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Original Paper

Determination of Calcium Sensing Receptor in the Scales of Goldfish and Induction of Its mRNA Expression by Acceleration Loading

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Abstract

It is known that the teleost scale regenerates after being removed. We previously reported that the osteogenesis in regenerating scales was very similar to that in calvarial bone, which suggests that regenerating scale can be used as a model for osteogenesis. In the present study, we determine calcium sensing receptor (CaSR) cDNA from the regenerating scales of goldfish. The determined partial sequence was coded as a 258-amino acid protein. The amino acid identity of this receptor to teleost CaSRs is 89 to 96%, lizard CaSR is 82%, and mammalian CaSR is 83 to 84%. These results indicate that CaSR is highly conserved in fish, lizard and mammals. Then, to examine the role of CaSR in bone metabolism, effect of acceleration loading (3 *G*) by vibration on the expression of CaSR mRNA in the regenerating scales of goldfish was investigated. We found that CaSR mRNA expression increased after acceleration loading by vibration in the regenerating scales. Significant difference between control and treatment group was observed at 6 and 24 h of incubation. Our cloned CaSR appears to act for the metabolism of bone in the regenerating scales. This is the first demonstration, to our knowledge, of an effect of hyper-loading on the induction of CaSR mRNA expression of hard tissues. ©2012 Jpn. Soc. Biol. Sci. Space; doi: 10.2187/bss.26.26

***Key words*: CaSR, regenerating scale, osteoblasts, acceleration loading by vibration**

Introduction

The calcium sensing receptor (CaSR), a G protein-coupled receptor that responds to extracellular calcium, was first determined from bovine parathyroid gland (Brown *et al.*, 1993). Therefore, it is considered that CaSR plays a central role in controlling systemic calcium homeostasis, predominately through its effects on the regulation of parathyroid hormone (PTH) secretion by the parathyroid glands and on urinary calcium excretion by the kidney (Brown *et al.*, 1993; Brown and MacLeod, 2001). However, it was reported that CaSR located in many tissues and was involved in cell proliferation differentiation, and apoptosis (Hofer and Brown, 2003; Kwak *et al.*, 2005; Smajilovic *et al.*, 2006; Xu *et al.*, 2012). In the MC3T3-E1 cells, an osteoblast-like cell line, high extracellular calcium stimulated cell proliferation (Yamaguchi *et al.*, 1998). As CaSR was expressed in the MC3T3-E1 cells, calcium signaling is seems to transmit *via* CaSR in the MC3T3-E1 cells (Yamaguchi *et al.*, 1998; Godwin and Soltoff, 2002). Therefore, it is considered that CaSR regulates osteoblastic functions such as bone development and mineralization (Theman and Collins, 2009; Dvorak-Ewell *et al.*, 2011). On the other hand, the bone matrix plays an important role for response to

physical stimuli (Harter *et al.*, 1995; Owan *et al.*, 1997; Hoffler *et al.*, 2006). In addition, Sun *et al.* (2012) reported that mechanical stretch induced calcium efflux from bone matrix and stimulated osteoblasts. As few techniques for culture system of bone cells including bone matrix have been developed, the function of CaSR under physical stimuli has not elucidated yet.

The teleost scale is a calcified tissue that contains osteoblasts, osteoclasts, and the bone matrix of two layers (bony layer: a thin, well-calcified external layer; a fibrillary layer: a thick, partially calcified layer) (Bereiter-Hahn and Zylberberg, 1993; Suzuki *et al.*, 2000; Yoshikubo *et al.*, 2005; Suzuki *et al.*, 2007; Ohira *et al.*, 2007). Furthermore, it is known that the teleost scale regenerates after being removed. In our previous study, we reported that the osteogenesis in regenerating scales was very similar to that in calvarial bone (Yoshikubo *et al.*, 2005). The response of osteoblasts to estrogen in regenerating scales was higher than that in developed normal scales (Yoshikubo *et al.*, 2005). Using the regenerating scales, we previously demonstrated that scale osteoblastic activity responses to low-loading (3 *G*) for 10 min by vibration increased (Suzuki *et al.*, 2009). As mammalian osteoblasts are activated by high-loading from 5 to 50 *G* (Gebken *et al.*, 1999; Saito *et al.*, 2003; Searby., 2005), the sensitivity to hyper-loading response in the regenerating scales possibly be high when compared with mammalian osteoblasts.

To examine the response of CaSR to hyper-loading, the cDNA of CaSR was cloned from the regenerating scales of goldfish and then the effect of acceleration loading by vibration on CaSR mRNA expression was investigated.

Materials and Methods

Animals

Both female and male goldfish (*Carassius auratus*) (20-30 g) were purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) and artificially fertilized. The hatched goldfish were kept in an aquarium at 25˚C under a daily photoperiod cycle of 12 h light: 12 h darkness. Goldfish were provided *ad libitum* diets every morning. After having adequate size (around 15 g), the female goldfish were used in the experiment because we previously reported that the sensitivity for a calcemic hormone, such as estrogen and calcitonin, was higher in mature female than in mature male goldfish (Suzuki *et al.*, 2000). All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

Cloning of CaSR from the scales of goldfish

Goldfish were anesthetized with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich, Inc., MO, USA), and the developed normal scales on the body were then removed to allow the regeneration of scales. On day 14, goldfish were anesthetized again, and the regenerating scales were removed and kept at -80˚C. Using the regenerating scales, CaSR was cloned.

Total RNAs were extracted from the regenerating scales of goldfish using a kit (Qiagen GmbH, Hilden, Germany). After cDNA synthesis (Qiagen GmbH), PCR was performed. The primers (5'-GGGATCAGYTTTGTTYTMTGCATC-3' and 5'-CCTCMACAGCYGARACAAACTT-3') were designed from conserved region among *Sparus aurata* (AJ289717), *Oreochromis niloticus* (XM_003446845), *Platichthys flesus* (FJ755006), *Salmo salar* (NM_001126231) and *Danio rerio* (XM_684005). Then, 5' rapid amplification of cDNA ends (RACE) were performed with nested primer (5'-CACCAGTAGGACACGATTGG-3' for 5'-RACE, bold and italic type in Fig.1) designed using the obtained sequence (BD SMART RACE cDNA amplification kit, BD Biosciences Clonetech, California, USA). The products were put into vector by TA cloning and then sequenced on ABI 310 sequencer (Applied Biosystems, California, USA). After determination of the sequences, the amino acid sequences were aligned using the clustal w program (Chenna *et al.*, 2003).

Acceleration-loading apparatus

Our loading apparatus has an acceleration-loading part and an acceleration-monitoring part. The detail was described in Suzuki *et al.* (2007). The acceleration-loading part, which consists of a sampling tube-loading stage and an aluminum plate with 2 vibration motors, is hung with 4 springs. Two vibration motors can provide a sinusoidal wave of acceleration ranging from 0.5 to 12 *G* in amplitude and from 8 to 50 Hz in frequency. The acceleration-monitoring part consists of a piezoelectric accelerometer, a charge amplifier, an A/D converter board, and a personal computer. A piezoelectric accelerometer, equipped on a sampling tube-loading stage, converts the loading acceleration into an analog electric signal. The signal was amplified with a charge amplifier (Yamco 4101, Yamaichi Electronics, Osaka, Japan) and transmitted *via* a 12-bit A/D converter board to a personal computer. Using software (LaBDAQ2000, Matsuyama Advan, Ehime, Japan), the PC allows us to monitor the real-time loading acceleration of the vibration. Therefore, we can load accurate gravity to the scales in a micro tube by confirming the amplitude and frequency of acceleration shown on the monitor screen.

Effect of acceleration loading by vibration on CaSR mRNA expression in the scales of goldfish

As described above, the regenerating scales were prepared and put into a 1.5 ml micro tube then 700 μl of Leibovitz's L-15 medium (Invitrogen, Grand Island, NY, USA) and a 1% penicillin-streptomycin mixture (ICN Biomedicals, Inc., OH, USA) were added. To fix the scales, a cotton ball (diameter 1 cm) was placed into a micro tube. The tube containing scales was loaded to 2 *G* acceleration by vibration for 10 min at room temperature. The loading times were determined according to our previous study (Suzuki *et al.*, 2007; Suzuki *et al.*, 2009). The lines on the left side were used as the treatment group, while those on the opposite side were used as the control group. In the loading, different parallel experiments using six goldfish were conducted. After loading for 10 min, the scales were incubated for 3-, 6-,

