Activation of gill Ca^{2+} -sensing receptor as a protective pathway to reduce Ca^{2+} -induced cytotoxicity

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Abstract

The expression of the Ca^{2+} -sensing receptor (Casr) in the endocrine gland known as the corpuscle of Stannius (CS) regulates the secretion of the hypocalcemic hormone stanniocalcin-1 (STC1) to inhibit gill Ca^{2+} uptake. Although numerous studies have reported the branchial expression of Casr and Stc1, the functions of these proteins in gills have not been elucidated yet. On the basis of recent findings regarding the autocrine/paracrine functions of STC1 in mammalian models, we proposed the hypothesis that branchial CaSR has an *in situ* 'sensing' function to regulate STC1 that maintains local Ca^{2+} homeostasis. In this study, we investigated Casr-mediated signaling and its regulation of Stc1 and cyclooxygenase-2 (Cox2) expression/function using a primary gill-cell culture model. The biochemical responses of gill cells isolated from Japanese eels to an increasing concentration of extracellular Ca^{2+} (0.1–1 mM) were tested. This stimulation led to a transient increase in phosphatidylcholine-phospholipase C (PC-PLC) activity, followed by activation of ERK and inositol 1,4,5-trisphosphate- Ca^{2+} /calmodulin-dependent protein kinase 2 (CaMK2) signaling pathways. Cotreatment with the calcimimetic R467 caused synergistic effects on Ca^{2+} -stimulated PC-PLC activity, ERK signaling, and CaMK2 signaling. The activation of the CaSR-PLC-ERK pathway was associated with increased expression levels of Stc1 and Cox2 as confirmed by the inhibition of Erk using a chemical inhibitor, PD98059. Functionally, Ca^{2+} /R-467 pretreatment was found to protect cells from thapsigargin-induced cell death. Inhibition of COX2 activity using NS398 abolished this protection, while transduction of STC1 lentiviral particles in the gill cells increased the protective effects. Collectively, our data revealed the expression of functional CaSR in gill tissues. The identification of the CaSR-STC1/COX2-mediated protective pathway in gill cells sheds light on a possible cellular protective mechanism against an increase in intracellular Ca^{2+} levels associated with transepithelial Ca^{2+} transport.

Key Words

- ▶ calcium signaling
- ▶ gene expression
- ▶ calcium
- ▶ cell culture

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Introduction

Calcium ions have a variety of beneficial roles in living organisms. Their function is pleiotropic, ranging from the regulation of signaling cascades in a single cell to

a wide variety of physiological functions at the organ level. Failure to maintain Ca^{2+} homeostasis affects a variety of molecular and cellular processes, ultimately

leading to a variety of pathological consequences. In mammals, Ca^{2+} homeostasis is maintained by the coordinated calcium (re)absorption that occurs in small intestines, kidneys, and bones, and it is under precise hormonal control (Khanal & Nemere 2008, Suzuki *et al.* 2008a). Disturbances in Ca^{2+} homeostasis have been linked to bone abnormalities, renal insufficiency, and malignancy-associated hypercalcemia (Suzuki *et al.* 2008a,b). The identification of Ca^{2+} -sensing receptor (Casr), a G-protein-coupled receptor that senses extracellular Ca^{2+} levels, has facilitated better understanding of hormonal regulation of Ca^{2+} homeostasis (Ruat *et al.* 1995). In fish, Casr has been identified in different species and is known to be expressed in both endocrine and non-endocrine tissues (Nearing *et al.* 2002, Loretz 2008). In endocrine tissues, the well-recognized function of CaSR involves the regulation of the secretion of calciotropic hormones, such as parathyroid hormone (PTH) from parathyroid glands in mammals (Chen & Goodman 2004) and stanniocalcin-1 (STC1) from the corpuscle of Stannius (CS) in teleosts (Greenwood *et al.* 2009). In non-endocrine tissues, such as gill epithelia, the function of CaSR was found to be associated with ion (Ca^{2+} , Mg^{2+} , and Na^{+}) sensing (Nearing *et al.* 2002). However, downstream responses following CaSR activation have not yet been elucidated.

