The Corpuscles of Stannius, Calcium-Sensing Receptor, and Stanniocalcin: Responses to Calcimimetics and Physiological Challenges

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This study has examined whether the calcium-sensing receptor (CaSR) plays a role in control of stanniocalcin-1 (STC-1), the dominant calcium regulatory hormone of fish, comparable with that demonstrated for CaSR in the mediation of ionized calcium regulation of PTH secretion in mammals. In a previous study, we have cloned flounder STC-1 from the corpuscles of Stannius (CS). Here, we report the cloning and characterization of the CS CaSR, and the *in vivo* responses of this system to altered salinity, EGTA induced hypocalcemia, and calcimimetic administration. Quantitative PCR analysis demonstrated, for the first time, that the CS are major sites of CaSR expression in flounder. Immunoblot analysis of CS proteins with CaSR-specific antibodies revealed a broad band of approximately 215–300 kDa under nonreducing conditions, and bands of approximately 215–300 kDa and approximately 120–150 kDa under reducing conditions. There were no differences in CS CaSR mRNA expression or plasma STC-1 levels between seawater and freshwater (FW)-adapted fish, although CS STC-1 mRNA expression was lower in FW animals. Immunoblots showed that glycosylated monomeric forms of the CaSR migrated at a lower molecular mass in CS samples from FW animals. The ip administration of EGTA rapidly induced hypocalcemia, and a concomitant lowering of plasma STC-1. Calcimimetic administration (1 mg/kg R-568) rapidly increased plasma STC-1 levels, and reduced plasma concentrations of calcium, phosphate, and magnesium when compared with S-568-treated controls. Together, these findings support an evolutionary conserved role for the CaSR in the endocrine regulation of calcium before the appearance of parathyroid glands in tetrapods. **(*Endocrinology* 150: 3002–3010, 2009)**

The concentration of calcium in extracellular fluid is precisely regulated throughout vertebrates from fish to mammals. The ability to sense changes in plasma-ionized calcium is mediated by the calcium-sensing receptor (CaSR), which is an integral component in regulating PTH secretion from the parathyroid glands of mammals (1). The CaSR has more recently been cloned and localized in a large number of tissue types in fish (2–4), many of which seem to have a functional relationship with the regulation of calcium (4–6). However, in fish, in the absence of parathyroid glands, there are currently no data describing a comparable role for the CaSR in the major calcium hormone regulatory systems, which in bony fish are dominated by stanniocalcin-1 (STC-1).

In contrast to mammals, in which calcium is obtained from dietary sources, in fish calcium is readily available from the surrounding aquatic environment. Accordingly, fish calcium regulation is predominantly mediated by the hypocalcemic hormone, STC-1. The corpuscles of Stannius (CS), which are teleost-specific endocrine glands associated with the kidneys, synthesize and secrete STC-1. Early studies showed that removal of these glands resulted in the rapid onset of hypercalcemia, clearly underscoring their role in calcium regulation (7). Actions of STC-1 on calcium homeostasis include inhibition of gill calcium transport (8–10), reduced intestinal calcium uptake (11), and stimulation of phosphate reabsorption by renal proximal tubules (12). A second STC, STC-2, has been identified recently in fish (13), but current evidence suggests that it does not play a significant role in calcium regulation (14). Synthesis and secretion of STC-1 by the CS have been shown to be sensitive to extracellular

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Abbreviations: CaSR, Calcium-sensing receptor; CNSS, caudal neurosecretory system; CS, corpuscles of Stannius; CT, calcitonin; FW, freshwater; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PNGase F, Peptide: N-Glycosidase F; RACE, rapid amplification of cDNA end; SH, sulfhydryl; STC-1, stanniocalcin-1; SW, seawater.

ionized calcium concentration both *in vitro* and *in vivo* (15–17), and it is suggested that regulation of STC-1 secretion from the CS in fish, similar to that for PTH and calcitonin (CT) secretion in mammals (18–20), involves the CaSR (21), though evidence to support this is lacking. Accordingly, the overall aim of the current study was to clarify the role of the CaSR in fish calcium regulation by first cloning the CaSR in CS and then investigating its expression in response to calcium challenge. These investigations have been performed in the euryhaline flounder, which can accommodate both calcium-rich seawater (SW) and relatively calcium-poor freshwater (FW), and from which we have also previously cloned STC-1 (22).

The function and expression of fish CaSRs have been assessed in transiently transfected human embryonic kidney cells, in which sensitivity to calcium, magnesium, and sodium ions has been demonstrated (4, 23), similar to that reported for mammalian CaSRs (24). Furthermore, previous investigations in fish found STC-1 producing cells to be metabolically more active in SW by comparison with FW-adapted fish (25), reflecting the 10- to 100-fold higher calcium content of SW. Therefore, one would predict higher levels of STC-1 secretion in SW compared with FW fish, with consequent modifications in calcium fluxes to secure stability of plasma composition. Interestingly, high levels of STC-1 mRNA expression have been observed in a second fish-specific endocrine tissue, the caudal neurosecretory system (CNSS) (26). Therefore, we also report here on the expression of CaSR along with STC-1 in the CS together with this novel expressing tissue (CNSS) in long-term SW and FW-adapted fish.

The cloning of the flounder CaSR revealed significant structural similarities with mammalian CaSRs, in particular, with respect to potential sites for calcimimetic interactions. Thus, it appeared likely that calcimimetics would also be effective in fish. Indeed, recently, Radman *et al.* (21) showed that the calcimimetic R-467 significantly enhanced the secretion of STC-1 in the rainbow trout, leading to a reduction in gill calcium transport. To further investigate CaSR activity in fish, we administered the calcimimetic R-568 or its enantiomer S-568. In mammals, administration of R-568 significantly decreases the secretion of PTH from the parathyroid *in vitro* and *in vivo* (20), and increases CT release from the thyroid gland (18). If the CaSR is central to fish calcium regulation, then one might expect calcimimetics to enhance CS sensitivity to calcium, if they act as in mammals, resulting in increased STC-1 secretion, with consequent effects on ion fluxes, and, thus, plasma and urine composition. The results of this study provide compelling evidence for a central role of the CaSR in systemic calcium regulation in fish.

