significantly different groups. All statistical analyses were performed with the statistical package SPSS (SPSS, Chicago, IL). Groups were considered significantly different at $P < 0.05$, unless stated otherwise.

RESULTS

Sea Bream STC Purification

A single STC-immunoreactive band of 25 kDa was identified by Western blot of the medium used for culture of sea

bream corpuscles of Stannius in vitro (Fig. 1). The sequences obtained from the mass spectrometry profile of the fragmented protein corresponded to 89 amino acids. A MASCOT blast search of the amino acid sequence obtained revealed that it shared greatest similarity with STC 1.

Sequence comparison shows a high degree of similarity between the partial sea bream STC 1 sequences and those from fish of diverse orders (Fig. 1). One of the features of the STC family is the presence of 11 cysteine residues important for protein dimerization, and after alignment of the sea bream tryptic peptides cysteine residues were identified in positions 99, 115 and 171; the latter is important for protein dimerization. Characteristic sites for protein kinase C phosphorylation (residues 102–104) and casein kinase II phosphorylation (residues 36–39 and 142–145) were also identified in the sequence of the tryptic peptides.

Stannius glands from 60 sea bream yielded 1,750 μg of secreted STC.

Bioelectrical Characterization of Sea Bream Intestine Under Voltage Clamp

The sea bream duodenum was mounted in Ussing chambers with the same saline (Table 1) bathing the apical and basolateral sides, generating a small potential difference of 1.13 ±