The fish gill is a multifunctional organ that is involved in gaseous exchange, acid-base balance, and ion-osmoregulation. It has also developed an efficient mechanism to cope with Ca^{2+} uptake from waters with different calcium content (0.01–10 mM). In response to an increase in blood Ca^{2+} level, the CaSR-mediated STC1 release from CS glands inhibits gill Ca^{2+} uptake to restore homeostasis (Greenwood *et al.* 2009, Lin *et al.* 2014). Intriguingly, a re-examination of *stc1* gene expression in fish tissues revealed the presence of *stc1* transcript in gills of trout (McCudden *et al.* 2001). Moreover, the expression of *stc1* in gills of Japanese flounder was found to be stimulated by water salinity and Ca^{2+} (Shin & Sohn 2008). These observations prompted us to investigate the local function of the CaSR-STC1 pathway in gill tissues. We developed the hypothesis that branchial CaSR plays an *in situ* Ca^{2+} -sensing role to maintain local Ca^{2+} homeostasis via the induction of STC1 expression in cells. In this study, we have provided compelling evidence to show the presence of functional CaSR in fish gills, in which phosphatidylcholine-phospholipase C (PC-PLC)-ERK and PLC-inositol 1,4,5-trisphosphate (IP_3)- Ca^{2+} /calmodulin-dependent protein kinase 2 (CaMK2) signaling were activated by Ca^{2+} or calcimimetic cotreatment. The PLC-ERK signaling

pathway was found to be associated with the induction of STC1 and cyclooxygenase-2 (Cox2) expression. A cytotoxicity assay was conducted to demonstrate the protective effects of CaSR-mediated COX2 and STC1 induction against thapsigargin (TG)-induced cell death.

Materials and methods

Animals and gill cell isolation

Japanese eels (*Anguilla japonica*) weighing 500–600 g were reared in fiberglass tanks supplied with charcoal-filtered, aerated tap water for at least 2 weeks. The fish were anesthetized with 0.1% MS-222 (Sigma). The gills were perfused with PBS (pH 7.7) and gill arches were excised, washed, and cut into small fragments for enzymatic digestion (Wong & Chan 1999, Tse *et al.* 2006). After cell dissociation, the resuspended gill cells were layered on a two-step Percoll gradient solution (1.03 and 1.09 g/ml in PBS) and centrifuged at 2000 *g* at 15 °C for 20 min. The isolated gill cells at the interface of 1.03–1.09 g/ml were collected and washed with PBS followed with Ca^{2+} - and Mg^{2+} -free Hank's balanced salt solution (HBSS, Gibco).

PC-PLC assay and western blotting

Isolated gill cells in Ca^{2+} - and Mg^{2+} -free HBSS medium were treated with i) 0, 0.1, 0.5, or 1 mM Ca^{2+} ; ii) 1 μM of the calcimimetics R467 or S467 (NPS Pharmaceuticals, Salt Lake, City, UT, USA); or iii) 1 μM of R467 or S467 + 0.1 mM Ca^{2+} for 5–30 min at 22 °C. For some experiments, the cells were pretreated for 5 min with an IP_3 inhibitor (40 μM 2-APB, Calbiochem, Darmstadt, Germany). The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris of pH 7.4, 150 mM NaCl, 1% NP40) at 5, 15, or 30 min post-treatment. After centrifugation at 13 000 *g* for 15 min at 4 °C, the supernatant was collected and the total protein concentration was determined by the Bradford method (Bio-Rad). The protein lysates (15 μg /sample) were analyzed for PC-PLC activity using an Amplex Red PC-PLC assay kit according to the manufacturer's instruction (Molecular Probes, Eugene, OR, USA). The reactions were incubated in darkness at 37 °C for 1 h. The fluorescence was measured with a micro-plate reader (VICTOR X4 Multilabel Plate Reader, Perkin-Elmer Life Sciences, Boston, MA, USA) with excitation at 530 nm and emission at 590 nm. The protein lysates containing 20–40 μg total cellular protein in RIPA buffer were subjected to electrophoresis on 10% polyacrylamide gels. The gels