Materials and Methods

Animals

The flounder, *Platichthys flesus*, were collected from Morecambe Bay (Cumbria, UK) and transported to the aquarium facilities at the University of Manchester. Flounder were of mixed sex and ranged in weight from 300–500 g. The flounder were maintained in SW (Natureland, Skegness, UK) or FW (tap water) tanks at 10–12 C under a 12-h light, 12-h dark cycle for at least 2 wk before experimentation. All experiments were performed in accordance with United Kingdom Home Office Regulatory requirements and local ethics committee approval.

Cloning of CaSR cDNA

Total RNA was extracted from tissues using TRIZOL reagent in accordance with the manufacturer's protocol (Invitrogen, Paisley, UK), and RNA yield was quantified using a NanoDrop (ND-1000) spectrophotometer (NanoDrop, Wilmington, DE). For cDNA synthesis, 1 μg total RNA was treated with deoxyribonuclease I (Invitrogen) and reverse transcribed using Invitrogen SuperScript II reverse transcriptase with random primers, as recommended by the manufacturer. The RT-PCRs were performed using Bioline reagents (Bioline, London, UK). The primer pairings of CaSR3F (5′-ACAGGATTGGATGTGCCGTTT-3′)-CaSR3R (5′-CGTTGGTCTTGAGGCTGATGG-3′) and CaSR4F (5′-CACCAGGCCACMGCCATG-3′)-CaSR4R (5′-AAKGTTTCCTCCCAAAACTC-3′) were used to clone small N-terminal and C-terminal portions of the flounder CaSR from CS cDNA. The PCR cycle conditions for CaSR3F-CaSR3R were: 94 C for 3 min, followed by 35 cycles of 94 C for 1 min, 59 C for 1 min, 72 C for 2 min, and finally 72 C for 10 min. The PCRs using CaSR4F-CaSR4R were performed, as described previously, except with an annealing temperature of 55 C. The intermediate segment between these fragments was amplified using primers CaSR5F (5′-CTATTGGCTTTGCTCTGAAGGC-3′)-CaSR5R (5′-CAAGGATGCACGAGATACAGAG-3′) and a cycling profile, as described previously, with an annealing temperature of 60 C.

Rapid amplification of cDNA ends (RACEs)

The 5′ and 3′ ends of the flounder CaSR were amplified using Clontech's SMART RACE cDNA Amplification Kit (Clontech, Oxford, UK). The cDNA for 5′ and 3′ RACE were synthesized from 1 μg CS total RNA. The 5′ and 3′ ends of the cDNA encoding the flounder CaSR were amplified using the primers CaSR5Race (5′-ATGCCACAGCAATGACCCAGTTCCACTCG-3′) and CaSR3Race (5′-GGATTGGATGTGCCGTTTACGCCAACCTGCCTTT-3′), respectively. A touchdown PCR protocol was used following the manufacturer's guidelines. The PCR products were ligated into pGEM-T vector (Promega, Southampton, UK).

Sequence analysis

Sequencing reactions were performed using Applied Biosystems (ABI, Warrington, UK) BigDye sequencing kit version 1.1. Amino acid sequence comparisons were performed using DNAMAN (Lynnon Biosoft, Quebec, Canada). The signal peptide was predicted using SignalP 3.0, potential regions of glycosylation using NetNGlyc 1.0, potential sites for the initiation of phosphorylation using NetPhos 2.0, and 7 transmembrane segments using TMpred (www.cbs.dtu.dk).

Tissue distribution of CaSR and STC-1 mRNA

Real-time quantitative PCR analysis

The tissue distribution of the CaSR and STC-1 mRNAs were analyzed in 18 tissues collected from six SW flounder using quantitative PCR. All primers and TaqMan probes were designed using Primer Express (ABI) and synthesized by Eurogentec (Liège, Belgium): CaSR_sense-2695F_(5′-CCATACTGGCATCCAGCTTTG-3′); CaSR_antisense-2773R_(5′-CGGGACGGTTTGAAAAGGA-3′); CaSR_TaqMan_probe-2721T_(5′-FAM-CTGGCCTGCATCTTCTTCAACAAGGTCT-TAMRA-3′); STC-1_sense-542F_(5′-AAGGTAAAACCTTTGTGAAGAAGAC-3′); STC-1_antisense-618R_(5′-GAAGTGAGCTGGTACCTGC-3′); STC-1_TaqMan_probe-571T_(5′-FAM-TTCAGTGCATCTCCCAGGGAATCTCCT-TAMRA-3′); Actin_sense-352F_(5′-AAGATGACCCAGATCATGTTCGA-3′); Actin_antisense-454R_(5′-GAGCCACCAATCCACACAGA-3′); and Actin _TaqMan_probe382T_(5′-FAM-AACACCCCCGCCATGTACGTTGC-TAMRA-3′).

The optimization and validation of primers and probes were performed using standard ABI protocols. PCRs were performed in triplicate as described by Lu *et al.* (27). For absolute quantification of CaSR and

STC-1 transcript copies, numbers were quantified as a comparison of sample CT values for each reaction compared with standard curves generated from linearized plasmid clones in accordance with ABI protocols. For the relative quantification of CaSR and STC-1 gene expression in SW *vs.* FW fish, the $2^{-\Delta\Delta Ct}$ method was used. The internal control gene used for these analyses was the housekeeping gene β-actin, though comparable results were also obtained with 18S.

Northern blot analysis

Ten micrograms of total RNA from SW flounder CS tissue were electrophoresed on a 1% denaturing agarose formaldehyde gel for 12 h at 25 V. The RNA samples were then blotted and subsequently fixed onto Hybond N^+ nylon membranes (Amersham Biosciences, Buckinghamshire, UK) as previously described (26). The original C-terminal CaSR fragment and full-length flounder STC-1 (22) were used as cDNA probes for Northern blotting.

Protein extraction

Tissues were obtained from SW (CS and CNSS) and FW (CS) acclimated flounder and homogenized in ice-cold buffer as described by Stewart *et al.* (28). For total protein extraction, homogenates were centrifuged at 2500 × *g* for 15 min at 4 C. For crude membrane fractions, the resulting supernatants from the initial purification were centrifuged at 200,000 × *g* for a further 30 min at 4 C. The supernatants were decanted and the resulting pellets containing crude membrane proteins resuspended in homogenization buffer. A Bio-Rad Protein Assay was used to determine the concentration of solubilized protein (Bio-Rad Laboratories, Inc., Hercules, CA).

