Effects of Chum Salmon Stanniocalcin, a Calcium Regulating Hormone in Teleosts and its Synthetic N-terminal Peptide Fragment on Bone Metabolism

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1996

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Ph. D. Thesis

by

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1996

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Professor Norihiko Maeda, Department of Oral Anatomy I for his friendship, generous support, guidance and interest throughout this study.

I am also very grateful for the generous support from and continued, fruitful discussions with Professor Takashi Uchida, Department of Oral Anatomy II, and Professor Yukio Kato, Department of Oral Biochemistry.

Many thanks are due to my coworkers, especially Dr. Yoshio Koide, associate professor of Laboratory of Molecular Endocrinology, School of Fisheries Sciences, Kitasato University, and Mr. Tetsuya Kosugi, Pharmaceutical Research Laboratories, POLA R and D Laboratories for their continuous guidances and invaluable supports .

My gratefulness is extended to Professor Hiroshi Kawauchi, Laboratory of Molecular Endocrinology, School of Fisheries Sciences, Kitasato University, and Dr. Hiromichi Sagitani, director of Pharmaceutical Research Laboratories, POLA R and D Laboratories, for their gainful encouragements.

My thanks are due to my colleagues in the Department of Oral Anatomy I.

APPENDIX

This thesis is based on the following paper:

Yoshiko, Y., Kosugi, T. and Koide, Y. Effects of a synthetic N-terminal fragment of stanniocalcin on the metabolism of mammalian bone *in vitro*. Biochimica et Biophysica Acta, in press.

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CONTENTS

1. INTRODUCTION

The predominant hypocalcemic hormone in bony fishes is stanniocalcin (STC), previously referred to as hypocalcin or teleocalcin (*e.g.* Wagner *et al.*, 1986, Lafeber *et al.*, 1988 and Wagner *et al.*, 1988). STC is synthesized and secreted by the corpuscles of Stannius (CS), that are located on the mesonephric kidney (*e.g.* Wagner *et al.*, 1986, Lafeber *et al.*, 1988, Wagner *et al.*, 1988 and Hirano, 1989) and first described by Stannius (1839). It has been proposed as the major active principle from the CS and it was purified from sockeye salmon (Wagner *et al.*, 1986), Australian eel (Butkus *et al.*, 1987), coho salmon (Wagner *et al.*, 1988), and rainbow trout (Lafeber *et al.*, 1988). It is a homodimeric glycoprotein with a molecular weight ranging from 39 (sockeye salmon) to 54 kDa (rainbow trout) in the native form and from 18-32 kDa under reducing conditions. The nucleotide sequences of STC cDNA from Australian eel (Butkus *et al.*, 1987) and coho salmon (Wagner *et al.*, 1992) have been elucidated. However, the complete amino acid sequence of STC has not yet been determined, except for the N-terminal region (Wagner *et al.*, 1986, 1988, Lafeber *et al.*, 1988 and Sundell *et al.*, 1992). STC exhibits no similarities to calcitonin (CT) or to parathyroid hormone (PTH) in terms of the amino acid sequence deduced from Australian eel cDNA (Butkus *et al.*, 1987). The target organs of STC appear to be the kidney (Lu and Wagner, 1994), the intestine (Sundell *et al.*, 1992) and the gills (So and Fenwick, 1977, 1979, Milet *et al.*, 1979, Ma and Copp, 1982 and Fenwick, 1989), which play an important role in calcium and potassium regulation in teleosts.

CT plays physiologically important roles in maintenance of calcium homeostasis and it is a potent inhibitor of osteoclastic bone resorption (Chambers *et al.*, 1983, 1985, 1986). In clinical studies, the specific activity of CT derived from bony fishes is

higher than that derived from mammals. However, the hypocalcemic effect is governed by STC rather than CT in bony fishes. Therefore, the biological functions of STC have been noted in the comparative endocrinology and the phylogeny of calcium metabolism. Possible biological functions for components of the CS and STC in mammals have been discussed, as has the possibility that the effects of STC are similar to those of PTH (Milet *et al.*, 1979, Milet *et al.*, 1982, Lopez *et al.*, 1984, Lafeber *et al.*, 1986, Lafeber *et al.*, 1989 and Stern *et al.*, 1991). Serum levels of calcium and magnesium are increased in rats after an intraperitoneal (i.p.) injection of an extract of silver eel CS (Milet *et al.*, 1982). A PTH-like molecule in silver eel plasma was identified from its cross-reactivity with an antiserum against bovine PTH (bPTH) and the immunoreactive substance was no longer detected after removal of the CS (Lopez *et al.*, 1984). The PTH-like substance was detected in all cells of the silver eel CS by immunocytochemistry using an antiserum against bPTH (Lopez *et al.*, 1984). In other studies, the administration (i.p.) to rats of an extract of silver eel CS resulted in the activation of osteoclastic bone resorption at the periosteum of the femur (Milet *et al.*, 1982). A bone resorption assay using organ cultures of fetal bones revealed that extracts of silver eel CS (Milet *et al.*, 1982) and of rainbow trout CS (Lafeber *et al.*, 1986), as well as purified STC from rainbow trout (Lafeber *et al.*, 1989), stimulate the release of calcium in a manner that resembles the release of calcium in response to stimulation by bPTH. However, Ma and Copp (1982) have reported that pacific salmon STC infusion has no significant effect on plasma calcium in rats, in contrast to hypocalcemic effect of the protein in fish and young chicks. Moreover, pure STC from coho salmon does not consistently stimulate bone resorption in a variety of rodent bone-culture systems and that it has limited PTH-like activity, if any, on calcium metabolism in a mammalian system (Stern *et al.*, 1991).