were then blotted onto PVDF membranes (Perkin-Elmer Life Sciences). Western blotting was carried out using mouse antibody against CaSR (Thermo Fisher Scientific, San Jose, CA, USA), rabbit antibody against ERK, pERK1/2, pCaMK2, COX2 (Cell Signaling, Danvers, MA, USA), or STC1 (Origene, Rockville, MD, USA) followed by incubation with (1:4000) HRP-conjugated goat anti-mouse/rabbit antibody (Bio-Rad). The specific bands were visualized using a chemiluminescent reagent (Western-lightening Plus, Perkin-Elmer Life Sciences). The blots were then washed in PBS with 0.5% Tween-20 and re-probed with mouse anti-actin serum (1:100; JLA20, Developmental Studies Hybridoma Bank, the University of Iowa, USA).

Gene expression assay

The isolated gill cells in Ca^{2+} - and Mg^{2+} -free HBSS medium were treated with i) 0, 0.1, and 0.5 mM Ca^{2+} ; ii) 1 μM R467 or S467; or iii) 1 μM R467 or S467 + 0.1 mM Ca^{2+} for 24 h at 22 °C. For some experiments, the cells were pretreated for 5 min with an IP_3 inhibitor (40 μM 2-APB, Calbiochem), ERK inhibitor (10 μM PD98059, Calbiochem), or intracellular Ca^{2+} chelator (10 μM BAPTA, Calbiochem). Total RNA was extracted from gill tissues using a TRI reagent (Molecular Research Centre, Oxford, UK). Those with an A_{260}/A_{280} ratio of 1.8–2.0 were used in this study. For cDNA synthesis, 0.5 μg total cellular RNA was reversed transcribed using a high capacity RNA-to-cDNA kit (Applied Biosystems). Real-time

PCRs were carried out using the StepOne real-time PCR system using power SYBR green PCR master mix (Applied Biosystems). The verified gene-specific primers (*gapdh*-F: GCGCCAGCCAGAACATCATC, *gapdh*-R: CGTTAAGCTCGGGGATGACC; *stc1*-F: CTCAGAGGTTTCAGGAGGAGT, *stc1*-R: GGTGCTGTAGTACCTGTTGG; *cox2*-F: TAGCCACCAACCTACAATG, *cox2*-R: TGAGCAAAAAAGGCAAACAC; *ecac1*-F: GCAGCCCAATAAAACCATCGC, *ecac1*-R: CCACCA-CATTGCCCTCTCTG) specific to transcripts of Japanese eels were used. The copy number of transcripts for each sample was calculated with reference to parallel amplification of known concentrations of the respective cloned PCR fragments. The occurrence of primer-dimers and secondary products was inspected using melting curve analysis. Our data indicated that amplification was specific for each individual set of primers. Control amplification was done either without reverse transcriptase or without RNA. The relative expression ratio of (*stc1*, *cox2*, or *ecac1*):*gapdh* was calculated according to the method described by Pfaffl (2001). The expression ratio = $E_{stc1}^{CP_{stc1}(\text{control} - \text{treatment})} / E_{gapdh}^{CP_{gapdh}(\text{control} - \text{treatment})}$, where $E = 10^{(-1/\text{slope})}$ and CP is the crossing point at which fluorescence rises above background levels.