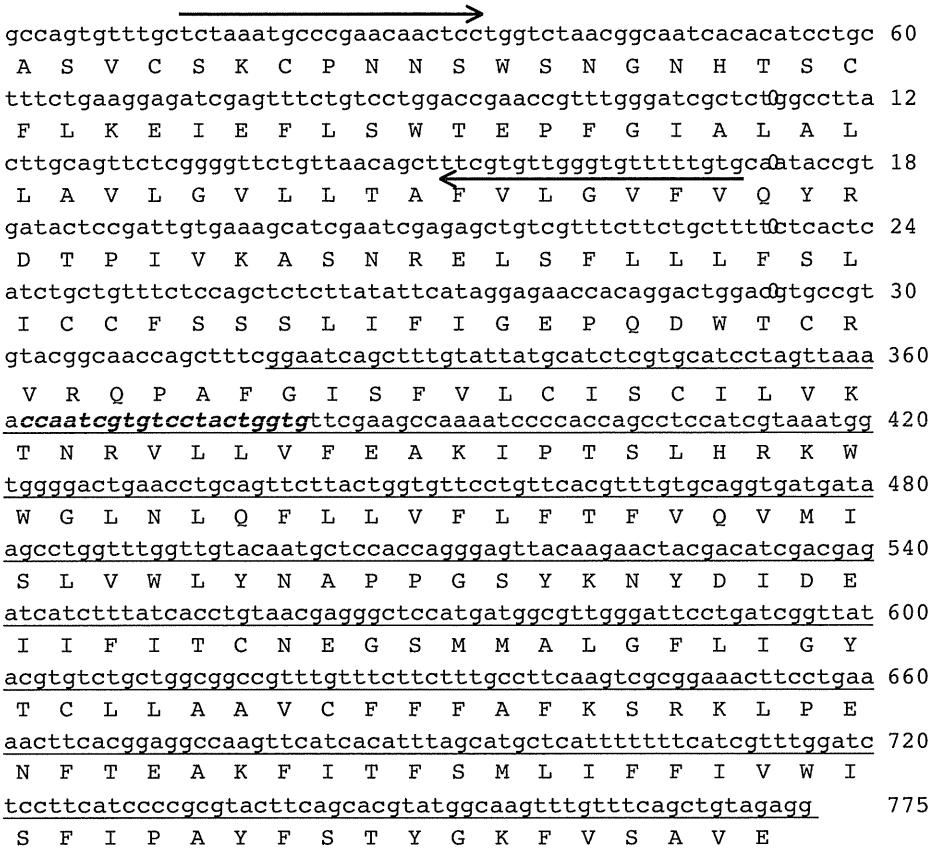

Fig. 1. Partial cDNA sequences of goldfish CaSR and the putative amino acid sequences. The underlined region is an obtained sequence by the first PCR. The CaSR mRNA expression in the scales of goldfish due to the effect of acceleration loading by vibration was examined using the primers shown by arrows. The primer for 5'-RACE indicated by bold and italic type. This data has been available under Genbank accession no. AB713518.

12-, and 24-h at 15˚C. After incubation, the scales were frozen at -80˚C until using for mRNA analysis. The loaded scales (3 *G*) were compared with unloaded (1 *G* control) scales.

After extraction of total RNAs, cDNA synthesis was performed using a kit (Qiagen GmbH). Gene-specific primers for CaSR (sense: 5'-TCTAAATGCCCGAACAACTCC-3'; antisense: 5'-CACAAAAACACCCAACACGA-3') (arrow in Fig.1) were design from the obtained sequence of goldfish CaSR. The amplification of β-actin cDNA was performed using a primer set (5'-CGAGCGTGGCTACAGCTTCA-3'; 5'-GCCCGTCAGGGAGCTCATAG-3') (Azuma *et al.*, 2007). The PCR amplification was analyzed by a real-time PCR apparatus (Mx3000p, Agilent Technologies, CA, USA) (Suzuki *et al.*, 2011). The annealing temperature for amplification of CaSR and β-actin was 60˚C. The mRNA levels were normalized to the β-actin mRNA level.

Statistical analysis

All results are expressed as the means ± SEM (n = 6). The statistical significance was assessed by paired *t*-test.