Immunoblotting

Five-fold concentrated Laemmli buffer solution [0.32 M Tris (pH6.8), 5% (wt/vol) sodium dodecyl sulfate, 25% (vol/vol) glycerol, and 1% (wt/vol) bromophenol blue] was added to the protein samples in a 1:4 ratio in the presence and absence of the sulfhydryl (SH) group reducing agent β-mercaptoethanol (10%). Protein samples were heated at 95 C for 10 min, and 5 μg/lane (CS) or 25 μg/lane (CNSS) of protein was fractionated on 6% (CaSR) or 15% (STC-1) sodium dodecyl sulfate polyacrylamide gels. Electrophoresis and immunoblotting were performed as previously described (29). Immunoblots were probed with anti-CaSR mouse monoclonal antibody (diluted 1–5,000), raised against amino acids 214–235 of the human CaSR (Affinity BioReagents, Inc., Golden, CO), or with antifish STC-1 (1–10,000) rabbit antiserum (from G.F.) (30). Immunoblots for STC-1 were stripped and reprobed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1–1000) rabbit antibody (Abcam, Cambridge, UK) to assess protein loading. Negative controls were performed by omission of the primary antibody. Protein deglycosylation was performed on 5 μg CS protein in the absence and presence of Peptide: N-Glycosidase F (PNGase F) following manufacturer's guidelines (New England BioLabs, Ipswich, MA).

Animal experiments

Long-term acclimation to SW and FW

To determine the steady-state conditions of fully acclimated animals, fish were studied after being held in SW or FW for 2 wk in October. Fish were removed from tanks and without anesthetic blood samples (3–6 ml) were collected within 90 sec into ammonium-heparinized syringes by needle puncture of the caudal blood vessels. Blood was aliquoted into ammonium-heparinized tubes and plasma separated by centrifugation for 5 min at 13,000 × *g*. The flounder were humanely killed, and tissues were removed and immediately snap frozen in liquid nitrogen.

Experimental induction of acute hypocalcemia

A total of 56 flounder was acclimated to FW for 2 wk in September before the induction of hypocalcemia by administration of a calcium-chelating agent. Fish were split into four experimental groups related to sampling time after treatment: 0.5, 1, 2, and 4 h. Fish were injected ip with a single dose of either 150 mmol/liter NaCl (control vehicle) or 30 μmol/100 g body weight EGTA in a volume of 0.1 ml/100 g body weight. At each time point, treated fish (n = 7) were sampled alongside time-matched vehicle-injected controls (n = 7). Blood samples (3–6 ml) were collected as described previously, and plasma was separated by centrifugation before analysis.

Effect of calcimimetics on plasma and urine composition

Flounder were acclimated to FW in May, and to achieve continuous administration of reagents and permit serial blood sampling, fish were implanted with an arterial cannula as previously described (31). After 48 h post-operation recovery, experimental animals were administered 1 mg/kg body weight of the calcimimetic agent R-568 via the implanted cannula, and controls given the enantiomer S-568 (Amgen Inc., South San Francisco, CA) in 200 μl 150 mmol/liter saline (n = 8 for each group). Serial blood samples (200 μl) were collected via the arterial cannula 0, 0.5, 1, 2, 4, and 8 h after injection. Plasma was separated by centrifugation, and red blood cells were resuspended in 150 mmol/liter saline (200 μl) and placed back into the fish through the cannula. At 8 h, animals were killed and urine samples collected directly from the bladder by needle puncture.

Plasma analysis

Osmolality was measured by freezing-point depression (Roebling Micro-Osmometer; Camlab, Cambridge, UK), sodium, potassium, magnesium, and total calcium concentrations were determined by atomic absorption spectrophotometry (Solaar; Thermo Elemental, Winsford Cheshire, UK), chloride concentrations were analyzed by electrode titration (Corning Chloride Analyzer 925; Corning, Inc., Corning, NY), and phosphate concentrations were determined using an autoanalyzer following the manufacturer's protocols (SANPlus Segmented Flow Analyzer; Skalar Analytical B.V., Breda, The Netherlands). The ionized calcium measures were determined using a Rapid 865 Blood Gas Analyzer (Bayer HealthCare, Leverkusen, Germany). Plasma STC-1 levels were determined in duplicate on 100-μl aliquots of plasma by RIA (17).

Statistical analysis

Statistical differences between experimental groups were evaluated using independent sample *t* tests. $P < 0.05$ was considered significant. Results are expressed as mean + SE.

Results

The CaSR from flounder CS comprised a full-length cDNA of 3302 bp with an open-reading frame of 2802 bp encoding a 934-amino acid protein (Fig. 1A). The predicted amino acid sequence shared highest sequence identity to sea bream (94%), tilapia (93%), and Fugu (91%) CaSRs, and lower sequence identity with the human (76%) and dogfish (75%) CaSRs (Fig. 1B).

The flounder CaSR contained characteristic features consistent with members of family 3 of the GPCRs, with a large extracellular domain of 596 amino acids, a 246-amino acid membrane spanning domain, and a small 92-amino acid C-terminal intracellular domain (Fig. 1A). The large extracellular domain included an 18-amino acid signal peptide, 11 potential sites for N-linked glycosylation, 18 conserved cysteines, a hydrophobic region, and a large number of conserved acidic residues. Acidic residues in the second and third extracellular loops of the transmembrane domain are recognized in mammalian CaSRs to be important in modulating the actions of calcium, gadolinium,