Cytotoxicity assay

Isolated gill cells in Ca^{2+} - and Mg^{2+} -free HBSS medium were pretreated with i) 0–0.1 mM Ca^{2+} , ii) 1 μM R467,

Figure 1

Detection of gill CaSR and effects of Ca^{2+} on PL-PLC-ERK signaling pathway. (A) A representative western blot shows that a CaSR MAB recognized a single band (~95 kDa) in gill cells. (B) The relative activity of PC-PLC in gill cells post-exposed to Ca^{2+} for the indicated durations. (C) A representative western blot illustrates the levels of pERK1/2 and

ERK1/2 in cells post-exposed to Ca^{2+} for the indicated durations. The band intensities were measured and quantified. Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple test ($P < 0.05$).

iii) 1 μM R467 + 0.1 mM Ca^{2+} , iv) COX2 inhibitor (2 μM NS-398, Cayman, Ann Arbor, MI, USA), or v) 2 μM NS-398 + 1 μM R467 + 0.1 mM Ca^{2+} for 24 h at 22 °C. The cells were then treated with TG (2 μM , Calbiochem) for 4 h. The culture medium was collected to measure the activity of lactate dehydrogenase (LDH) using the LDH Cytotoxicity Assay Kit (Cayman) according to the manufacturer's instructions. The absorbance at 490 nm was detected using a microplate reader (Bio Tek, ELX800 (Bio Tek, Winooski, VT, USA)).

STC1 expression constructs and lentiviral transduction in primary gill cell culture

To prepare the pLenti6.3/TO/V5-DEST-STC1 plasmid, eel cDNA encoding WT full-length Stc1 transcript without the stop codon was amplified by PCR and cloned into pENTR/SD/D-TOPO (Invitrogen) according to the manufacturer's instructions. The STC1 insert was transferred from pENTR/SD/D-TOPO into the expression vector pLenti6.3/TO/V5/-DEST (Invitrogen) using the Gateway LR Clonase™ II Plus Enzyme Mix (Invitrogen). The sequence of the STC1 insert was verified by DNA sequencing.

A primary gill cell culture was prepared as described previously (Tse *et al.* 2008, Chow & Wong 2011). After enzymatic digestion of gill tissues, the cell suspension was washed and resuspended in Leibovitz's L-15 medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 1% penicillin/streptomycin, and 0.5% fungizone (Gibco, Invitrogen). The cells were seeded at a density of 2×10^6 cells/cm² onto a collagen-coated culture plate (Iwaki, Tokyo, Japan). The culture was incubated at 22 °C in a growth chamber with humidified air. For lentiviral transduction, lentivirus was first prepared by co-transfection of HEK293FT cells with ViraPower™ packaging mix (Invitrogen) and either pLenti6.3/TO/V5-DEST-STC1 (pLenti-STC1) or pLenti6.3/TO/V5/-DEST using Lipofectamine 2000. Viral supernatants were harvested at 48 h after transfection. The viral supernatant was filtered through a 0.45 μm filter to eliminate cellular debris followed by precipitation in a 1:4 solution of PEG-it Virus precipitation solution and at 4 °C for overnight incubation. After centrifugation (1500 *g* for 30 min), the precipitated lentiviral particles were resuspended in Leibovitz's L-15 medium. The primary gill cells were transduced with 2 ml of (a) pLenti-Ctrl or (b) pLenti-STC1 virus containing medium with 6 $\mu\text{g}/\text{ml}$ of polybrene (Sigma). After 24 h incubation at 22 °C, the cells were washed and replaced using the fresh Leibovitz's L-15 medium. The cells were then treated with 2 μM TG for 4 h.

The culture medium was collected to measure the activity of LDH. The overexpression of Stc1-V5 in gill cells was verified by western blotting using V5 antibody. Total RNA was collected for real-time PCR assay of *stc1* and *ecacl* transcripts.