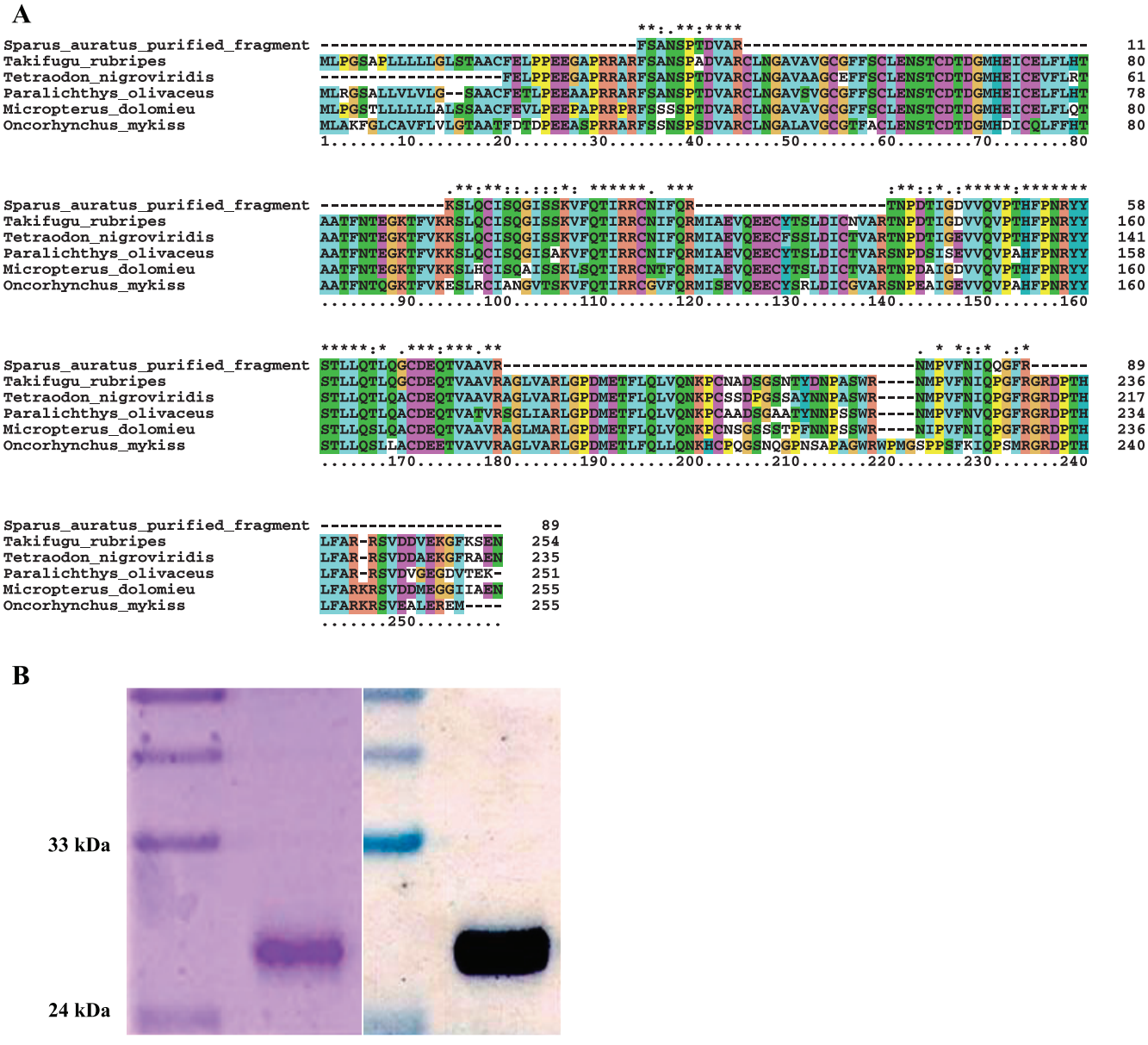

Fig. 1. *A*: multiple sequence alignment (ClustalX) of protein fragments identified by mass spectrometry analysis of gel-extracted putative sea bream stanniocalcin 1 (STC 1). This fragmented sequence was used to search the NCBI database with the BLASTP 2.2.15 protocol to confirm identity of the protein sequence as STC 1. The NCBI accession numbers of STC sequences used for the alignment with the sea bream STC 1 fragments were the following: *Takifugu rubripes*, NP_001072056; *Tetraodron nigroviridis*, ACA35029; *Paralichtys olivaceus*, ABI64157; *Microptreus dolomieu*, BAD99603; and *Oncorhynchus mykiss*, NP_001117762. *B*: protein profile of a Coomassie blue-stained acrylamide gel (*left*) in which the single protein band represents the purified sea bream STC 1; identity is confirmed by Western blot (*right*) using an anti-trout antiserum (see MATERIALS AND METHODS for further details).

Table 2. *Bioelectrical variables of sea bream anterior intestine mounted in Ussing chambers under voltage clamp*

V_t, mV	1.13 ± 0.12
I_{sc}, μA/cm^2	−9.69 ± 0.94
G_t, mS/cm^2	10.86 ± 0.67

Results are shown as means ± SE (n = 21). V_t, spontaneous open circuit potential before voltage clamping; I_{sc}, short-circuit current; G_t, conductance. I_{sc} and G_t were measured under voltage clamp to 0 mV.

Table 3. *Basal variables of sea bream anterior intestine mounted in Ussing chambers under current-clamp conditions*

V_t, mV	−8.10 ± 0.47 (34)
G_t, mS/cm^2	8.72 ± 0.52 (34)
BCS, nmol·cm^{-2}·h^{-1}	461.47 ± 52.8 (34)
Net Ca^{2+} uptake, nmol·cm^{-2}·h^{-1}	11.20 ± 3.02 (10)

Results are shown as means ± SE for number of individuals given in parentheses. BCS, bicarbonate secretion. V_t was measured under current clamp to 0 μA/cm^2.

0.12 mV (mucosa reference). To voltage clamp the tissue to 0 mV, the injection of a small but consistent current (I_{sc}) of around −9 μA/cm^2 was required (Table 2).

Control preparations sustained constant I_{sc} (μA/cm^2; Fig. 2*A*) and conductance (G_t, mS/cm^2; Fig. 2*B*) for the duration of the experimental periods. To test the effects of PTHrP(1–34) and STC 1 in I_{sc}, the hormones were applied to the basolateral side of the preparation as a single dose of 1 μg/ml and the I_{sc} was followed up to 1 h after addition. The application of PTHrP(1–34) basolaterally had a small effect on I_{sc}, with a significant decrease (more negative) after 40 min of application (Fig. 2*C*). No change was observed in G_t in response to PTHrP(1–34) (Fig. 2*D*). The application of sea bream STC 1 resulted in a slow but consistent increase/inversion of the I_{sc} ($P < 0.01$, 1-way ANOVA) from 25 min after addition up to the end of the recording period (Fig. 2*E*). In addition, G_t was slightly, but significantly, increased after the application of basolateral STC 1 (Fig. 2*F*).

Current-Clamped Sea Bream Intestine

Basal variables of sea bream duodenum under current-clamp conditions are shown in Table 3.

Manipulation of bicarbonate availability in the basolateral saline significantly modified apical BCS in the sea bream duodenum (Fig. 3). The combination of 10 mM HEPES and O_2 (0 CO_2, 0 mM HCO_3^-) resulted in an apical BCS of 183 ± 26 nmol·cm^{-2}·h^{-1}, which is not significantly different from the