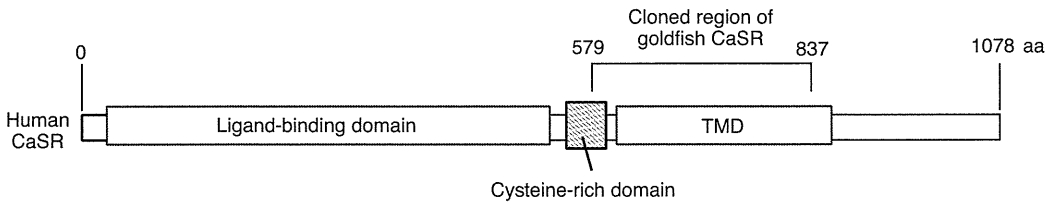

Fig.2. The molecular structure of human CaSR and the cloned region of goldfish CaSR. Human CaSR consists of 1078-amino acids (aa) and has ligand-binding domain (20-530 aa), cysteine-rich region (538-591 aa) and the transmembrane domain (622-860 aa).

```
goldfish    ASVCSKCPNNSWSNGNHTSCFLKEIEFLSWTEPFGIALALLAVLGVLLTAFVLGVFVQYRDTPIVKASNRELSFLLLFSLICCFSSSLIFIGEPQDWTCR
zebrafish   ASFCVKCPNNSWSNGNHTSCFLKQIEFLSWTEPFGIALALFAVLGVLLTAFVLGVFVQFRDTPIVKASNRELSFLLLFSLICCFSSSLIFIGEPQDWTCR
salmon      ASVCTKCPNDSWSNENHTSCFLKEIEFLSWTEPFGIALALCSVLGVFLTAFVMGVFIKFRNTPIVKATNRELSYLLLFSLICCFSSSLIFIGEPQDWTCR
lizard      ASACEKCAENFWSNENHTVCIPKQIEFLSWTEPFGIALTLFAILGIFLTSFVLGVFTRFRNTPIVKATNRELSYLLLFSLLCCFSSSLFFIGEPQDWNCR
pig         ASACDKCPDDFWSNENHTSCIAKEIEFLSWTEPFGIALTLFAVLGIFLTAFVLGVFIKFRNTPIVKATNRELSYLLLFSLLCCFSSSLFFIGEPQDWTCR
mouse       ASACDKCPDDFWSNENYTSCIAKEIEFLAWTEPFGIALTLFAVLGIFLTAFVLGVFIKFRNTPIVKATNRELSYLLLFSLLCCFSSSLFFIGEPQDWTCR
human       ASACNKCPDDFWSNENHTSCIAKEIEFLSWTEPFGIALTLFAMLGIFLTAFVLGVFIKFRNTPIVKATNRELSYLLLFSLLCCFSSSLFFIGEPQDWTCR
            ** * **    *** * * *  * **** ********* *   **  ** ** ***   * ****** ***** ****** ******* ******** **

goldfish    VRQPAFGISFVLCISCILVKTNRVLLVFEAKIPTSLHRKWWGLNLQFLLVFLFTFVQVMISLVWLYNAPPGSYKNYDI-DEIIFITCNEGSMMALGFLIG
zebrafish   VRQPAFGISFVLCISCILVKTNRVLLVFEAKIPTSLHRKWWGLNLQFLLVFLFTFVQVMICVVWLYNAPPGSYKNYDI-DEIIFITCNEGSMMALGFLIG
salmon      LRQPAFGISFVLCISCILVKTNRVLLVFEAKIPTSLHRKWWGLNLQFLLVFLFTFVQVMICVVWLYNAPPASYRNHDI-DEIIFITCNEGSMMALGFLIG
lizard      LRQPAFGISFVLCISCILVKTNRVLLVFEAKIPTSLHRKWWGMNLQFLLVFLCTFVQIVICVIWLYTAPPSSFRNHELEDEIIFITCNEGSLMALGFLIG
pig         LRQPAFGISFVLCISCILVKTNRVLLVFEAKIPTSFHRKWWGLNLQFLLVFLCTFMQIVICAIWLYTAPPSSYRNHELEDEIIFITCHEGSLMALGFLIG
mouse       LRQPAFGISFVLCISCILVKTNRVLLVFEAKIPTSFHRKWWGLNLQFLLVFLCTFMQIVICIIWLYTAPPSSYRNHELEDEIIFITCHEGSLMALGSLIG
human       LRQPAFGISFVLCISCILVKTNRVLLVFEAKIPTSFHRKWWGLNLQFLLVFLCTFMQIVICVIWLYTAPPSSYRNQELEDEIIFITCHEGSLMALGFLIG
            ********************************** ****** ********* ** *  *   *** *** *  *    ******** *** **** ***

goldfish    YTCLLAAVCFFFAFKSRKLPENFTEAKFITFSMLIFFIVWISFIPAYFSTYGKFVSAVE
zebrafish   YTCLLAAICFFFAFKSRKLPENFTEAKFITFSMLIFFIVWISFIPAYFSTYGKFVSAVE
salmon      YTCLLAAICFFFAFKSRKLPENFTEAKFITFSMLIFFIVWISFIPAYFSTYGKFVSAVE
lizard      YTCLLAAICFFFAFKSRKLPENFNEAKFITFSMLIFFIVWISFIPAYASTYGKFVSAVE
pig         YTCLLAAICFFFAFKSRKLPENFNEAKFITFSMLIFFIVWISFIPAYASTYGKFVSAVE
mouse       YTCLLAAICFFFAFKSRKLPENFNEAKFITFSMLIFFIVWISFIPAYASTYGKFVSAVE
human       YTCLLAAICFFFAFKSRKLPENFNEAKFITFSMLIFFIVWISFIPAYASTYGKFVSAVE
            ******* *************** ********************** ***********
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Fig. 3. Sequence alignment of goldfish CaSR with other CaSRs (zebrafish: XP_689097; salmon: NP_001119703; lizard: XP_003223807; pig: XP_003132690; mouse: AAD28371; human: DQ327728). The asterisks indicate identical amino acid residues in all the sequences.

The selected significance level was $P<0.05$.

Results

Cloning of CaSR from the scales of goldfish

PCR products (457 bp, underlined region in Fig.1) were obtained by PCR amplification. Using the obtained PCR product, RACE cloning was performed. As a result, the partial cDNA sequence of CaSR was determined (Fig. 1). The molecular structure of human CaSR and the cloned region of goldfish CaSR were shown in Fig. 2. Human CaSR consists of 1078-amino acids (aa) and has ligand-binding domain (20-530 aa), cysteine-rich region (538-591 aa) and transmembrane domain (622-860 aa). The cloned region of goldfish CaSR (579-837 aa) contained most of the transmembrane domain.

Structural comparison of goldfish CaSR with other CaSRs (zebrafish: XP_689097; salmon: NP_001119703; lizard: XP_003223807; pig: XP_003132690; mouse: AAD28371; human: DQ327728) was indicated in Figure 3. Most of amino acids were conserved among these vertebrates.

Effect of acceleration loading by vibration on CaSR mRNA expression in the scales of goldfish

The results of acceleration loading by vibration on CaSR mRNA expression are shown in Figure 4. The mRNA expression of CaSR was increased by hyper-loading in the regenerating scales of goldfish. Significant difference between control and experimental group was observed at 6 and 24 h of incubation.

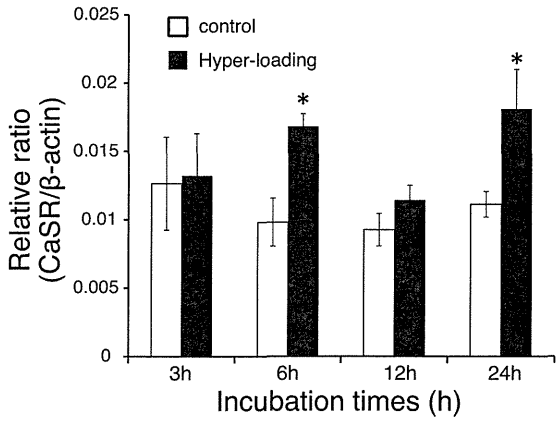

Fig. 4. Effect of acceleration loading by vibration on CaSR mRNA expression in the regenerating scales of goldfish. Each column and vertical line represent the mean ± S.E.M. (n = 6 samples; one sample from one goldfish). *P<0.05 versus control by paired *t*-test.

Discussion

We successfully determined the partial sequence of CaSR in the scales of goldfish. The putative goldfish CaSR of 258-amino acids has high sequence identity to zebrafish CaSR (XP_689097, 96%), salmon CaSR (NP_001119703, 92%), gilt-head bream CaSR (CAC41352, 91%), tilapia CaSR (XP_003446893, 90%), fugu CaSR (BAA26122, 89%), flounder CaSR (FJ755006, 89%), lizard CaSR (XP_003223807, 82%), cattle CaSR (DAA33414, 84%), pig CaSR (XP_003132690, 84%), mouse CaSR (AAD28371, 83%), and human CaSR (DQ327728, 84%). Among these vertebrates, we found that CaSR sequences are well-conserved at least in the region of 258-amino acids and suggesting that CaSR sequences possess evolutionary conserved property which may have important biological function in vertebrates.

A regulatory mechanism(s) governing CaSR mRNA expression is essential to understand the physiological significance of goldfish CaSR. Recently, Sun *et al.* (2012) reported that mechanical stretch induced calcium efflux from bone matrix and stimulated osteoblasts, and suggested that bone matrix acts as an intermediate mechanochemical transducer which converts the mechanical strain into a chemical signal in terms of the calcium efflux. There are several reports which show that the teleost scale contains osteoblasts, osteoclasts, and the bone matrix (Bereiter-Hahn and Zylberberg, 1993; Suzuki *et al.*, 2000; Yoshikubo *et al.*, 2005; Suzuki *et al.*, 2007; Ohira *et al.*, 2007). As the scale is equipped with the bone matrix, osteoblasts in the scales sensitively may respond to acceleration loading.