Statistical analysis

Drug treatments were carried out in triplicate and each experiment was repeated at least three times. All data are represented as mean $\pm$ S.E.M. Statistical significance was

Figure 2

Effects of Ca^{2+} and calcimimetics on the PL-PLC-ERK signaling pathway. Isolated gill cells in Ca^{2+} - and Mg^{2+} -free HBSS medium were incubated with Ca^{2+} (0.1 or 0.5 mM), R467, and/or S467 (1 μM) for 5 min. (A) The activity of PC-PLC in gill cells post-exposed to Ca^{2+} , R467 (R), and/or S467 (S) for 5 min. (B) Analysis of a representative western blot illustrates the levels of pERK1/2 and ERK1/2 in cells post-exposed to Ca^{2+} , R467, and/or S467 for 5 min. A synergistic effect of Ca^{2+} /R467 cotreatment was observed for the activation of ERK1/2 pathway. The band intensities were measured and quantified. Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple test ($P < 0.05$).

assessed using Student's *t*-test or one-way ANOVA followed by Duncan's multiple range tests. Groups were considered significantly different for $P < 0.05$.

Results

Detection of gill CaSR and the effects of Ca^{2+} and/or calcimimetic treatment on the activation of the PC-PLC-ERK signaling pathway in isolated gill cells

The presence of CaSR in gill cells was revealed by western blotting (Fig. 1A). The treatment of gill cells with Ca^{2+} (0.1–1 mM) stimulated PC-PLC activity in a dose-dependent manner (Fig. 1B). The stimulation was rapid but transient. The stimulated activity returned to levels similar to those in controls within 30 min. The western blotting analysis of PC-PLC downstream signaling revealed the activation of the ERK pathway in a dose- and time-dependent manner (Fig. 1C).

The treatment of cells with calcimimetics (i.e., R467 or S467) alone for 5 min had no noticeable stimulatory effects on PC-PLC activity compared with the effects of treatment with 0.1 or 0.5 mM Ca^{2+} (Fig. 2A). However, cotreatment of R467 with 0.1 mM Ca^{2+} caused synergistic effects on Ca^{2+} -stimulated PC-PLC activity. Similarly, the synergistic effect of Ca^{2+} /R467 cotreatment was observed in the activation of ERK pathway (Fig. 2B). The synergistic effect was not detected with S467/ Ca^{2+} cotreatment.

Effects of Ca^{2+} and/or calcimimetic treatment on the activation of CaMK2 in isolated gill cells

Treatment of cells with Ca^{2+} (0.1–1 mM) for 5 min stimulated phosphorylation of CaMK2 in a dose-dependent manner (Fig. 3A). No significant effects were observed with calcimimetic (i.e., R467 or S467) treatment compared with the effects of 0.1 or 0.5 mM Ca^{2+} (Fig. 3B). Cotreatment with 0.1 mM Ca^{2+} and either R467 or S467 caused synergistic effects on Ca^{2+} -stimulated CaMK2 phosphorylation. The effect of R467 was significantly greater than that of S467 in the cotreatment. The Ca^{2+} or Ca^{2+} /calcimimetics-induced phosphorylation of CaMK2 was blocked by the addition of the IP_3 inhibitor 2-APB (Fig. 3C).

Effects of Ca^{2+} and/or calcimimetic treatment on *stc1* and *cox2* mRNA and protein expression in isolated gill cells

Treatment of gill cells with Ca^{2+} (0.1 or 0.5 mM) resulted in greater cell viability (Supplementary Figure 1, see section on supplementary data given at the end of this

Figure 3

Effects of Ca^{2+} and/or calcimimetics on the CaMK2 signaling pathway. Isolated gill cells in Ca^{2+} - and Mg^{2+} -free HBSS medium were incubated with Ca^{2+} (0.1–1 mM), R467, S467 (1 μM), and/or IP_3 inhibitor (40 μM 2-APB) for 5 min. The representative western blots illustrate the levels of pCaMK2 in cells post-exposed to (A) Ca^{2+} for the indicated durations; (B) Ca^{2+} , R467 (R), and/or S467 (S); (C) Ca^{2+} , R467 (R), S467 (S), and/or 2-APB. The band intensities were measured and quantified. Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple test ($P < 0.05$).