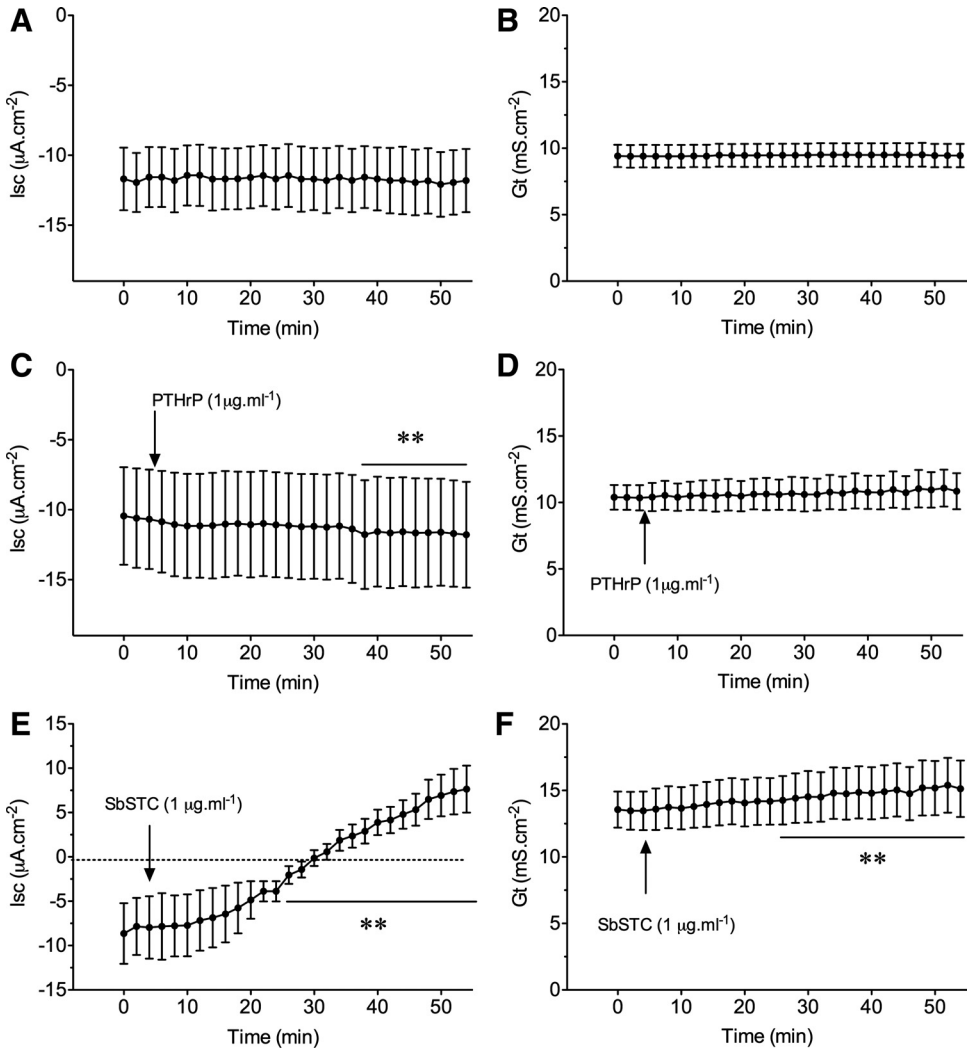

Fig. 2. Short-circuit current (I_{sc}) and conductance (G_t) in sea bream duodenum in control preparations (*A* and *B*) and in response to basolateral application of 1 μg/ml parathyroid hormone-related protein (PTHrP)(1–34) (*C* and *D*) or 1 μg/ml purified sea bream STC 1 (*E* and *F*). Each point represents the average ± SE of 7 individuals. **Significant difference from pretreatment control (1-way ANOVA, $P < 0.01$).

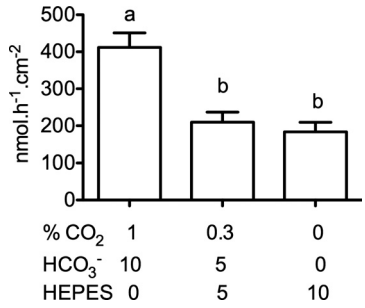

Fig. 3. Bicarbonate secretion (BCS) in sea bream duodenum obtained with different combinations of HEPES, HCO_3^-, and gas CO_2 levels as shown. Each column represents mean ± SE (n = 10 individuals/treatment). Different superscript letters indicate significant differences from other groups ($P < 0.01$, 1-way ANOVA, Bonferroni post hoc test).

secretion obtained in the presence of 5 mM HEPES, 5 mM HCO_3^-, and 0.3% CO_2. In contrast, apical BCS was significantly higher ($P < 0.01$, 1-way ANOVA) in the presence of 10 mM HCO_3^- with 1% CO_2. The latter combination of saline was used in the basolateral chamber for all further experimentation under current clamp, thus allowing transcellular HCO_3^- movements to be represented in the total BCS measured.