Calcium is an essential mineral to maintain cell viability and, ultimately, animal life. In all vertebrates, blood calcium levels are strictly kept at a constant concentration (around 2.5 mM) in spite of changing the internal milieu or external environment (Dacke, 1979). To regulate the blood calcium in teleosts, the scales have an important role because teleost scales are known to work as a potential internal calcium reservoir, similarly to those in endoskeletons of mammals, especially during increasing demand of calcium, such as sexual maturation or starvation (Yamada, 1961; Berg, 1968; Bereiter-Hahn and Zylberberg, 1993; Mugiya and Watabe, 1977). We recently detected cathepsin K and tartrate-resistant acid phosphatase mRNA expression in the scale osteoclasts (Azuma *et al.*, 2007). In osteoblasts as well, we detected osteoblast-specific markers, such as runt-related transcription factor 2, osterix, type 1 collagen, alkaline phosphatase, osteocalcin, and the receptor activator of the NF-κB ligand (Thamamongood *et al.*, 2012). Therefore, the features of osteoclasts and osteoblasts in scales are quite similar to those in mammals. Calcium signal is essential for the proliferation and differentiation in mammalian osteoblasts (Theman and Collins, 2009; Dvorak-Ewell *et al.*, 2011), suggesting that extracellular calcium ion production occurs from the bone matrix of goldfish scales for conducting calcium signaling to osteoblasts *via* CaSR. We are currently developing an original array system for goldfish on the basis of Expressed Sequence Tag analysis. In the future study, we will be able to examine the detail mechanism including calcium signaling on osteogenesis using our original array system.

In conclusion, goldfish scales express CaSR like other vertebrates, and the expression of CaSR is regulated by loading. The expression of CaSR by loading suggests that scale is a good model for the analysis of bone metabolism under various gravity conditions.

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Prostaglandin E_2 Increases Both Osteoblastic and Osteoclastic Activity in the Scales and Participates in Calcium Metabolism in Goldfish

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Using our original in vitro assay system with goldfish scales, we examined the direct effect of prostaglandin E_2 (PGE_2) on osteoclasts and osteoblasts in teleosts. In this assay system, we measured the activity of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) as respective indicators of each activity in osteoblasts and osteoclasts. ALP activity in scales significantly increased following treatment at high concentration of PGE_2 (10^{-7} and 10^{-6} M) over 6 hrs of incubation. At 18 hrs of incubation, ALP activity also significantly increased in the PGE_2 (10^{-9} to 10^{-6} M)-treated scale. In the case of osteoclasts, TRAP activity tended to increase at 6 hrs of incubation, and then significantly increased at 18 hrs of incubation by PGE_2 (10^{-7} to 10^{-6} M) treatment. At 18 hrs of incubation, the mRNA expression of osteoclastic markers (TRAP and cathepsin K) and receptor activator of the NF-κB ligand (RANKL), an activating factor of osteoclasts expressed in osteoblasts, increased in PGE_2 treated-scales. Thus, PGE_2 acts on osteoblasts, and then increases the osteoclastic activity in the scales of goldfish as it does in the bone of mammals. In an in vivo experiment, plasma calcium levels and scale TRAP and ALP activities in the PGE_2-injencted goldfish increased significantly. We conclude that, in teleosts, PGE_2 activates both osteoblasts and osteoclasts and participates in calcium metabolism.

Key words: prostaglandin E_2, osteoblasts, osteoclasts, goldfish scales, plasma Ca, RANKL

INTRODUCTION

Prostaglandin E_2 (PGE_2) is an important local regulator in bone and promoter of osteoclastogenesis (Kaji et al., 1996; Gardner, 2007; Kaneko et al., 2007). The bone-resorbing activity of mature osteoclasts in osteoblast-containing mouse bone cell cultures is increased by PGE_2 although it does not affect osteoclast-like cell formation in osteoblast-free mouse spleen cell cultures (Kaji et al., 1996). The function of PGE_2 must therefore be examined using a co-culture system of osteoclasts and osteoblasts.

In fish, the osteoblastic function of PEG_2 has been examined using salmon (Ytteborg et al., 2010). However,

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there has been no report on the osteoclastic function of PGE_2 due to a lack of a suitable in vitro assay system. Scales are calcified tissue that contains osteoclasts and osteoblasts (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Suzuki et al., 2007). Scales have been reported to be a better potential internal calcium reservoir than body skeletons, jaws, and otoliths, as examined in a ^{45}Ca-labeling study for the calcified tissues of goldfish and killifish (Mugiya and Watabe, 1977). In the scale, as in mammalian bone, type I collagen (Zylberberg et al., 1992), bone γ-carboxyglutamic acid protein (Nishimoto et al., 1992), osteonectin (Lehane et al., 1999; Redruello et al., 2005), and hydroxyapatite (Onozato and Watabe, 1979) are present. Moreover, we have previously demonstrated that the osteogenesis of regenerating scale is very similar to that of mammalian membrane bone, and serves as a good model of osteogenesis (Yoshikubo et al., 2005). Thus, the teleost scale has a number of features in common with mammalian bone.

Using goldfish scales, we recently developed a novel in vitro assay system (Suzuki et al., 2000; Suzuki and Hattori, 2002), as the scale is a very active tissue of calcium regulation in fish, as described above. Using this system in the present study, we examined the effect of PGE_2 on scale osteoblasts and osteoclasts. In addition, the mRNA expression of osteoclastic markers (tartrate-resistant acid phosphatase (TRAP) and cathepsin K) and the receptor activator of the NF-κB ligand (RANKL), an activating factor of osteoclasts expressed in osteoblasts, was investigated in vitro. To confirm the results of the in vitro experiment, furthermore, an in vivo experiment was performed.

To our knowledge, our study is the first to indicate the effect of PGE_2 on osteoclasts in teleosts.

MATERIALS AND METHODS

Animals

In a previous study (Suzuki et al., 2000), we indicated that the sensitivity to a calcemic hormone, such as estrogen and calcitonin, was higher in mature female than in mature male goldfish (*Carassius auratus*). Mature female goldfish were purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) and used in the scale in vitro assay. To examine the effect of PGE_2 on the calcium metabolism, immature goldfish (4–6 g), in which the endogenous effects of sex steroids are negligible, were used for the in vivo study. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

Effect of PGE_2 on ALP and TRAP activities in the cultured scales of goldfish