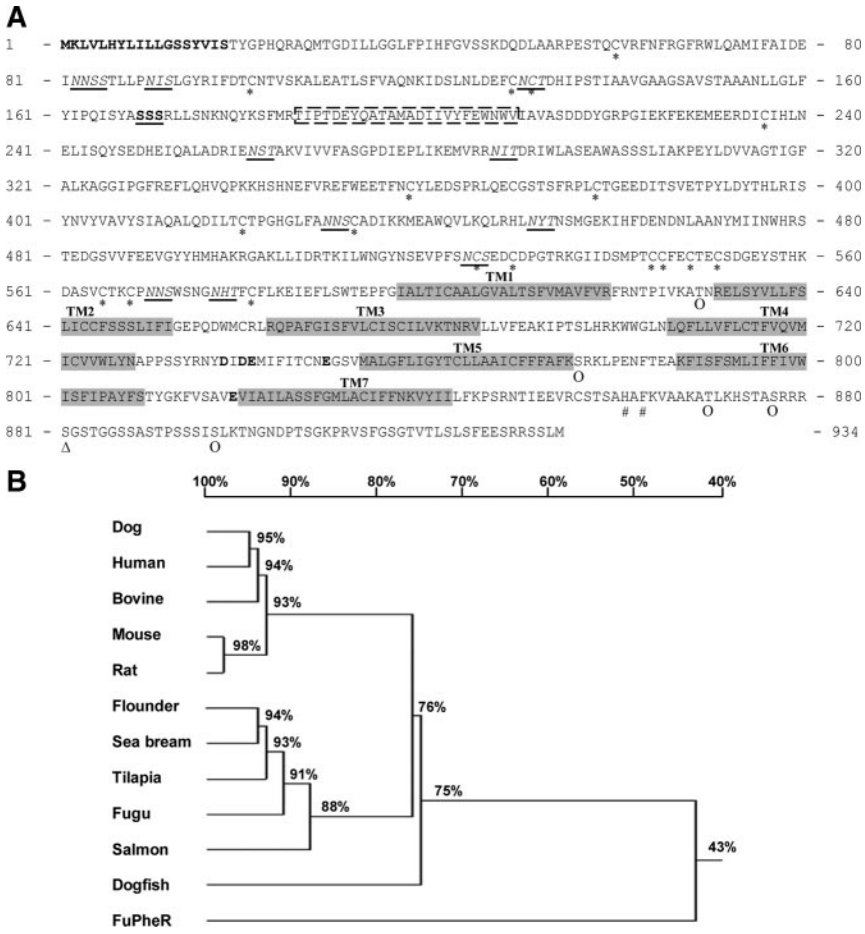

FIG. 1. Deduced amino acid sequence of flounder CaSR and homology tree for vertebrate CaSR sequences. A, Deduced amino acid sequence of flounder CaSR. The location of the signal peptide (*bold print*), potential N-linked glycosylation sites (*underlined italics print*), conserved cysteine residues (*), serine residues implicated in receptor activation (*underlined bold print*), and a 24-amino acid hydrophobic segment (*boxed*) are indicated. The transmembrane helices of the predicted seven transmembrane domain are *shaded*. Potential sites of protein kinase C (O) and protein kinase A (Δ) phosphorylation are indicated *below the sequence*. The *hash symbols* in the intracellular domain indicate amino acids (H and F) implicated in trafficking of the receptor to the cell membrane (38). Amino acids shown to be involved in the binding of allosteric modulators R-568 and NPS 2143 in mammalian CaSRs are shown in *bold print*. B, Homology tree of selected fish and mammalian CaSR genes. The Fugu pheromone receptor (FuPheR) has been used as the out-group sequence for this analysis. The sequence alignment and identity analysis were performed using DNAMAN software using distance matrix and Neighbor Joining methods. Dog, NP_001074978; human, NP_000379; bovine, NP_776427; mouse, NP_038831; rat, NP_058692; flounder, ACN62418; sea bream, CAC41352; tilapia, AAT06805; Fugu, BAA26122; salmon, NP_001119703.1; dogfish, AAM77700; FuguPheR, BAA26126.

allosteric modulators such as R-568, and organic polycations (32, 33). Five of the six acidic residues in extracellular loops 2 and 3 were found at corresponding positions in the flounder CaSR, suggesting that these residues may play important roles in the regulation of receptor activation. The intracellular domain of the flounder CaSR contained a number of consensus phosphorylation sites and amino acids involved in receptor trafficking.

Tissue mRNA distribution

Real-time quantitative PCR

The sensitive technique of quantitative PCR was used to determine CaSR and STC-1 mRNA expression levels in a wide range of SW tissues. Expression of CaSR mRNAs was not restricted to the CS, with moderate copy numbers detected in the CNSS, testis, bladder, kidney, and gill, and detectable levels in all other tissues analyzed (Fig. 2Ai). The STC-1 copy number was considerably higher in the CS by comparison with other tissues (Fig. 2Bi). However, in this case a second major site of STC-1 mRNA expression was also identified, with the CNSS expressing significantly higher copies of STC-1 by comparison with all other tissues analyzed with the exception of the CS.

Northern blotting

CaSR mRNA transcripts were only detectable by Northern blot in the CS, where two main transcripts of approximately 3 and 4.5 kb were identified in 10 μg total RNA (Fig. 2Aii). The broad band of approximately 3 kb is consistent with the predicted major band size based on the full-length cDNA of the flounder CaSR. The identity of the weaker band of approximately 4.5 kb that was observed is unknown but may represent an alternatively spliced form of the flounder CaSR. The high level of CaSR mRNA expression in the CS corresponded with high levels of STC-1, as demonstrated by Northern analysis (Fig. 2Bii). Bands of approximately 2 kb, closely matching the size of full-length flounder STC-1, and 3 kb were detected.

Characterization of the flounder CaSR

The CaSR monoclonal antibody recognized a single broad band in CS immunoblots under denaturing conditions, with lower molecular mass bands evident after addition of the reducing agent β-mercaptoethanol (Fig. 3A). There was a reduction in the intensity of the broad band of 215–300 kDa, with the appearance of an additional intense immunoreactive band migrating at a lower molecular mass of 120–150 kDa after the addition of β-mercaptoethanol. When CS protein was incubated in the absence and presence of PNGase F enzyme, the resulting immunoblots showed that the original 215–300 and 120–150 kDa bands were both reduced by deglycosylation to lower molecular masses of 200–240 and 90–120 kDa, respectively (Fig. 3B). Further immunoblot analysis showed that the CaSR was predominantly located in the membrane-enriched fraction rather than the supernatant containing cytosolic proteins (Fig. 3C). In the CNSS, CaSR-specific bands of approximately 300, 140, and 104 kDa were also detected in crude membrane (25 μg) proteins (Fig. 3D). In contrast to the CS, the intensity of the unglycosylated CaSR monomeric form (~104 kDa) represented the greatest CaSR signal in the CNSS.