The application of basolateral PTHrP(1–34) (1 μg/ml) resulted in a significant decrease in BCS, to values less than half those of the control period (Fig. 4). In contrast, the application of basolateral STC 1 (1 μg/ml) resulted in a more than twofold significant increase in BCS ($P < 0.01$, 1-way ANOVA). In control preparations, BCS was determined in consecutive periods in the absence of hormones showing no significant variation, confirming the specificity of the hormonal effects (Fig. 4).

Calcium fluxes determined in current-clamped sea bream duodenum are shown in Fig. 5. Application of basolateral PTHrP(1–34) (1 μg/ml) caused a significant increase in calcium uptake ($P < 0.01$, 1-way ANOVA) and a slight but not significant decrease of calcium efflux, with the net result of an about fivefold increase in calcium uptake (Fig. 5). In turn, application of basolateral STC 1 (1 μg/ml) had no effect in calcium uptake and induced a significant increase of about twofold in calcium outflux ($P < 0.01$, 1-way ANOVA), which resulted in a significant decrease of net calcium transport ($P < 0.01$, 1-way ANOVA), which became outside directed (Fig. 5).

DISCUSSION

The present study establishes for the first time the role of the calcitropic hormones STC 1 and PTHrP in both the regulation of calcium movements and BCS in the intestine of marine fish. These results support the hypothesis that calcium-regulating factors may have, in addition to their calcitropic actions, a role in the general osmoregulatory process of marine fish. Assuming the premise that the formation of $CaCO_3$ aggregates in the intestine drives fluid processing and subsequent water absorption (13), the endocrine regulation of BCS by calcitropic factors may have a central role in this process.

The short-term effects of both PTHrP and STC 1 in the regulation of calcium transport are in good agreement with previous studies. PTHrP achieves its hypercalcemic action in

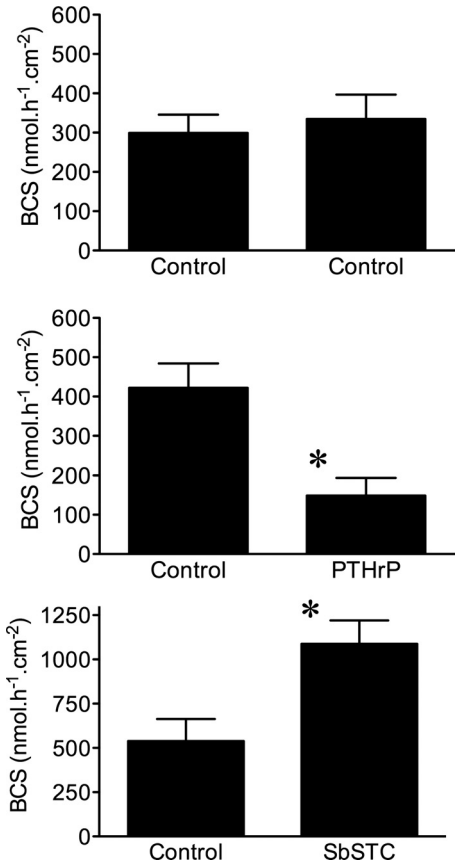

Fig. 4. BCS in sea bream duodenum in consecutive 1.5-h periods in response to no treatment (control, n = 6; *top*), PTHrP(1–34) (1 μg/ml, n = 8; *middle*), or sea bream STC 1 (SbSTC, 1 μg/ml, n = 10; *bottom*) applied in the second period. Each column represents mean ± SE. *Significant difference from pretreatment control (1-way ANOVA, $P < 0.01$).

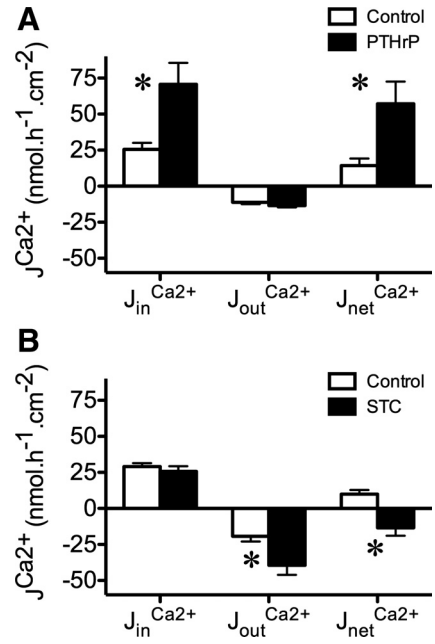

Fig. 5. Calcium uptake (J_{in}^{Ca2+}), efflux (J_{out}^{Ca2+}), and net flux (J_{net}^{Ca2+}) in sea bream duodenum in consecutive 1.5-h periods in response to 1 μg/ml PTHrP(1–34) (*A*; n = 4) or 1 μg/ml STC 1 (*B*; n = 6) applied in the second period. Each column represents mean ± SE. *Significant difference from pretreatment control (1-way ANOVA, $P < 0.05$).

the intestine by the combination of a threefold increase in calcium uptake and a slight increase of calcium efflux that results in a threefold increase of net calcium uptake (Fig. 5). The results obtained are in agreement with previous in vivo (2, 10, 11, 19) and in vitro (9) results and indicate a mechanism consistent with hypercalcemic actions of PTHrP in fish.