Scales collected from goldfish after anesthesia with ethyl 3-aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich, Inc., MO, USA) were cut into two approximately equal pieces. One piece was treated with PGE_2 (Cayman Chemical, MI, USA, 10^{-11} to 10^{-6} M), and the other was used as a control. Using these scales, we examined the effects of PGE_2 on the osteoblasts and osteoclasts with alkaline phosphatase (ALP) and TRAP as markers because, in mammals, the effects of hormones and some bioactive substances on osteoclasts and osteoblasts have been investigated using ALP and TRAP as respective markers (Vaes, 1988; Dimai et al., 1998; Suda et al., 1999). Sixteen scales (ALP activity: eight scales; TRAP activity: eight scales) were used for the evaluation of each concentration of PGE_2. The pieces of scales were incubated for 6 and 18 hrs in Eagle's modified minimum essential medium (MEM; ICN Biomedicals Inc., OH, USA). HEPES (20 mM) was added to MEM. The pH was adjusted to 7, and the incubation temperature was 15°C.

The measurement methods of ALP and TRAP activity reported by Suzuki and Hattori (2002) were modified as follows. The incubated scale was transferred to its own well in a 96-well microplate after washing with saline. An aliquot of 100 μl of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM $MgCl_2$; 0.1 mM $ZnCl_2$) was added to each well. This microplate was frozen at –85°C immediately and then kept at –20°C until analysis. After thawing, an aliquot of 100 μl of 20 mM para-nitrophenyl-phosphate in an alkaline buffer was added to each well. This plate was then incubated at 20°C for 30 min while being shaken. After incubation, the reaction was stopped by adding 50 μl of a 3 N NaOH-20 mM EDTA solution. Aliquots of 150 μl of a colored solution were transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced para-nitrophenol (pNP) using a standard curve for pNP. After measurement of the absorbance, the ALP activity was normalized by the dry weight (mg) of each goldfish scale. The results are shown as the means ± SE of eight scales.

TRAP activity was measured using an acid-tartrate buffer (20 mM tartrate in a 0.1 M sodium acetate buffer (pH 5.3)). Other conditions were the same as those for the measurement of ALP activity.

Changes in TRAP, cathepsin K and RANKL mRNA expressions in PGE_2-treated goldfish scales

Scales were collected from goldfish under anesthesia with ethyl 3-aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich) and cut into halves. One half of a piece was then put into a microtube in MEM (1 ml) supplemented with PGE_2 (10^{-6} M). The other half was also put into another microtube in a PGE_2-free medium as a control. To examine changes in TRAP, cathepsin K and RANKL mRNA expressions in response to PGE_2, the scales were incubated for 6 and 18 hrs in MEM (containing an antibiotic and 20 mM HEPES) at 15°C. The mRNA expression in the control and experimental scales in the same individual was compared. After incubation, the scales were frozen at –85°C for mRNA analysis.

Total RNAs were prepared from goldfish scales using a total RNA isolation kit for fibrous tissue (Qiagen GmbH, Hilden, Germany). Complementary DNA synthesis was performed using a kit (Qiagen GmbH). Gene-specific primers for TRAP (sense: 5′-AACTTCCGCATTCCTCGAACAG-3′; antisense: 5′-GGCCAGCCACCAGGAGATAA-3′) (Azuma et al., 2007), cathepsin K (sense: 5′-GCTATGGAGCCACACCAAAAGG-3′; antisense: 5′-CTGCGCTTCCAGCTCTCACAT-3′) (Azuma et al., 2007), and RANKL (sense: 5′-GCGCTTACCTGCGGAATCATATC-3′; antisense: 5′-AAGTGCAACAGAATCGCCACAC-3′) (Suzuki et al., 2011a) were used. The amplification of β-actin cDNA using a primer set (5′: CGAGCGTGGCTACAGCTTCA; 3′: GCCCGTCAGGGAGCTCATAG) (Azuma et al., 2007) was performed. PCR amplification was analyzed by real-time PCR apparatus (Mx3000p, Agilent Technologies, CA, USA) (Suzuki et al. 2011a). The annealing temperature of TRAP, cathepsin K, RANKL, and β-actin was 60°C. The TRAP, cathepsin K and RANKL mRNA levels were normalized to the β-actin mRNA level.

Changes in the plasma calcium and scale TRAP and ALP activities in PGE_2-injected goldfish (in vivo experiment)

In the experimental group, goldfish (body weight: 4–6 g) were anesthetized in the same manner as above, and PGE_2 (500 ng/g body weight) was then injected intraperitoneally. The goldfish in the control group were injected with saline (0.9% NaCl) in the same manner as experimental goldfish. These goldfish were kept in the aquarium for one and two days, respectively (each n = 8). During the experimental periods, these goldfish were not given any food to exclude intestinal calcium uptake from diets. Each day after injec-

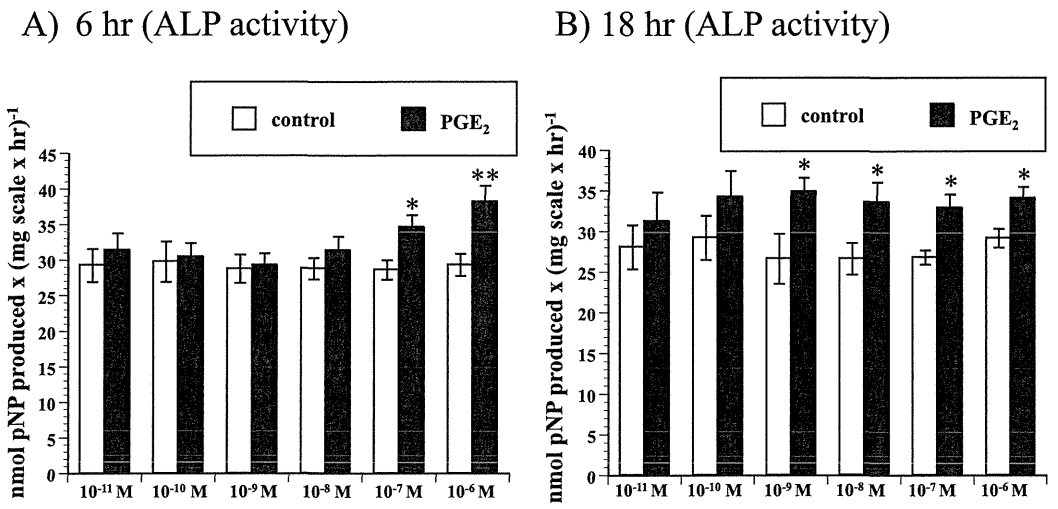

Fig. 1. Effect of PGE_2 (10^{-11} to 10^{-6} M) on ALP activity in cultured scales incubated for 6 **(A)** and 18 **(B)** hrs. * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, from the values in the control scales ($n = 8$).

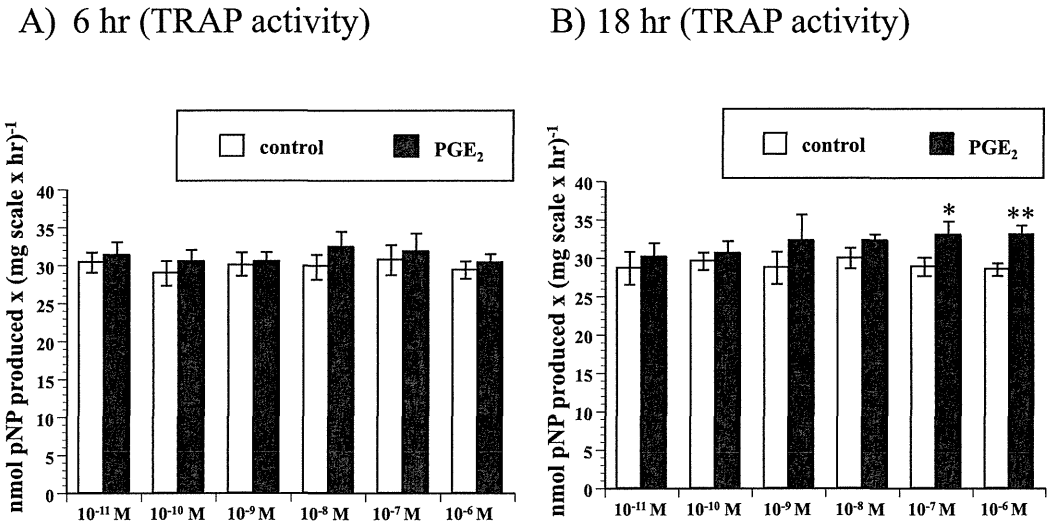

Fig. 2. Effect of PGE_2 (10^{-11} to 10^{-6} M) on TRAP activity in cultured scales incubated for 6 **(A)** and 18 **(B)** hrs. * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, from the values in the control scales ($n = 8$).

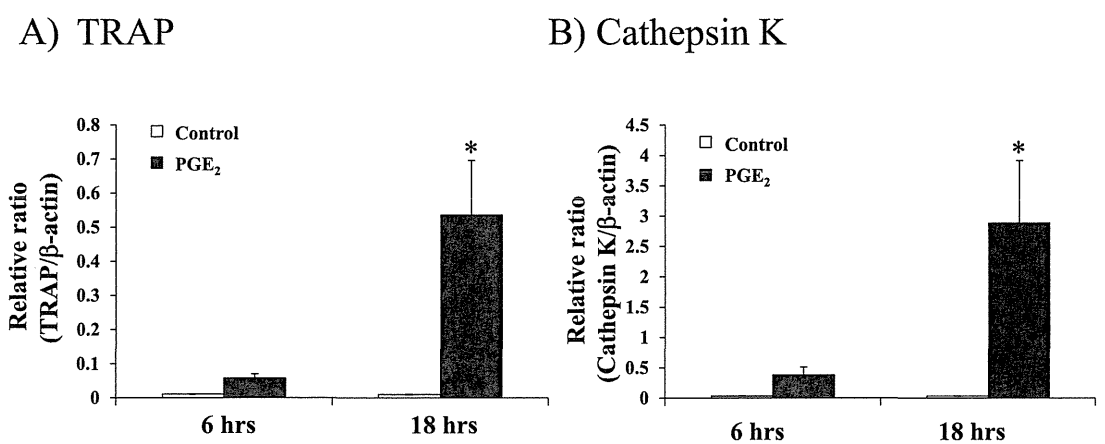

Fig. 3. Expression analysis of osteoclastic markers: TRAP **(A)** and cathepsin K **(B)** mRNAs in the PGE_2 (10^{-6} M)-treated scale. The TRAP and cathepsin K mRNA levels were normalized to the β-actin mRNA level. * indicates a statistically significant difference at $P < 0.05$ from the values in the control scales ($n = 8$).

tion, blood samples were collected from the gill using a heparinized capillary from individual, anesthetized goldfish. After centrifugation at 15,000 rpm for 3 min, the plasma was immediately frozen and kept at –80°C until use. The plasma total calcium level (mg/100 ml) was determined using an assay kit (Calcium C, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Then, we measured the ALP and TRAP activities in the scale. As the scales were very small, the respective marker enzyme activity was normalized by the surface area (mm^2) of each goldfish scale (Suzuki et al., 2009).

Statistical analysis

All results are expressed as the means ± SE (n = 8). The statistical significance between control and experimental group was assessed by Student's t-test or paired t-test. In all cases, the selected significance level was $P < 0.05$.

RESULTS

Effect of PGE_2 on ALP activity in the cultured scales of goldfish

PGE_2 significantly increased the ALP activities of the scales by 6 hrs of incubation ($P < 0.05$ for 10^{-7} M; $P < 0.01$ for 10^{-6} M) (Fig. 1A). At 18 hrs of incubation, the ALP activities in the PGE_2-treated scales also significantly increased by PGE_2 treatment ($P < 0.05$ for 10^{-9} to 10^{-6} M) (Fig. 1B).

Effect of PGE_2 on TRAP activity in the cultured scales of goldfish