Figure 4

Effects of Ca^{2+} and/or calcimimetics on the expression levels of STC1 and COX2. Isolated gill cells in Ca^{2+} - and Mg^{2+} -free HBSS medium were incubated with Ca^{2+} (0.1 or 0.5 mM), R467, S467 (1 μM), and/or ERK inhibitor (10 μM PD98059, PD) for 24 h. The (A) mRNA and (B) protein

expression levels of STC1 and Cox2 are shown. Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple test ($P < 0.05$).

article) and significant increases in the mRNA (Fig. 4A) and protein (Fig. 4B) expression levels of Stc1 and Cox2. Calcimimetic treatment had no significant effects on the expression levels of these genes. Cotreatment with 0.1 mM Ca^{2+} and either R467 or S467 caused synergistic stimulation of *stc1* and *cox2* mRNA expression. The effect of R467 was greater than that of S467 in the context of cotreatment. The stimulatory effects of Ca^{2+} or Ca^{2+} /calcimimetics on Stc1 and Cox2 expression were abolished by cotreatment with the ERK inhibitor PD98059. In addition, we tested the effects of an IP_3 inhibitor (2-APB)

and an intracellular Ca^{2+} chelator (BAPTA) on Ca^{2+} and/or calcimimetic-induced mRNA expression of *stc1* and *cox2* in gill cells. However, no significant effects were observed (data not shown).

Effects of CaSR activation, STC1 overexpression, and COX2 inhibition (NS-398) on TG-induced cell death

TG treatment is commonly used to examine the cytotoxic effects of increased intracellular free Ca^{2+} levels. Pretreatment of gill cells with Ca^{2+} and R467 significantly reduced

Figure 5

Effect of Ca^{2+} and/or R467 on thapsigargin (TG)-induced cellular cytotoxicity. Isolated gill cells in Ca^{2+} - and Mg^{2+} -free HBSS medium were incubated with Ca^{2+} (0.1 mM) R467 (1 μM), ERK inhibitor, and/or COX2 inhibitor (2 μM NS-398) for 24 h followed by 2 μM TG treatment for 4 h. Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple test ($P < 0.05$).

TG-induced cytotoxic effects (Fig. 5). Treatment with the COX2 inhibitor NS398 increased basal LDH activity and abolished the protective effects. The viability of gill cells incubated in Ca^{2+} - and Mg^{2+} -free HBSS medium for 24 h was less than that of the cells maintained in Ca^{2+} -containing medium.

Transduction with pLenti-STC1 reduced TG-induced cytotoxicity and *ecac1* mRNA expression levels (Fig. 6A and B). The expression of high levels of STC1 was confirmed in pLenti-STC1 transduced cells, using an antibody against the V5 epitope (Fig. 6C).

Discussion

The expression and function of CaSR has been known for nearly 20 years. Most studies on CaSR have focused on mammalian models to identify its role in the regulation of calciotropic hormones and maintenance of extracellular Ca^{2+} homeostasis. Altered CaSR expression has been shown to be associated with many pathological conditions, including bone abnormalities, renal insufficiency, and malignancy-associated hypercalcemia (Suzuki *et al.* 2008a,b). The cloning and characterization of CaSR expression were reported by Nearing *et al.* (2002) using piscine models. The expression of CaSR in renal proximal tubules, gill cells, pituitary gland, and olfactory lobe was reported for tilapia and Japanese eels (Loretz *et al.* 2009). Surprisingly, little is known about the functional roles of CaSR in fish gills, which are the major organs involved