The effect of purified sea bream STC 1 on calcium transport in vitro demonstrates its bioactivity (Fig. 5) and indicates a mechanism for its hypocalcemic action demonstrated in other in vivo and in vitro models (3, 28, 34, 36). In our in vitro system, the intestinal absorptive calcium pathway was unaffected by application of STC 1 (Fig. 5), which is surprising since among the targets of STC 1 are the apical epithelial calcium channels, the limiting step for calcium internalization in the cell, as revealed by the stimulation of epithelial channels in morpholino knockdown of STC 1 in zebrafish embryos (31). A possible explanation for this divergence would be the relatively minor role of the intestine in freshwater zebrafish larvae compared with its importance in the marine sea bream (18), in which the extra intestinal component of calcium uptake varies as a function of salinity from >90% at 0–10% seawater to 40–50% in full-strength seawater. In other model systems such as the mammalian intestine, the addition of STC 1 to in vitro intestinal preparations induces a net decrease in calcium absorption, which surprisingly is the result of simultaneous increases in both absorptive and secretory calcium pathways (22).

The hypocalcemic effect of STC 1 in the intestine of the sea bream in vitro is not achieved by inhibition of the uptake component, but instead by a threefold stimulation of calcium efflux (Fig. 5). As a consequence, the net calcium transport becomes negative and the intestine becomes primarily secretory in relation to calcium (Fig. 5). This result is surprising because it happens against a fivefold concentration gradient (Table 1) and is achieved by an as yet unidentified molecular mechanism, although the secretory pathway Ca^{2+}-ATPase (SPCA) is the most likely candidate. The SPCA functions in models in which calcium transfer for secretion is functionally important, such as the mammary gland (32), and in which the epithelial cells must transport large amounts of calcium against a large concentration gradient, which is similar to the situation in the fish intestine. In addition, the SPCA is present in all the segments of the gastrointestinal system of mammals (33), where calcium is primarily processed. While calcium efflux has been routinely described in several studies, the calcium secretory pathway remains uncharacterized at both mechanistic and regulatory levels in fish. Database searches with the mammalian SPCA sequence (NCBI accession no. NM_001001487) identified expressed sequence tags with high homology in piscine species such as the euryhaline killifish (*Fundulus heteroclitus*, accession nos. DR441449 and DR441450), euryhaline rainbow trout (*Oncorhynchus mykiss*, accession nos. DT957416 and DT956043), the freshwater stickleback (*Gasterosteus aculeatus*, accession nos. DT957416 and DT956043), the euryhaline medaka (*Oryzias latipes*, accession no. DK011462), and the freshwater zebrafish (*Danio rerio*, accession no. EH541307). This further supports the notion that this pathway is also active in fish.

The sea bream intestine secretes bicarbonate at a rate in the range of 185–450 nmol·cm^{-2}·h^{-1} depending on the combination of CO_2 and HCO_3^- in the basolateral saline (Fig. 3). In the saline used for most of the experiments in this study (1% CO_2-10 mM HCO_3^-), roughly 50% of BCS is likely driven via transcellular pathways, while the other 50% is likely produced by hydration of CO_2 in the epithelial cell (as shown in preparations devoid of CO_2/HCO_3^-). The values of intestinal BCS obtained in this study in the sea bream are well within the range of those described for other marine species by pH-stat methods (1, 14). Validation of the robustness of the preparation of sea bream intestine was provided by consecutive 1.5-h measurements with virtually the same BCS rates (Fig. 4). In addition, BCS in the sea bream intestine is hormonally regulated, as shown by the effects of PTHrP and STC 1. Accordingly, while the dose of PTHrP used (1 μg/ml) significantly reduces BCS, STC 1 addition raises BCS between two- and threefold (Fig. 4). To our knowledge this is the first report on the effect of calcitropic hormones in intestinal BCS in vertebrates.