The TRAP activity of the PGE_2-treated scales by 6 hrs of incubation tended to be higher than that in the control scales, although there was no significant difference between the PGE_2-treated and control scales (Fig. 2A).

However, the TRAP activity in the PGE_2-treated scales was significantly higher by 18 hrs ($P < 0.05$ for 10^{-7} M; $P < 0.01$ for 10^{-6} M) of incubation relative to the values of the control scales (Fig. 2B).

Changes in TRAP, cathepsin K and RANKL mRNA expressions in PGE_2-treated goldfish scales

The results of TRAP and cathepsin K are shown in Fig. 3. The mRNA expression of osteoclastic markers (TRAP and cathepsin K) in the PGE_2-treated scales tended to

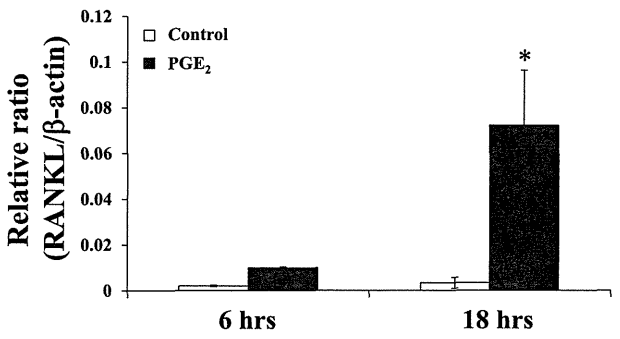

Fig. 4. Expression analysis of RANKL mRNA in the PGE_2 (10^{-6} M)-treated scale. The RANKL mRNA levels were normalized to the β-actin mRNA level. * indicates a statistically significant difference at $P < 0.05$ from the values in the control scales (n = 8).

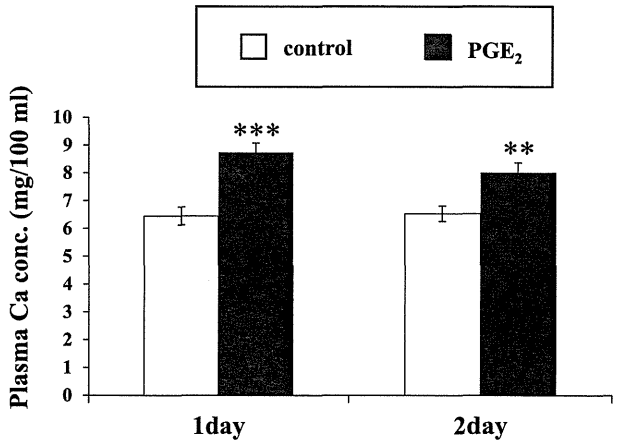

Fig. 5. Effects of PGE_2 on plasma calcium (mg/100 ml) in an in vivo experiment using immature goldfish. ** and *** indicate statistically significant differences at $P < 0.01$ and $P < 0.001$, respectively, from the values in the control (n = 8).

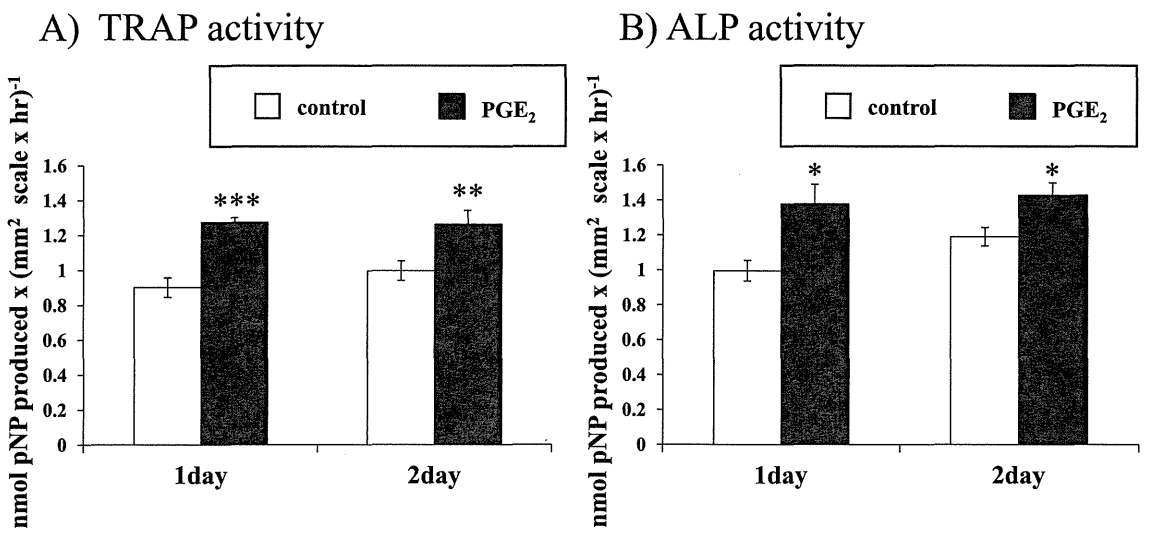

Fig. 6. Effects of PGE_2 on scale TRAP **(A)** and ALP **(B)** activity in an in vivo experiment using immature goldfish. *, **, and *** indicate statistically significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, from the values in the control (n = 8).

increase at 6 hrs of incubation compared with those in control scales. At 18 hrs incubation, mRNA expression of both osteoclastic markers increased significantly following PGE_2 treatment.

Similar results were obtained in RANKL. The mRNA expression of RANKL, an activating factor of osteoclasts expressed in osteoblasts, increased significantly by PGE_2 treatment at 18 hrs of incubation although RANKL mRNA in PGE_2-treated scales tended to be higher than that in the control scales at 6 hrs of incubation (Fig. 4).

Changes in the plasma calcium and scale TRAP and ALP activities in PGE_2-injected goldfish (in vivo experiment)

The plasma calcium levels increased significantly at both one and two days after PGE_2 injection (Fig. 5). In addition, the scale TRAP and ALP activities in PGE_2-injected goldfish were significantly promoted on both days promoted (Fig. 6).

DISCUSSION

The present study is the first to demonstrate that PGE_2 promoted both osteoblastic and osteoclastic activity in the scales of goldfish in an in vitro experiment. Furthermore, we found that mRNA expression of osteoclastic markers, such as TRAP and cathepsin K, was significantly promoted in PGE_2-treated scales. An in vivo experiment reconfirmed the findings from the in vitro experiments. In teleosts, the functions of calcemic hormones in both osteoblasts and osteoclasts have not been elucidated because of the lack of a suitable in vitro assay system. Using our system, we first demonstrated that calcitonin, a hypocalcemic hormone, suppressed osteoclastic activity in teleosts as well as in mammals (Suzuki et al., 2000) and that melatonin, a major hormone secreted from the pineal gland, suppresses the functions in both osteoclasts and osteoblasts (Suzuki and Hattori, 2002). Osteoblasts in the scale responded to estrogen as they do in mammals (Yoshikubo et al., 2005). The effects of endocrine disrupters, such as bisphenol-A (Suzuki and Hattori, 2003) and tributyltin (Suzuki et al., 2006), and heavy metals, i.e., cadmium (Suzuki et al., 2004a), organic mercury (Suzuki et al., 2004a), and inorganic mercury (Suzuki et al., 2011b), on osteoblasts and osteoclasts have also been sensitively evaluated. Considering these results with the present data, our scale assay system may be useful for the analysis of various substances, including hormones, in bone metabolism.

RANKL produced by cells in the osteoblast lineage binds to the receptor activator of NF-κB (RANK) in mononuclear hemopoietic precursors and promotes the formation and activity of multinucleated osteoclasts (Teitelbaum, 2000). Our present study indicated that RANKL mRNA expression was promoted by PGE_2 treatment. In mammals, it has been reported that PGE_2 promotes osteoclastogenesis via the RANK-RANKL system (Gardner, 2007; Kaneko et al., 2007; Blackwell et al., 2009). We therefore strongly believe that, in teleosts as well as mammals, osteoclastogenesis is induced by the RANK-RANKL pathway.

In our in vivo experiment, the plasma calcium levels increased significantly at both one and two days after PGE_2 injection. In the reproductive period, the plasma calcium level in female teleosts increases remarkably (Watts et al., 1975; Yamauchi et al., 1978; Norberg et al., 1989; Suzuki et al., 2004b; Guerreiro et al. 2007). This calcium is bound to vitellogenin, which is a major component of egg protein and the calcium-binding protein (Tinsley, 1985; Kwon et al., 1993). We previously demonstrated, in an in vivo experiment with goldfish, that fugu PTH1-induced hypercalcemia results from an increase of both osteoblastic and osteoclastic activity in the scale as well as the decrease of scale calcium contents after fugu PTH1 injection (Suzuki et al., 2011a). In estrogen-injected goldfish, the scale TRAP and ALP activities (Suzuki and Hattori, 2003) and plasma calcium level (Suzuki et al., 2003) increased. In estrogen-injected goldfish, we demonstrated that estrogen promoted calcitonin secretion and that estrogen directly acts on the ultimobranchial gland (UBG), a CT-secreting organ since the estrogen receptor was detected in UBG (Suzuki et al., 2004b). Our preliminary data indicate that, during reproductive periods, the plasma calcium level in female goldfish increases corresponding to the plasma level of PGE_2. In addition, the plasma TRAP level also increased at this period (N Suzuki Personal Communication). There may therefore be an interaction among calcemic hormones, such as PGE_2, PTH, estrogen, and calcitonin, in the scales of goldfish. As a result of the hormonal interaction, scale resorption may occur. We are currently developing an original array system for goldfish on the basis of EST analysis for goldfish scales. In the future, we will examine the interaction among calcemic hormones and describe in detail mechanism of teleost bone metabolism.

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トピックス 特集：細胞や微生物に関わる最近の話題

魚類のウロコを用いた評価系の開発と骨代謝研究への応用

Development of an Assay System Using Fish Scales and Its Application to the Study of Bone Metabolism