Animal experiments

Long-term acclimation to SW and FW

The impact of environmental salinity on ion regulatory systems was confirmed by analysis of plasma ion composition.

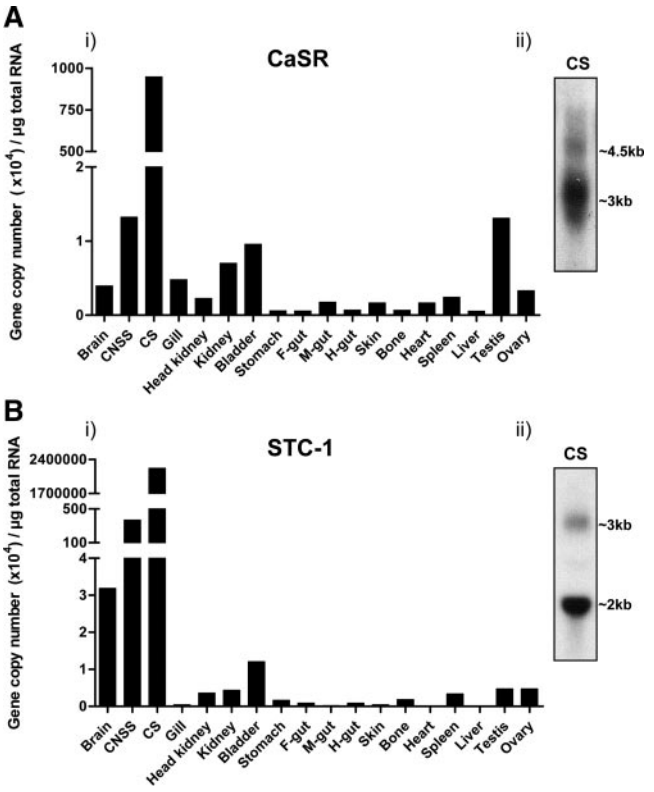

FIG. 2. Tissue distribution of CaSR (Ai) and STC-1 (Bi) mRNA. Results of real-time quantitative PCR analysis of tissues from six SW-acclimated adult flounder. Values are mean mRNA copies in 1 μg total RNA calculated from a calibration curve produced from known concentrations of recombinant plasmid DNA. A, CS > CNSS > testis > bladder > kidney > gill > brain > ovary > spleen > head kidney > mid (M)-gut > heart > skin > hind (H)-gut > bone > stomach > fore (F)-gut > liver. B, CS > CNSS > brain > bladder > testis > ovary > kidney > head kidney > spleen > bone > stomach > fore-gut > hind-gut > gill > skin. STC-1 mRNA was not detectable in mid-gut, heart, and liver after 40 amplification cycles. Aii, and Bii, Northern blots illustrating the mRNA transcript size of CaSR and STC-1 in total RNA samples (10 μg) from the CS of three (pooled RNA samples) SW-acclimated flounder. Blots were exposed to film at −80 C for 2 (STC-1) and 48 h (CaSR). The approximate size of each band on each blot is indicated in kilobases (kb).

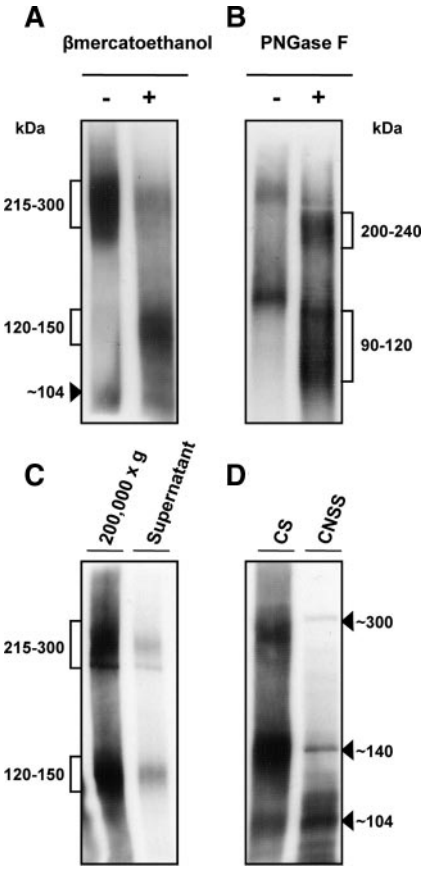

FIG. 3. Immunoblots of CaSR immunoreactivity in CS protein samples and tissues of SW-adapted flounder using a CaSR-specific mouse monoclonal antibody. A, Immunoblot of CS total protein (5 μg/lane) in the absence (−) and presence (+) of the SH-reducing agent β-mercaptoethanol demonstrating that almost all CaSR immunoreactivity migrates as a broad band of 215–300 kDa in the absence of β-mercaptoethanol. The addition of β-mercaptoethanol to the Laemmli buffer during denaturation resulted in a reduction of the intensity of the 215–300 kDa band together with the appearance of lower molecular mass (120–150 and 104 kDa) immunoreactive bands. B, Deglycosylation of CaSR protein. Immunoblot of CS total protein (5 μg/lane), treated at 37 C for 1 h with (+) or without (−) PNGase F, probed with CaSR antibody. The control signals were reduced to lower molecular masses by deglycosylation. C, Immunoblot of CS proteins (5 μg/lane) after serial centrifugation. Each lane contains different subcellular fractions: 200,000 × *g*, crude membrane proteins; supernatant, cytosolic proteins. The CaSR signals were strongest in the 200,000 × *g* fractions and virtually absent in the cytosolic protein fraction. D, CaSR-specific immunoreactive bands were also identified in CNSS crude membrane protein fractions (25 μg/lane) at approximately 300, 140, and 104 kDa. No positive staining was observed after the omission of the primary antibody. Molecular mass marker (kDa).