in Ca^{2+} uptake from aquatic environments. In the present study, our western blotting analysis data support the presence of Casr expression in gill tissues of Japanese eels. The molecular size of the eel Casr protein is similar to the expected molecular mass of non-glycosylated Casr core protein in tilapia (Loretz *et al.* 2004). Moreover the treatment of isolated gill cells with Ca^{2+} or Ca^{2+} /calcimimetics activated the conserved CaSR-mediated pathways to increase PLC activity and ERK- and IP_3 /CaMK2-signaling. The R-enantiomer used in this study, R467, is classified as a type II calcimimetic and functions as a positive allosteric modulator of CaSR to amplify receptor sensitivity to Ca^{2+} (Harrington & Fotsch 2007). The S-enantiomer S467 is known to be less active

Figure 6

Effects of *stc1* overexpression on thapsigargin-induced cell death and *ecac1* mRNA expression. Primary gill cells were transduced with pLenti-Ctrl or pLenti-STC1 virus-containing medium and were treated with 2 μM TG for 4 h. (A) The culture medium was collected for the measurement of LDH activities. (B) The cells were lysed for total RNA extraction for real-time PCR analysis. The transduction of cells with pLenti-STC1 reduced TG-induced cytotoxicity and the mRNA expression levels of *ecac1*. (C) The overexpression of *Stc1*-V5 in gill cells was verified by real-time PCR (the left panel) and western blotting using anti-V5 antibody (the right panel). Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple test ($P < 0.05$).

Figure 7

A schematic to illustrate the activation of the CaSR-mediated signaling pathway, stimulation of STC1 and COX2 expression, and the possible downstream effects on the reduction of intracellular free Ca^{2+} levels. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0060>.

(Nemeth *et al.* 2004). Since R467 is not a CaSR agonist, it had no significant effect on the activation of PLC/ERK pathway. Nevertheless, the stimulation of CaSR with Ca^{2+} and/or calcimimetics increased the expression levels of Stc1 and Cox2 in gill cells. Experimental studies demonstrated that CaSR-induced STC1 and COX2 expression protected cells from Ca^{2+} -induced cytotoxicity upon TG treatment. The present study supports the notion that branchial expression of functional CaSR facilitates the *in situ* regulation of cellular Ca^{2+} homeostasis.

The Ca^{2+} -sensing capability of CaSR relies on its large extracellular domain (Brown & MacLeod 2001, Loretz 2008), which is known to be the target of a variety of metabolic signaling molecules, including divalent and multivalent cations, aromatic L-amino acids, polycations, and polyamines (Brown & MacLeod 2001, Hofer & Brown 2003, Breitwieser *et al.* 2004, Riccardi & Maldonado-Perez 2005, Conigrave & Hampson 2006). In mammalian models, sensitivity of CaSR to Ca^{2+} was affected by local ionic strength and pH (Quinn *et al.* 1998, 2004), where the activation of CaSR results in the stimulation of PLC and Ca^{2+} mobilization (Hofer & Brown 2003). A number of studies have reported the expression of CaSR in ion-transporting tissues (i.e., gill, kidney, and intestine) in shark (Nearing *et al.* 2002), tilapia (Loretz *et al.* 2004), and flounder (Greenwood *et al.* 2009). Although the expression of CaSR was found to be associated with ion (Ca^{2+} , Mg^{2+} , and Na^{+}) sensing (Nearing *et al.* 2002), downstream responses following CaSR-activation were not known. This prompted us to study the role of CaSR