The mechanisms responsive to both PTHrP and STC 1 in the regulation of calcium and BCS so far remain unknown. The key calcium transport mechanisms involved in the calcium transport cascades (5–7, 20, 26, 27), i.e., apical epithelial calcium channels (31) and basolateral Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase, could adjust BCS by indirect regulation of calcium availability. In addition, there is a long list of potential candidates involved in bicarbonate transport in mammalian models, such as Na^+/H^+ exchangers, H^+-ATPase, Na^+-HCO_3^- cotransporters, anion exchangers, carbonic anhydrase, and even the apical cystic fibrosis transmembrane regulator (CFTR) (29). In the marine fish intestine only recently have some of the key elements of BCS been functionally confirmed: H^+-ATPase (V type), Na^+/H^+ exchanger 3, Na^+-HCO_3^- cotransporter 1, and two carbonic anhydrase isoforms (15–17). In addition, the presence and functional analysis (in *Xenopus* oocytes) of apical Slc26a6A and Slc26a6B (putative Cl^-/HCO_3^- exchangers) and basolateral NBCe1 (Na^+-HCO_3^- cotransporter) confirm the apical mechanisms and the existence of transcellular bicarbonate movement (21). On the other hand, the timing and I_{sc} reversal obtained in voltage-clamp experiments in response to STC (Fig. 2) resemble the CFTR response to ionomycin obtained in the intestine of seawater *F. heteroclitus* (23). Considering the relationship between CFTR and the SLC26 family of Cl^-/HCO_3^- exchangers in higher vertebrates (12), it would be tempting to suggest a regulatory action of STC on CFTR to explain enhanced I_{sc} in voltage-clamp experiments and BCS in the present study.

Interestingly, PTHrP and STC 1 regulate calcium movements and BCS in opposite directions. Although the experimental design precludes measurement of both variables in the same fish, there is a very strong negative correlation between the mean rates of net calcium flux and BCS, including hormone-stimulated (PTHrP or STC 1) and nonstimulated measurements. This reinforces the notion of common endocrine control mechanisms in both processes and strengthens the hypothesis that luminal calcium may regulate BCS (39). When less calcium is available in the lumen under PTHrP regulation, lower BCS rates are observed. In contrast, when more calcium is made available to the lumen under STC 1 regulation, BCS is stimulated. Assuming that larger amounts of water are made available for absorption (13) when BCS is stimulated, the significance of calcitropic hormones for fluid regulation becomes clear. This idea is substantiated by the decrease of drinking rates observed in sea bream larvae in response to

water-borne PTHrP (19) and the increase of drinking rates in seawater larvae challenged with increased water calcium (18). Both effects on water ingestion are in good agreement with our observations on BCS, and they reinforce the idea of its importance to favor water absorption in the intestine (13).

Perspectives and Significance

The present study provides evidence to further reinforce the relationship between epithelial calcium movements and BCS in the intestine of marine fish. The ionic status of the animal modulates the formation of intestinal carbonate aggregates (13, 37, 38) by influencing calcium and bicarbonate availability, and in the present study the role of the endocrine hormones in this process is revealed. The antagonistic action of the endocrine factors PTHrP and STC 1 tested in our experiments indicates that calcitropic hormones play a key role at both ends of formation of intestinal carbonate aggregates in the intestine of marine fish: the regulation of both the alkalinizing component and calcium availability. It will be of importance to establish whether the molecular and cellular mechanisms targeted by both calcitropic hormones in the formation of carbonate aggregates are common, or if the transporters in the calcium or bicarbonate routes are differentially regulated. In this context, the identification of STC 1 receptors will be an essential step. It will be important to determine the role of other endocrine factors in the regulation of epithelial calcium movements and BCS in the intestine. In addition, considering the previously demonstrated role of PTHrP in drinking (19), the link between regulation of drinking, bicarbonate, and fluid processing requires more detailed study.

ACKNOWLEDGMENTS

The authors acknowledge Adriana Silva for fish maintenance and Liliana Anjos for valuable help in the isolation of STC.

GRANTS

This work was supported by Ministry of Science and Higher Education and European Social Funds through the Portuguese National Science Foundation Projects POCTI/CVT/55683/2004 and PTDC/MAR/104008/2008 to J. Fuentes.