Plasma sodium (SW, 154.52 ± 1.76; FW, 141.80 ± 2.92), chloride (SW, 144.36 ± 1.39; FW, 121.50 ± 2.60), magnesium (SW, 2.80 ± 0.14; FW, 0.46 ± 0.02) concentrations (mmol/liter), and osmolality (SW, 311 ± 4; FW, 278 ± 5 mosmol/liter) were significantly higher ($P < 0.05$) in SW *vs.* FW-adapted fish, whereas plasma potassium, phosphate, total, and ionized calcium concentrations remained unchanged (data not shown).

This long-term acclimation of flounder to SW or FW did not affect CaSR mRNA expression in the CS or CNSS (Fig. 4A). Although there were no salinity related differences in CaSR mRNA expression, STC-1 mRNA expression levels were significantly lower in both the CS and CNSS (Fig. 4A) of FW *vs.* SW-adapted flounder. Notably, STC-1 mRNA expression in the CNSS of FW fish was approximately 4-fold lower by comparison to SW-adapted flounder, consistent with a potential role for STC-1 secretion from this neuroendocrine tissue in the markedly different ion regulatory demands of SW and FW fish. To determine the effect of salinity on CaSR and STC-1 protein expression, CS protein samples from SW and FW fish were analyzed by immunoblotting under reducing conditions (Fig. 4B). In FW CS, the intensity of the CaSR-specific 215–300 kDa complex was markedly reduced in intensity in comparison to SW CS glands. Furthermore, the 120–150 kDa complex evident in SW CS glands appeared as a 115–145 kDa complex in FW CS glands. Four different molecular mass forms of STC-1 between 22 and 29 kDa were identified in CS extracts from SW fish. In contrast, only three molecular mass forms were identified in CS protein samples from FW fish. The highest molecular mass band was absent in FW CS extracts, whereas densitometry confirmed a reduction in the intensity of the two lower molecular mass bands in comparison to SW samples (data not shown). Reprobing the STC-1 blot with the loading control GAPDH showed that the higher expression levels in SW fish were not due to errors in protein load-

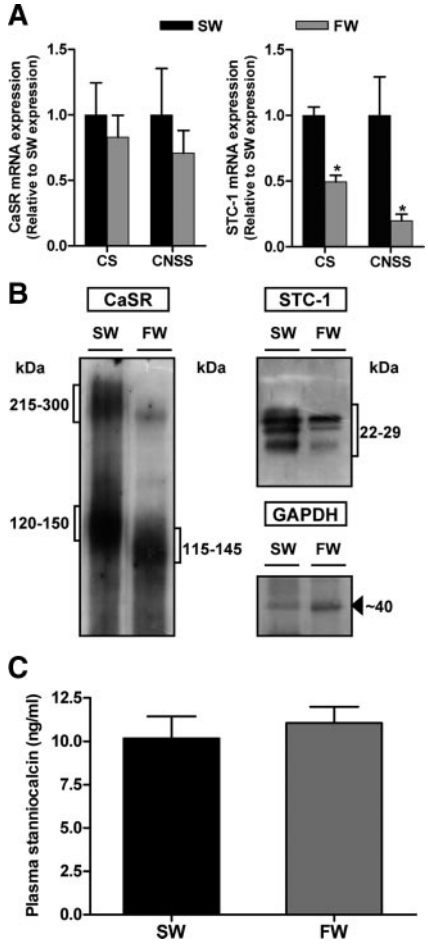

FIG. 4. Analysis of CaSR and STC-1 expression in CS and CNSS and plasma immunoreactive STC-1 of flounder long term adapted to SW or FW. A, Tissue expression levels were analyzed by real-time quantitative PCR with β-actin as the reference gene. There was no significant difference in CS or CNSS CaSR relative mRNA expression when comparing SW and FW fish. In FW fish, STC-1 relative mRNA expression was found to be significantly lower in CS and CNSS compared with SW fish. B, CS total protein samples (5 μg/lane) from SW and FW fish (n = 3) were denatured in Laemmli buffer in the presence of the reducing agent β-mercaptoethanol. Immunoblots were probed with either anti-CaSR mouse monoclonal antibody or anti-STC-1 polyclonal antiserum. For STC-1 immunoblots, protein loading was assessed by stripping and reprobing the membrane with anti-GAPDH polyclonal antibody. C, Plasma STC-1 immunoreactivity in SW and FW flounder plasma samples was measured by RIA. Independent sample *t* tests have been used to assess differences between SW and FW flounder. *Asterisks* indicate significant difference (*, $P < 0.05$). Values are means + SE of eight to 10 fish.

ing. The differences in STC-1 mRNA and protein levels in SW and FW CS did not translate into changes in circulating levels of immunoreactive STC-1 (Fig. 4C).

Experimental EGTA induction of acute hypocalcemia

To establish a link between circulating levels of ionized calcium and plasma STC-1, fish were injected with EGTA. The bolus injection of 30 μmole/100 g body weight of EGTA significantly reduced circulating levels of ionized calcium in groups sampled at 0.5, 1, 2, and 4 h, by comparison with the respective time-matched control groups (Fig. 5A), in which ionized calcium measures remained similar for all time points. These lower ionized calcium measures were associated with reduced plasma levels of immunoreactive STC-1 in the 1, 2, and 4-h sample groups compared with paired time-matched controls (Fig. 5B). All other plasma ion measures remained similar to controls during the experimental period.

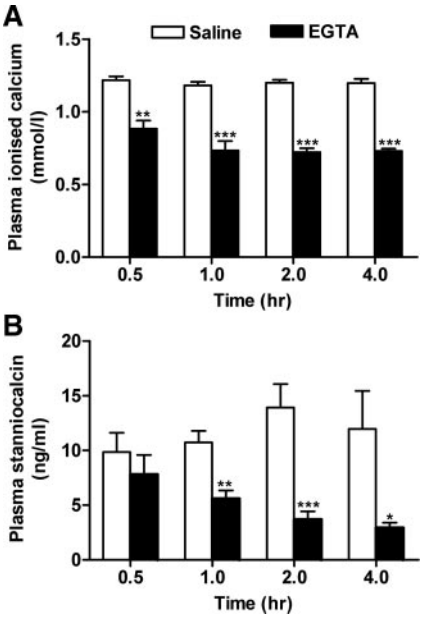

FIG. 5. The effects of single bolus ip injection of EGTA on plasma-ionized calcium and immunoreactive STC-1 in FW flounder. A, EGTA administration significantly lowered plasma-ionized calcium at all time points by comparison with paired time-matched saline controls. B, Plasma immunoreactive STC-1 levels were significantly reduced 1, 2, and 4 h (hr) after EGTA injection by comparison with paired time-matched saline controls. Results are means + SE (n = 6–7 for each group). *Asterisks* represent significant difference from paired time-matched controls (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) by independent sample *t* tests.