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Magnetic fields are known to enhance bone formation. However, basic data concerning the mechanism are limited because there are no satisfactory model systems of human bone, which consists of osteoblasts, osteoclasts, and a bone matrix. The bone matrix, consisting of substances including hydroxyapatite and type I collagen, has an important function in the response to physical stress. Few techniques to co-culture osteoclasts and osteoblasts that include the bone matrix have been developed. Fish scales are calcified tissue that contains osteoclasts, osteoblasts, and a bone matrix, similar to human bone. We recently developed an *in vitro* assay system using fish scales and then examined the effects of physical stimuli, such as hypergravity, microgravity, and magnetic fields, on bone metabolism. We describe here our recent results regarding bone metabolism.

Key words: extremely low-frequency magnetic fields, fish scales, hyper-gravity, micro-gravity, bone metabolism

1. はじめに

ヒトの骨には骨を作る細胞である骨芽細胞と骨を壊す細胞である破骨細胞があり，それらが骨形成を調節している．骨芽細胞は細胞株があるため培養は容易であるが，破骨細胞は多核の活性型に誘導する必要があるため，培養は難しい．さらに磁場や重力等の物理的な刺激には，骨基質が重要な役割を果たしていることも報告されている[1),2)]．したがって，人体の骨組織を再現させるためには，これらすべて共存させて培養する必要がある．しかしながら，現時点ではこのような共存培養は困難な状況である．また骨粗鬆症などの骨疾患に対する治療薬を開発するためには，卵巣を除去し，骨が折れやすくしたラットを用いて，時間と多額の費用を投資して開発している．そこで磁場のような物理的な刺激により，骨疾患の治療を行うことができれば，薬を使用する必要がなく，さらに副作用もなく，高齢者には適した治療になる．しかしながら，磁場の刺激に対する骨形成機構の研究は，モデルシステムの欠如によって遅れ，そのメカニズムに関する基礎的なデータも少なく，骨形成に最適な磁場の条件は決定されていない．

魚類のウロコは，直接化骨をする点で哺乳類の頭蓋骨などの膜性骨に似ており，I型コラーゲンからなる線維層と，I型コラーゲンとハイドロキシアパタイトからなる石灰化層の二つを主体とする(Fig. 1)．そして石灰化層の上に，骨芽細胞と破骨細胞が共存し(Fig. 2)，添加的石灰化による骨形成や破骨細胞による骨吸収を行っている．

進化のうえで，初めて骨の原型を備えた動物は，古生代の海に生息した甲冑魚（甲皮類）である．甲冑魚の体制は極めて原始的で，顎もなく，歯もなく，体内の骨もまだ軟骨で形成されていたが，外表を覆う装甲（甲皮）は，構造的には象牙質と骨の性格を併せ持つ硬組織で，その主成分はハイドロキシアパタイトであった．甲皮類はやがて棘魚類を経て硬骨魚（＝現生の魚類）へと進化するが，この過程で甲皮も形を変え，頭部では膜性骨として残り，体表部では運動性向上と軽量化のためにウロコとなったと考えられている．また，顎では歯が，体内部では硬骨（軟骨性化骨）もできるようになった．このように，ウロコは甲皮の直系の子孫器官である点で，進化的に興味深い硬組織である．

このような骨のモデルとなりうるウロコを用いて評価システムを開発した[3),4)]．本稿では，①ウロコを用いた評価システムの特徴を記載し，②過重力，③擬似微小重力，④微小重力（宇宙），さらに⑤磁場に対する応答解析について述べる．

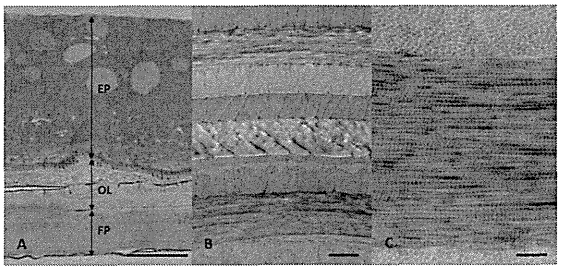

Fig. 1 Morphological characteristics of goldfish scales. Light-microscopic image of goldfish scales by toluidine blue stain (A). The main parts of the scale are the osseous layer (OL) and the fibrillary plate (FP), and they are partially covered by the epithelium (EP). The electron microscopic image shows a sheetlike structure composed of multiple layers of lamellae within FP (B); each of the layers is filled with parallel collagen fibers, and their orientation differs from that of the adjacent lamellae (C). The bars shown in Figs. lA, l B, and lC are 50 μm, 2 μm, and 500 nm, respectively.

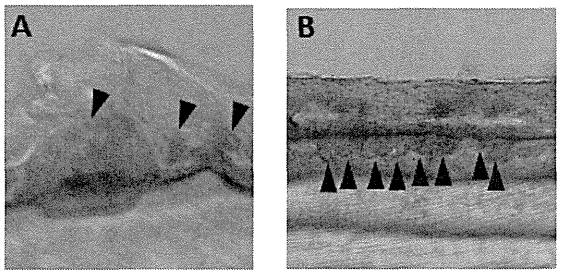

Fig. 2 TRAP for osteoclast (A) and ALP for osteoblast (B) staining of goldfish scales.

2. ウロコを用いた評価システムの特徴

ウロコには破骨細胞と骨芽細胞が共存し，その骨質層からカルシウムを出し入れして，血液中のカルシウム濃度を調節している[5]．したがってウロコを用いることで，従来の材料や方法では難しい骨芽細胞と破骨細胞の相互作用を，生体内に近い状態で再現できる．そこでウロコを用いた培養系を確立し，これを用いた新規測定系を開発した[3),4)]．以下にこの系の利点を挙げる．

1) ウロコは容易に抜去でき，培地の中に抜去したウロコを入れて培養するのみで，非常に簡便である．
2) 破骨細胞や骨芽細胞はウロコ表面に存在するため，そのまま培養に用いることができる．コラゲナーゼなどの処理による細胞の調整が不要なため，細胞の状態は極めて良い．
3) 短時間の培養には血清が不要であり，低コストである．炭酸ガスも要らない．
4) キンギョでは，1個体からほぼ同じ細胞活性をもつウロコを約100枚取れ，同時にさまざまな条件で検討できる．また，キンギョ（主として用いる）はラットやマウスに比べ，格段に安価な実験材料である．
5) 短時間（6時間から24時間）の培養で結果が得られ，迅速に解析できる．
6) ウロコ1枚でも，破骨細胞や骨芽細胞の活性測定が可能である．培養実験後のウロコをマイクロプレート（96穴）に1枚ずつ入れ，破骨細胞の場合は酒石酸抵抗性酸フォスファターゼ(TRAP)，骨芽細胞の場合はアルカリフォスファターゼ(ALP)を指標として，それぞれの酵素活性をプレートリーダーで容易に測定することができる．

3. 遠心機の過重力に対する応答解析

水棲生物用の麻酔薬(MS-222)で麻酔したキンギョ(*Carassius auratus*)からウロコを取り，そのウロコを半分に切り，片方を実験群とし，他方をコントロール群(1 G)とした．これらのウロコを96穴のマイクロプレートに入れた．次に，そのwellにHEPES (20 mM), (pH 7.0)および抗生物質(1%)を含む培地(MEM, ICN Biomedicals Inc.)を200 μL加えた．このマイクロプレートを2, 4および7 Gで遠心機（LIX-130，トミーデジタルバイオロジー(株)）で5および10分間遠心した後，6および24時間培養し，ウロコの骨芽細胞および破骨細胞の活性を測定した．なお，それぞれの実験は3回行い，ウロコは各群とも12枚使用した．

骨芽細胞の活性は，2 Gという低強度の重力負荷でも応答し，5および10分間処理により，その活性が上昇した．4 Gでは2 Gとあまり変わらないが，7 Gでは顕著に骨芽細胞の活性が上昇した．さらに，破骨細胞の活性も2 Gで5分間処理しても応答し，その活性が低下した．破骨細胞活性の低下率は，強度が上がるにつれて下がり，7 Gでは破骨細胞の活性が上昇する傾向にあった．

以上のように，非常に弱い強度で短時間の過重力処理でも，ウロコの骨芽細胞と破骨細胞は応答することが判明した．

4. 3次元クリノスタットによる擬似微小重力に対する応答解析

次に擬似微小重力に対する応答を解析した．培養細胞に対する影響を3次元クリノスタットで調べる場合は，培地の流動が細胞活性に影響を及ぼす可能性がある．そこで筆者らは，エッペンドルフチューブ内に綿球を詰めてウロコを固定し，ウロコの動きを抑え，かつ培地の流動をできるだけ低減する工夫を施した．結果として，ウロコは非常に感度よく3次元クリノスタットに応答した．

キンギョ（8個体）を使用して，過重力と同様にウロコを抜き，エッペンドルフチューブに入れ，綿球を詰めて，

擬似微小重力下で6および24時間培養した．その結果，骨芽細胞の活性は，6時間の処理により8個体中6個体において低下し，24時間の処理により8個体中7個体において有意に下がった．一方，破骨細胞の活性は，6時間の処理により8個体中5個体において上昇し，24時間の処理により8個体中6個体において有意に上がった．

次に1軸（2次元）でクリノスタットを6時間回転させ，骨芽細胞および破骨細胞の応答を解析した．その結果，骨芽細胞の活性は3次元では低下するのに，2次元では変化しなかった．また破骨細胞の活性は3次元ではその活性が上昇したが，2次元では変化しなかった．

したがって，3次元クリノスタットに対するウロコの骨芽細胞および破骨細胞に現れた現象は，微小重力に特異的な反応である可能性が高い．

5. ウロコを用いた宇宙実験

擬似微小重力に対する応答が見られ，微小重力に対する応答を解析する優れた骨モデルであると評価され，「きぼう」の国際宇宙ステーションを用いた宇宙実験を2010年5月に実施することができた．

日本でパッキングした試料を冷蔵状態で米国NASAケネディ宇宙センターに輸送した．試料は冷蔵を保ったまま，スペースシャトルアトランティスSTS-132によって国際宇宙ステーション(ISS)に向けて2010年5月14日に打ち上げられ，5月16日にISSに到着，同日のうちに「きぼう」内の細胞培養装置にセットされ，22℃で86時間培養された(Fig. 3)．5月20日，野口宇宙飛行士により細胞培養装置から取り出された培養試料は，冷凍保存および化学固定後，冷蔵保存により，地上に帰還した．

これらのウロコを解析した結果，破骨細胞の活性が上昇し，また，細胞が多核化するという形態学的な変化が引き起こされた．このことは，微小重力がウロコの骨吸収を引き起こしたことを示している．これは，長期間の低温保管も可能であり，かつ物理的刺激に対して応答性の良いウロコだからこそ達成することができた快挙である．

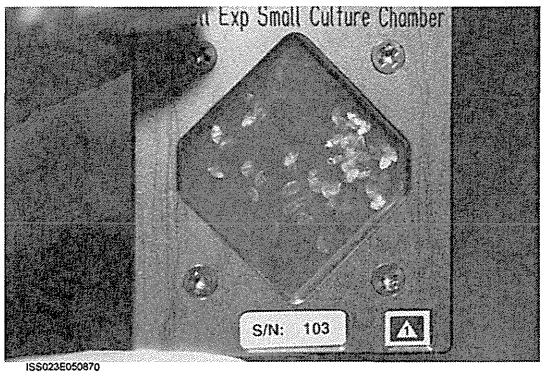

Fig. 3 JAXA astronaut Soichi Noguchi took a shot of cultured goldfish scales in KIBO of the International Space Station. The photograph indicates scales being cultured in space.

6. 極低周波磁場による破骨および骨芽細胞に及ぼす影響

以上のように，過重力および微小重力に対して非常に感度よく応答する骨モデルを用いて，60 Hzの極低周波磁場に対する影響を解析した．

麻酔したキンギョからウロコを取り，2 mL用のエッペンドルフチューブ（BM機器）に入れた．次に，そのチューブにHEPES (20 mM), (pH 7.0)および抗生物質(1%)を含む培地(MEM, ICN Biomedicals Inc.)を500 μL加えた．そのチューブを，3，5，10および30 mTの極低周波磁場(60 Hz)に15℃，24時間暴露し，破骨および骨芽細胞の活性に及ぼす影響を調べた．磁場発生装置の詳細は，Miyakawa *et al.*[6]に示してある．本研究では，破骨細胞の活性の指標としてTRAPを用い，骨芽細胞の活性の指標としてALPを使用し，磁場の骨組織に対する作用を調べた．また，培地中に放出された酵素活性も同様にして測定した．さらに，30 mTで処理したウロコからRNAを抽出して，遺伝子発現解析も行った．

3 mTの磁場により，ウロコの破骨細胞の活性が有意に低下した．また，細胞の活性と同様に培地中のTRAP活性もコントロールに比べて有意に低下していた．一方，ウロコの骨芽細胞の活性は変化しなかったが，培養液中のALP活性が，コントロールと比較して上昇していた．したがって，3 mTの磁場刺激でも骨形成が進行中であると推測される．また，5 mTでも3 mTと同様な変化が見られた．

10および30 mTでは，骨芽細胞の活性が上昇し，それに伴い破骨細胞の活性も上昇していた．これら2種類の細胞は，密接に連絡しており，骨芽細胞で発現しているリガンドであるRANKLと破骨細胞にあるレセプターであるRANKが結合することにより，破骨細胞が活性化し，多核の活性型の破骨細胞に分化する[7]．したがって，10および30 mTで24時間暴露することにより，骨芽細胞が活性化し，RANK-RANKLを通して破骨細胞も活性化された可能性が高い．

さらに，30 mTで処理したウロコの遺伝子発現解析を調べると，膜レセプターであるカルシトニンレセプターmRNAの発現は上昇したが，核レセプターであるエストロゲンレセプターmRNAの発現は変化しなかった．カルシトニンレセプターは破骨細胞に特異的に発現している遺伝子なので，細胞活性と一致している．以上のことから，膜に何らかの影響を与えて，磁場は骨形成を促している可能性が高い．

7. おわりに

ウロコは，非常にシンプルで骨モデルとして有効である

ことがわかった．ウロコは，60 Hzの極低周波磁場に対しても非常に感度よく応答して，磁場強度より異なった応答をしていることがわかった．今後，磁場に対する応答を解析するため，キンギョのオリジナルアレイも開発中である．さらにこのモデルを用いて，物理的刺激のほか，骨代謝に関与するホルモン（カルシトニン[3]，副甲状腺ホルモン[8]，メラトニン[4]，プロスタグランジンE_2[9]）に対する応答も解析している．今後，ホルモンと磁場との相互作用についても解析していく予定である．