DISCLOSURES

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

REFERENCES

1. **Ando M, Subramanyam MVV.** Bicarbonate transport-systems in the intestine of the seawater eel. *J Exp Biol* 150: 381–394, 1990.
2. **Canario AVM, Rotllant J, Fuentes J, Guerreiro PM, Teodosio HR, Power DM, Clark MS.** Novel bioactive parathyroid hormone and related peptides in teleost fish. *FEBS Lett* 580: 291–299, 2006.
3. **Fenwick JC, So YP.** A perfusion study of the effect of stanniectomy on the net influx of calcium 45 across an isolated eel gill (1). *J Exp Zool* 188: 125–131, 1974.
4. **Flanagan JA, Power DM, Bendell LA, Guerreiro PM, Fuentes J, Clark MS, Canario AVM, Danks JA, Brown BL, Ingleton PM.** Cloning of the cDNA for sea bream (*Sparus aurata*) parathyroid hormone-related protein. *Gen Comp Endocrinol* 118: 373–382, 2000.
5. **Flik G, Klaren PHM, Schoenmakers TJM, Bijvelds MJC, Verbost PM, Bonga SEW.** Cellular calcium transport in fish: unique and universal mechanisms. *Physiol Zool* 69: 403–417, 1996.
6. **Flik G, Schoenmakers TJ, Groot JA, van Os CH, Wendelaar Bonga SE.** Calcium absorption by fish intestine: the involvement of ATP- and sodium-dependent calcium extrusion mechanisms. *J Membr Biol* 113: 13–22, 1990.
7. **Flik G, Vandervelden JA, Dechering KJ, Verbost PM, Schoenmakers TJM, Kolar ZI, Wendelaar Bonga SE.** Ca^{2+} and Mg^{2+} transport in gills and gut of tilapia, *Oreochromis mossambicus*: a review. *J Exp Zool* 265: 356–365, 1993.
8. **Fuentes J, Eddy FB.** Drinking in freshwater, euryhaline and marine teleosts. In: *Ionic Regulation in Animals*, edited by Hazon N, Eddy FB, Flik G. Heidelberg: Springer, 1997, p. 135–149.
9. **Fuentes J, Figueiredo J, Power DM, Canario AVM.** Parathyroid hormone-related protein regulates intestinal calcium transport in sea bream (*Sparus auratus*). *Am J Physiol Regul Integr Comp Physiol* 291: R1499–R1506, 2006.
10. **Fuentes J, Guerreiro PM, Modesto T, Rotllant J, Canario AVM, Power DM.** A PTH/PTHrP receptor antagonist blocks the hypercalcemic response to estradiol-17beta. *Am J Physiol Regul Integr Comp Physiol* 293: R956–R960, 2007.
11. **Fuentes J, Haond C, Guerreiro PM, Silva N, Power DM, Canario AVM.** Regulation of calcium balance in the sturgeon *Acipenser naccarii*: a role for PTHrP. *Am J Physiol Regul Integr Comp Physiol* 293: R884–R893, 2007.
12. **Gray MA.** Bicarbonate secretion: it takes two to tango. *Nat Cell Biol* 6: 292–294, 2004.
13. **Grosell M.** Intestinal anion exchange in marine fish osmoregulation. *J Exp Biol* 209: 2813–2827, 2006.
14. **Grosell M, Genz J.** Ouabain-sensitive bicarbonate secretion and acid absorption by the marine teleost fish intestine play a role in osmoregulation. *Am J Physiol Regul Integr Comp Physiol* 291: R1145–R1156, 2006.
15. **Grosell M, Genz J, Taylor JR, Perry SF, Gilmour KM.** The involvement of H^+-ATPase and carbonic anhydrase in intestinal HCO_3^- secretion in seawater-acclimated rainbow trout. *J Exp Biol* 212: 1940–1948, 2009.
16. **Grosell M, Gilmour KM, Perry SF.** Intestinal carbonic anhydrase, bicarbonate, and proton carriers play a role in the acclimation of rainbow trout to seawater. *Am J Physiol Regul Integr Comp Physiol* 293: R2099–R2111, 2007.
17. **Grosell M, Mager EM, Williams C, Taylor JR.** High rates of HCO_3^- secretion and Cl^- absorption against adverse gradients in the marine teleost intestine: the involvement of an electrogenic anion exchanger and H^+-pump metabolon? *J Exp Biol* 212: 1684–1696, 2009.
18. **Guerreiro PM, Fuentes J, Flik G, Rotllant J, Power DM, Canario AVM.** Water calcium concentration modifies whole-body calcium uptake in sea bream larvae during short-term adaptation to altered salinities. *J Exp Biol* 207: 645–653, 2004.
19. **Guerreiro PM, Fuentes J, Power DM, Ingleton PM, Flik G, Canario AVM.** Parathyroid hormone-related protein: a calcium regulatory factor in sea bream (*Sparus aurata* L.) larvae. *Am J Physiol Regul Integr Comp Physiol* 281: R855–R860, 2001.
20. **Hoenderop JGJ, Nilius B, Bindels RJM.** Calcium absorption across epithelia. *Physiol Rev* 85: 373–422, 2005.
21. **Kurita Y, Nakada T, Kato A, Doi H, Mistry AC, Chang MH, Romero MF, Hirose S.** Identification of intestinal bicarbonate transporters involved in formation of carbonate precipitates to stimulate water absorption in marine teleost fish. *Am J Physiol Regul Integr Comp Physiol* 294: R1402–R1412, 2008.
22. **Madsen KL, Tavernini MM, Yachimec C, Mendrick DL, Alfonso PJ, Buergin M, Olsen HS, Antonaccio MJ, Thomson ABR, Fedorak RN.** Stanniocalcin: a novel protein regulating calcium and phosphate transport across mammalian intestine. *Am J Physiol Gastrointest Liver Physiol* 274: G96–G102, 1998.
23. **Marshall WS, Howard JA, Cozzi RRF, Lynch EM.** NaCl and fluid secretion by the intestine of the teleost *Fundulus heteroclitus*: involvement of CFTR. *J Exp Biol* 205: 745–758, 2002.
24. **Musch MW, Orellana SA, Kimberg LS, Field M, Halm DR, Krasny EJJ, Frizzell RA.** Na^+-K^+-$2Cl^-$ co-transport in the intestine of a marine teleost. *Nature* 300: 351–353, 1982.
25. **Power DM, Ingleton PM, Flanagan J, Canario AVM, Danks J, Elgar G, Clark MS.** Genomic structure and expression of parathyroid hormone-related protein gene (PTHrP) in a teleost, *Fugu rubripes*. *Gene* 250: 67–76, 2000.
26. **Schoenmakers TJ, Flik G.** Sodium-extruding and calcium-extruding sodium/calcium exchangers display similar calcium affinities. *J Exp Biol* 168: 151–159, 1992.
27. **Schoenmakers TJ, Klaren PH, Flik G, Lock RA, Pang PK, Bonga SE.** Actions of cadmium on basolateral plasma membrane proteins involved in calcium uptake by fish intestine. *J Membr Biol* 127: 161–172, 1992.