in gill tissues. In this study, we provided evidence that the treatment of cells with Ca^{2+} or Ca^{2+} /calcimimetics activated PLC-ERK and IP_3 -CaMK2 pathways. The data on PLC-ERK activation are consistent with reports on ectopic expression of tilapia or human CaSR in embryonic kidney cells (Loretz *et al.* 2004, Davies *et al.* 2006) or in bovine parathyroid cells respectively (Kifor *et al.* 2001). Although the activation of CaSR-induced PLC- IP_3 signaling in fish tissues has not been reported previously, a recent study using isolated mouse juxtaglomerular cells described this post-receptor IP_3 pathway (Ortiz-Capisano *et al.* 2013). Accordingly, stimulation of CaSR-activated PLC resulted in an increase in IP_3 and cytosolic Ca^{2+} levels. As CaMK2 is a well-known target of cytosolic free Ca^{2+} (Famulski *et al.* 2003, Francescotto *et al.* 2010), the activation of CaSR-mediated PLC- IP_3 signaling stimulates CaMK2 through phosphorylation. Our data verified that this conserved pathway was activated in the gill-cell model. Taken together, the data collected in this study illustrated the evolutionary conservation of Ca^{2+} sensing-mediated signaling pathways in fish gills. Little was previously known about downstream targets of CaSR in gill cells.

Activation of the CaSR pathway is known to regulate the release of calciotropic hormones in mammals (i.e., PTH, calcitonin) and fish (i.e., STC1). These hormones act on their target tissues to regulate transepithelial Ca^{2+} transport (Radman *et al.* 2002, Greenwood *et al.* 2009, Brown 2013, Lin *et al.* 2014). In fish, the endocrine function of STC1 from the CS gland is known to reduce

serum Ca^{2+} levels via an inhibition of Ca^{2+} uptake across gills (Yeung *et al.* 2012). As fish gill is not an endocrine tissue, the identification of branchial expression of *Stc1* prompted us to further investigate their roles in local Ca^{2+} transport and homeostasis (McCudden *et al.* 2001). In this study, the activation of CaSR was found to be associated with increased expression levels of COX2 and STC1. This stimulation was found to be mediated by PLC-ERK signaling but not by the PLC-IP₃ pathway. To elucidate the functional significance of this signaling, we adopted a TG-induced cell death model, wherein the treatment is known to increase intracellular free Ca^{2+} level and cause cell apoptosis (Carlberg *et al.* 1996, Zeiger *et al.* 2011). Our results indicated that Ca^{2+} and/or calcimimetic treatment in gill cells significantly reduced TG-induced cell death. The protective effects were abolished by the addition of the COX2 inhibitor NS-398. The overexpression of lentivirus-driven STC1 protected the cells from TG-induced cytotoxicity and reduced the expression levels of epithelial Ca^{2+} channel (*ecacl*), a gatekeeper of Ca^{2+} uptake that plays an active role in apical entry of transcellular Ca^{2+} . This observation was in agreement with results from a STC1-morpholino study in zebrafish, indicating a negative regulatory effect of STC1 on gene expression of *ecacl* (Tseng *et al.* 2009). These observations were consistent with results from studies on mammals in illustrating the roles of COX2 (Johnson *et al.* 2002, Choudhary *et al.* 2003, Ogata *et al.* 2006) and STC1 (Yeung *et al.* 2012) in the regulation of intracellular Ca^{2+} homeostasis and apoptosis. Therefore, STC1 and COX2 may play important roles in cytosolic Ca^{2+} homeostasis in fish-gill tissues.

Life has evolved from aquatic environments. The study described here provides a fundamental understanding of the importance of *in situ* Ca^{2+} -sensing in the non-endocrine tissue, fish gill epithelia. The results of this study may provide a new perspective to reveal the functions of CaSR in the mammalian system. In summary, the results of this study demonstrated that branchial CaSR-mediated extracellular Ca^{2+} and/or calcimimetic activation of PLC-ERK and PLC-IP₃-CaMK2 signaling pathways (Fig. 7). Moreover, the activation of the CaSR-PLC-ERK signaling axis was responsible for the stimulation of *stc1* and *cox2* gene expression, which protected gill cells from Ca^{2+} -mediated cell injury and cell death. The identification of this CaSR-STC1/COX2-mediated protective pathway in the Ca^{2+} transport epithelium of gill tissue sheds light on a possible cellular protective mechanism associated with transcellular Ca^{2+} transport.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0060>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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