The effect of calcimimetics on plasma and urine composition

At time zero, ionized and total plasma calcium concentrations were similar between the two groups. The injection of R-568 significantly lowered total calcium at 0.5, 1, and 2 h (Fig. 6A), and ionized calcium levels at 1 and 2 h (Fig. 6B) when compared with time-matched S-568-treated control fish. At 4 and 8 h after R-568 injection, there was some recovery in total calcium concentration toward time zero levels. At time zero, plasma phosphate levels were similar between the two groups. The injection of R-568 resulted in the rapid onset of hypophosphatemia (Fig. 6C). Accordingly, plasma phosphate levels were significantly lower after 30 min and 1 h in R-568 compared with S-568-treated fish. Furthermore, administration of R-568 induced a steady decline in plasma magnesium levels (Fig. 6D). Plasma magnesium concentrations were similar at time zero between the two groups but were significantly reduced at the 0.5, 1, 2, 4, and 8-h sampling points. The early onset of hypocalcemia and hypophosphatemia after R-568 administration corresponded to significantly elevated levels of plasma immunoreactive STC-1 at 30 min compared with S-568-treated animals (Fig. 6E). Plasma STC-1 levels were similar in R-568 and S-568 treatment groups 8 h after administration (Fig. 6E), when both plasma calcium and phosphate measures were also similar to time-matched controls. No differences in plasma concentrations of sodium, chloride, and potassium ions or osmolality were observed over the experimental period.

The reduced plasma levels of both freely ionized and total calcium were associated with significantly higher calcium levels

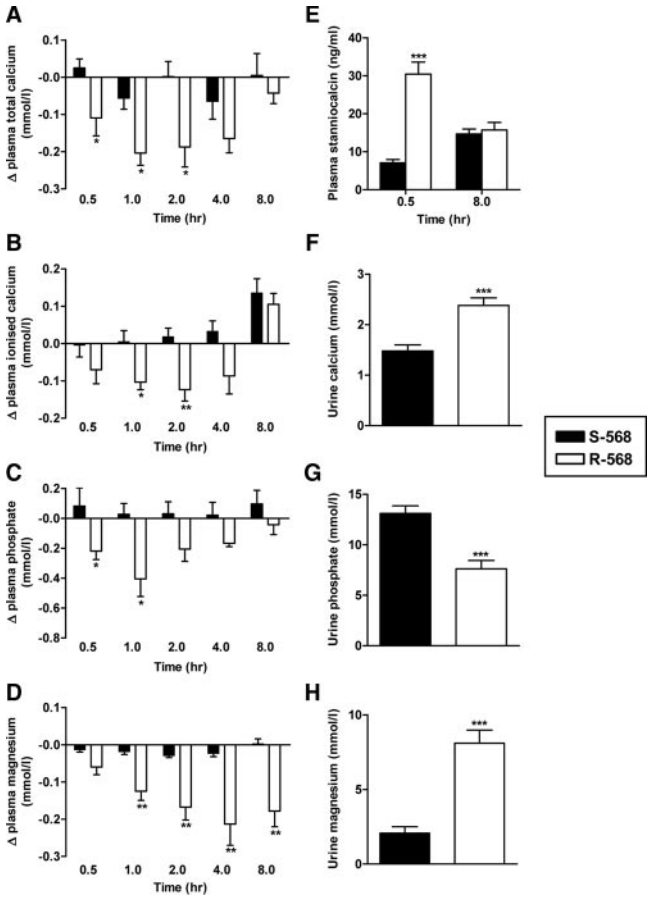

FIG. 6. The effect of a single arterial injection of 1 mg/kg R-568 (*clear bars*) or S-568 (*filled bars*) on plasma total calcium (A), ionized calcium (B), phosphate (C), total magnesium (D), STC-1 (E), and urine composition (F–H) in FW flounder. The data (A–D) are shown as changes (Δ) in plasma ion concentration from time zero measures for serial samples collected at 0.5, 1, 2, 4, and 8 h (hr) after R-568 or S-568 administration. E, Plasma STC-1 levels for two separate groups of fish terminally sampled 0.5 and 8 h after R-568 or S-568 administration. F–H, Urine composition for flounders sampled 8 h after calcimimetic administration. S-568-administered fish have been used as controls for these analyses. Significant differences by comparison with S-568-treated flounder in time-matched samples were assessed by independent sample *t* tests (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Values are means + SE (n = 8 fish per group).

in the urine of R-568 compared with S-568-treated fish 8 h after injection (Fig. 6F). Similarly, urine magnesium levels were found to be 4-fold higher (Fig. 6H), whereas urine phosphate levels were 50% lower in R-568 urine samples compared with S-568 urine measures (Fig. 6G). Sodium and potassium urine measures and osmolality were similar between the treatment groups.

Discussion

To our knowledge this is the first study in which the regulation of CaSR expression has been investigated in the CS. The flounder CaSR possessed several structural features that assisted in the assignment of this molecule as a typical CaSR. Notably, cysteines corresponding to Cys129 and Cys131 in the extracellular domain of the human CaSR are critical for receptor dimerization (34, 35). A similar orientation of these cysteines has been confirmed in the tilapia CaSR (23), suggesting that, like the human receptor, teleost CaSRs may function as disulfide-linked dimers (29). Moreover, 11 potential N-linked glycosylation sites in the extracellular domain of the flounder CaSR were found to exist at the same positions corresponding to those in mammalian CaSRs (1, 24). Therefore, the posttranslational processing of the flounder CaSR may be similar to mammalian CaSRs, with the variable addition of sugars possibly resulting in a series of different molecular mass forms (36).