28. **So YP, Fenwick JC.** In vivo and in vitro effects of Stannius corpuscle extract on the branchial uptake of ^{45}Ca in stanniectomized North American eels (*Anguilla rostrata*). *Gen Comp Endocrinol* 37: 143–149, 1979.
29. **Steward MC, Ishiguro H, Case RM.** Mechanisms of bicarbonate secretion in the pancreatic duct. *Annu Rev Physiol* 67: 377–409, 2005.
30. **Sundell K, Bjornsson BT.** Kinetics of calcium fluxes across the intestinal mucosa of the marine teleost, *Gadus morhua*, measured using an in vitro perfusion method. *J Exp Biol* 140: 171–186, 1988.
31. **Tseng DY, Chou MY, Tseng YC, Hsiao CD, Huang CJ, Kaneko T, Hwang PP.** Effects of stanniocalcin 1 on calcium uptake in zebrafish (*Danio rerio*) embryo. *Am J Physiol Regul Integr Comp Physiol* 296: R549–R557, 2009.
32. **VanHouten JN, Neville MC, Wysolmerski JJ.** The calcium-sensing receptor regulates plasma membrane calcium adenosine triphosphatase isoform 2 activity in mammary epithelial cells: a mechanism for calcium-regulated calcium transport into milk. *Endocrinology* 148: 5943–5954, 2007.
33. **Vanoevelen J, Dode L, Van Baelen K, Fairclough RJ, Missiaen L, Raeymaekers L, Wuytack F.** The secretory pathway Ca^{2+}/Mn^{2+}-ATPase 2 is a Golgi-localized pump with high affinity for Ca^{2+} ions. *J Biol Chem* 280: 22800–22808, 2005.
34. **Wagner GF, Dimattia GE.** The stanniocalcin family of proteins. *J Exp Zool A Comp Exp Biol* 305A: 769–780, 2006.
35. **Walsh PJ, Blackwelder P, Gill KA, Danulat E, Mommsen TP.** Carbonate deposits in marine fish intestines: a new source of biomineralization. *Limnol Oceanog* 36: 1227–1232, 1991.
36. **Wendelaar Bonga SE, Pang PK.** Control of calcium regulating hormones in the vertebrates: parathyroid hormone, calcitonin, prolactin, and stanniocalcin. *Int Rev Cytol* 128: 139–213, 1991.
37. **Wilson RW, Grosell M.** Intestinal bicarbonate secretion in marine teleost fish—source of bicarbonate, pH sensitivity, and consequences for whole animal acid-base and calcium homeostasis. *Biochim Biophys Acta* 1618: 163–174, 2003.
38. **Wilson RW, Millero FJ, Taylor JR, Walsh PJ, Christensen V, Jennings S, Grosell M.** Contribution of fish to the marine inorganic carbon cycle. *Science* 323: 359–362, 2009.
39. **Wilson RW, Wilson JM, Grosell M.** Intestinal bicarbonate secretion by marine teleost fish—why and how? *Biochim Biophys Acta* 1566: 182–193, 2002.