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Distribution of PAHs in the northwestern part of the Japan Sea

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ABSTRACT

Surface water samples were collected at 13 stations in the Japan Sea and filtered through a glass fiber membrane (pore size 0.5 μm). The vertical distribution of PAHs (nine points from depths between 2 m and 3300 m) were measured at one station. Thirteen PAHs having 3–6 rings in the dissolved and particulate phases were determined by HPLC with fluorescence detection. The mean concentration of total PAHs on the seawater surface was 8.5 ng/L. The mean concentrations in the dissolved and particulate phases for surface seawater were 5.6 ng/L and 2.7 ng/L, respectively. In the dissolved phase, 3-ring PAHs were the largest contributor, and 5-ring or more PAHs were in low ratio. Also, concentrations of PAHs in the particulate phase were dominated by 3-ring and 4-ring PAHs, but ratio of 3-ring PAHs was lower than in dissolved phase. Maximum of concentrations of PAHs in the dissolved (6.5 ng/L) and particulate (10.6 ng/L) phases were found for a depth 300 m. The possible source of PAHs is the atmosphere.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) containing from 2 to 8 fused aromatic rings are persistent organic pollutants. These hydrocarbons may be released into the environment as a result of human activity, and as a result of natural processes. PAHs may be formed by a variety of mechanisms: pyrogenic, petrogenic, biogenic and diagenic (Neff, 2002). As a result of human activity, most of these hydrocarbons are formed during the incomplete combustion of organic matter (coal, oil, timber, tobacco, etc.) (WHO, 2000).

The physical properties of these compounds vary widely depending on the number of benzene rings and chemical structures (McGowin, 2006). The data on the persistence of these substances are different. For the process of partial destruction of PAHs average half-life ranges from 2 to 700 days (Haritash and Kaushik, 2009; Johnsen et al., 2005; Shuttleworth and Cerniglia, 1995). Also important property of PAHs is the bioaccumulation (D'Adamo et al., 1997). Some PAHs (benzo[a]pyrene, benzo[b]-fluoranthene, etc.) are known to be carcinogenic and mutagenic (WHO, 2001), and therefore, the distribution and fate of these substances in the environment should be determinated. Additionally PAHs are excellent markers to determine their origin, ways of transport and transformation processes of these substances in different environmental conditions.

The main sources of PAHs in seas and oceans are mainly river runoff and atmospheric input (Dachs et al., 1997; Dickhut et al., 2000; Lipiatou and Saliot, 1991).

The Japan Sea is a relatively closed ocean system because the each strait connecting the Japan Sea with ocean and other seas is narrow and shallow, and the maximum and average depths of Japan Sea are 3796 and 1350 m, respectively (Kosuke et al., 2005). So pollutants may accumulate long-term in the bottom of the Japan Sea and the pollution can be assumed to be of serious proportions. The surface circulation of the sea is well described in many works (e.g., Talley et al., 2006). The main circulation features are the strait inflows (Tsushima Strait and Tatar Strait) and outflows (Tsugaru Strait and Soya Strait), major currents (including the western and eastern boundary currents), the Subpolar Front and vigorous eddies.

The Amur River's waters are imported into the Japan Sea by Primorye (Liman) Current through Tatar Strait. Depending on the season the Amur River runoff are going to the Sea of Okhotsk (summer period) or to the Japan Sea (winter period). Approximately one-third of the annual Amur River discharge flow into the Japan Sea that is half of total river runoff (Dobrovol'skii and Zalogin, 1982; Ogi et al., 2001; Yakunin, 1978).

The Japan Sea is connected with waters of the East China Sea by Tsushima Warm Current and East Korean Warm Current through Tsushima Straight (Talley et al., 2006). The total concentrations of PAHs in surface waters of the East China Sea were observed from 70.22 to 120.29 ng/L (Ren et al., 2010).

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Monsoon climate is typical for the Japan Sea, south-eastern winds in summer and north-western winds in winter are dominant over the sea (Kaneyasu et al., 2000). The winter season is characterized by the transfer of air masses from China to the waters of the sea (Hayakawa et al., 2007; Tang et al., 2005). The last 20 years China's economy has been developing rapidly, which resulted in the significant growing consumption of energy derived mainly from fuel combustion (Feng et al., 2006; Huang et al., 2009). Hence during the heating season, air masses over China are most heavily contaminated with PAHs (Yang et al., 2007). Spatial distribution of atmospheric PAHs from the Bohai Sea to the high Arctic was studied by Ding et al. (2007). They have demonstrated a decreasing latitude trend for gas-phase PAHs and have noted higher levels of total concentration of atmospheric PAHs occurred in Far East Asia (34–48N, 122–148E) including the Japan Sea with an arithmetic mean of 47.9 ng/m^3.

There are a few investigations and reports of PAHs in the seawater of the Japan Sea. Nemirovskaya (2007) has reported total concentrations of PAHs in the Amurskiy Bay (Peter the Great Bay, northwestern part of the Japan Sea) widely varied, from 5 to 85 ng/L. There is a gap in our knowledge about PAHs contents in the open part of the Japan Sea. This paper presented data about dissolved and particular forms of PAHs contents in seawater samples collected in late summer in 2010.

2. Materials and methods

2.1. Sampling site

Surface water samples in all stations were collected in the Japan Sea from August 11 to September 5, 2010 by cruise of R/V "Akademik M.A. Lavrent'ev". Also on the station D1 the vertical distribution of PAHs were studied. The sampling sites are shown in Fig. 1. All stations are located in northern from Subpolar Front. Five liters of sea water was collected by a Niskin bottle and conductivity and temperature depth profiler (CTD), respectively, were monitored.

2.2. Sample treatment

Water samples were filtered through a 0.5 μm pore glass fiber filter (Advantec GC50) to separate the dissolved phase from the particulate phase. The filters containing SPM samples were dried in air conditions for 1 h in the dark.

2.3. PAH extraction

Prior to HPLC analysis, PAHs in the dissolved phase (DP) and in the particulate phase (PP) were extracted by different methods.

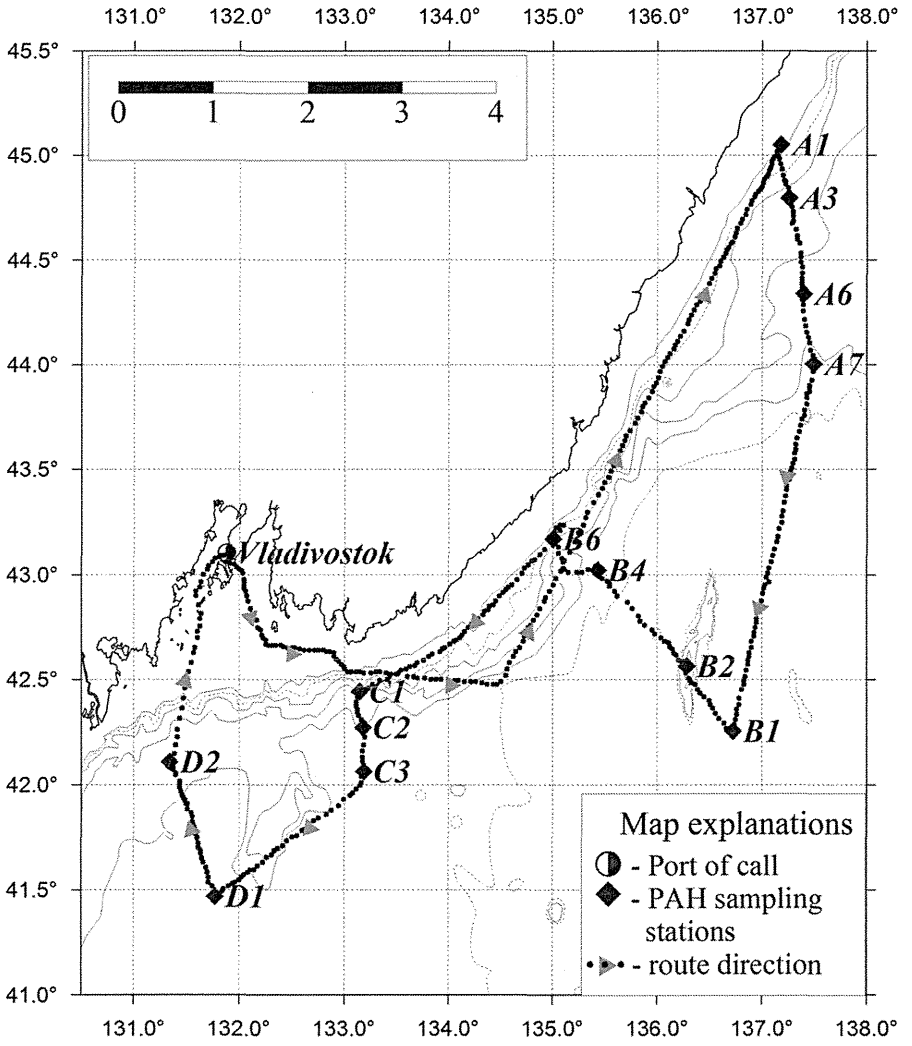

Fig. 1. The map of the cruise Lav-51.