The differences in molecular mass of the CaSR depending on the protein denaturation conditions reported here have been described previously in mammals (29). On SDS-PAGE under nonreducing conditions, the flounder CaSR exists in several forms with molecular masses greater than 215 kDa. The higher molecular mass forms likely represent dimeric species being greater than twice the predicted molecular mass of the flounder CaSR monomer. Addition of the SH-reducing agent β-mercaptoethanol diminished the intensity of the 215–300 kDa band with a concomitant appearance of CaSR reactive bands between 120 and 150 kDa, similar to those previously described in membrane proteins prepared from the bovine parathyroid glands under denaturing and reducing conditions (36). The 215–300 and 120–150 kDa bands for flounder were shown to be sensitive to PNGase F, similar to findings in mammals (37, 38). Together, these observations confirm that the CS CaSR exists as a disulfide-linked dimer in the cell membranes of CS cells with varying degrees of glycosylation.

The flounder CaSR mRNA was only detectable by Northern blots of total RNA from the CS among the various flounder tissues sampled. In mammalian Northern blots, CaSR mRNAs have been found to be most abundant in parathyroid cells (1). This very high level of CaSR expression in flounder CS is consistent with the proposed function of the CaSR in regulating STC-1 secretion (21). Although this is not the first report of CaSR expression in flounder CNSS, because we (39) previously identified CaSR immunoreactivity in the perikarya of Dahlgren cells and axons of the spinal cord, the high expression of the CaSR mRNA in the CNSS described in the current study was shown to correspond with high levels of STC-1 mRNA expression as for the CS. Thus, these observations lead to the notion that calcium regulation could also be modulated through the release of STC-1 from the CNSS in addition to the CS, perhaps providing a rapid reacting component through this neuroendocrine tissue. In support of this, previous unpublished work from our laboratory has shown that plasma total calcium levels were elevated (2.69 ± 0.24 *vs.* 1.96 ± 0.25 mmol/liter) 24/48 h after CNSS removal in flounder by comparison with sham-operated fish (n = 4).

The lower STC-1 mRNA expression in CS and CNSS from fish adapted to FW by comparison with SW provides further evidence of the effects of environmental salinity on CS STC-1 synthesis (22, 25). The difference in STC-1 mRNA expression between SW and FW fish occurred independently of any changes in CaSR mRNA expression or circulating levels of ionized calcium. This raises the possibility that changes in STC-1 mRNA expression and protein synthesis in fish exposed to different salinities may result from altered CaSR functional activity. Supporting this theory is the diminished expression of putative dimeric CaSR protein forms, together with altered levels of gly-

cosylation of the receptor monomer in FW compared with SW fish CS. These differences potentially occur from changes in plasma ionic strength between SW and FW fish because ionic strength alters the sensitivity of both fish and mammalian CaSRs when expressed in human embryonic kidney cells (4, 40). Despite detecting differences in mRNA expression and the molecular mass protein forms of STC-1 in CS samples of SW and FW fish, circulating levels of immunoreactive STC-1 were similar. Therefore, plasma STC-1 levels do not reflect the apparent higher rates of hormone synthesis and secretion of STC-1 from CS of SW fish in the present study. It has been shown that the metabolic clearance rate of STC-1 is significantly higher in SW by comparison with FW fish, indicating more target tissue and/or increased degradation of STC-1 in clearance organs (41), which may explain why plasma STC-1 levels were not higher in SW-adapted flounder. On the other hand, EGTA-induced hypocalcemia rapidly lowered circulating levels of STC-1, providing a strong link between circulating levels of ionized calcium and STC-1 secretion. In support of this view, EGTA has also been successfully used to induce hypocalcemia in mammals, resulting, in this case, in increased synthesis and secretion of PTH (42). The present study complements previous converse *in vivo* investigations in which increasing plasma calcium through calcium chloride administration resulted in increased circulating levels of STC-1 (17). Therefore, acute alterations in circulating levels of ionized calcium initiate appropriate modifications in circulating levels of STC-1, similar to the relationship between PTH and ionized calcium in mammals (43).

As predicted from the CaSR structure, calcimimetics were effective in flounder. There have been no previous reports of calcimimetic actions on either plasma or urine composition in fish, though R-467 administration has stimulated STC-1 secretion and reduced gill calcium uptake in rainbow trout (21). The calcimimetic, R-568, has consistently reduced PTH secretion from parathyroid cells, leading to reduced plasma levels of total and ionized calcium, phosphate and magnesium in mammals (19, 20, 44, 45). Here, we report similar responses to R-568 administration in the flounder, with a comparable rate of onset and level of hypocalcemia, hypophosphatemia, and hypomagnesia. The increased calcium and magnesium content of the urine in R-568-treated fish suggest that the changes in plasma composition occur, at least in part, through increased renal excretion of these ions. Similar to these effects of R-568 on phosphate excretion in flounder, studies in rats also reported lower levels of phosphate excretion resulting from calcimimetic administration (45). Lu *et al.* (12) have already shown that salmon STC-1 has a dose-dependent stimulatory effect on phosphate reabsorption in flounder proximal tubule primary cultures. One possible explanation for these coordinated effects is that STC-1 is promoting phosphate reabsorption to chelate the excess calcium in the extracellular fluid compartment, which subsequently may lead to increased deposition of calcium and phosphate in bone and scales. Thus, it is likely that all of the reported responses to R-568 administration potentially result from calcimimetic-induced increases in plasma STC-1 levels, evident here already 30 min after injection. This accords with the CS being a major site of CaSR expression in fish and, therefore, the primary site of calcimimetic actions.

In summary, it has been demonstrated that the CaSR is highly expressed in fish CS cells comparable with mammalian parathyroid gland cells, consistent with the evidence presented for calcium-stimulated STC-1 release in fish. The experimental series reported here provide evidence of associations between environmental salinity, CaSR, and STC-1 expression in the CS, and strongly support a role for ionized calcium in the regulation of STC-1 secretion. The reported effects of the calcimimetic are likely mediated predominantly by increased secretion of STC-1 from the CS via CaSR activation, as is the case for PTH and CT secretion in mammals. Therefore, the CaSR represents a key consistent component of the major calcium regulatory systems in fish and mammals in that they both appear to be regulated via the CaSR, albeit hypocalcemic (STC-1) mechanisms dominate in fish, whereas hypercalcemic (PTH) systems predominate in mammals.

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