Evidence was obtained for the existence of STC-like immunoreactivity in sera of several vertebrates and in human kidney, but the corresponding gene was not been

cloned (Wagner *et al.*, 1995). However, Chang *et al.*, (1995) was recently obtained a human cDNA clone coding for a protein highly homologous to fish STC. The mRNA is expressed in human tissues, with the highest levels in ovary, prostate and thyroid. Human cell (SUSM-1 cells, immortalized liver fibroblasts) culture studies show that the mRNA is positively regulated by extracellular Ca^{2+} in the medium.

Some investigators have described the activities of fragments of the STC molecule *in vivo* (Butkus *et al.*, 1989, Milliken *et al.*, 1990, Verbost *et al.*, 1993, Fenwick and Verbost., 1993 and Verbost and Fenwick, 1995). In fish, it has been found that an N-terminal peptide fragment of Australian eel (Milliken *et al.*, 1990, Verbost *et al.*, 1993 and Verbost and Fenwick, 1995) and sockeye salmon (Milliken *et al.*, 1990) STC has an inhibitory effect on whole-body calcium uptake, with the effect resembling that of the intact molecule. There is no agreement about the function of the C-terminal region (Milliken *et al.*, 1990, Verbost *et al.*, 1993, Fenwick and Verbost., 1993 and Verbost and Fenwick, 1995). The actions of six peptide fragments of Australian eel STC have been tested in sheep, and plasma potassium levels were affected by either the N-terminal region or a mid- sequence fragment of the STC (Butkus *et al.*, 1989).

Identification of the active site of the STC molecule in mammalian systems helped to clarify the actions of STC in mammals, as well as the relationship between STC and PTH. Therefore, we completely sequenced and characterized of STC from chum salmon. We also characterized the effects of the native form of STC and its synthetic N-terminal fragment, which includes the region that functions as the active site in fish, on the PTH-sensitive aspects of the metabolism of mammalian bone *in vitro*.

2. MATERIALS AND METHODS

2.1. Preparation of STC

The CS were collected from mature female chum salmon, *Oncorhynchus keta*, and STC was prepared according to Sundell *et al.* (1992). The CS were homogenized and extracted with 35% ethanol-10% ammonium acetate, pH 8.0, containing 5 mM EDTA and 1.5 mM phenylsulfonylfluoride at 4 °C, then mixed into cold ethanol. The precipitate was fractionated on a DE-52 column (Whatman, Kent, UK) and a Superdex 75 column (Pharmacia LKB, Uppsala, Sweden) and subsequently purified by reversed phase high performance liquid chromatography (rpHPLC) on a TSK gel ODS-120T column (Tosoh, Tokyo, Japan).

The protein peak in rpHPLC were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12 % polyacrylamide gel at pH 8.8, essentially as described by Laemmli (1970) with and without 1% β-mercaptoethanol and stained with Coomassie brilliant blue.

2.2. Intestinal Ca^{2+} influx of Atlantic cod

To determine the effects of STC on the unidirectional intestinal Ca^{2+} influx (J^{Ca}) of the Atlantic cod (*Gadus morhua*), isolated intestinal preparation was used as described in detail by Sundell and Björnsson (1990). The intestine was perfused through the coeliac artery with a cod Ringer's solution containing 0.1% bovine serum albumin (BSA; Sigma, St Louis, MO). The outflow from the intestinal vein was collected in fractions throughout the experiment. The intestinal lumen was perfused with a BIS based on ion levels measured in the intestinal fluid (Sundell and Björnsson,

1990). $^{45}CaCl_2$ (specific activity 0.85 ± 0.06 MBq/μmol; Amersham International, Buckinghamshire, UK) was added to the BIS. All perfusions were carried out for 3 h. The first hour was to allow the ^{45}Ca appearance rate to reach a steady state across the intestinal mucosa. During the second hour, the venous outflow was collected and the basal J^{Ca} for each preparation calculated. During the third hour, purified protein or vehicle was added to each preparation. J^{Ca} was calculated as the ratio of total radioactivity of each fraction to the original radioactivity level of the tracer added to the perfusion-solution as well as for 60-min periods with the flow linearly collected to the body weight (Fig. 1).

2.3. Preparation of peptide fragments of STC

The STC was reduced and pyridyl-ethylated (RPE), according to Friedman *et al.* (1970). STC dissolved in 1 M Tris-HCl buffer (pH 8.3) containing 6 M guanidine-HCl and 2 mM EDTA was reduced with dithiothreitol and subsequently alkylated with 4-vinylpyridine. The protein solution was loaded onto a TSK gel ODS-120T column. STC was also reduced with dithiothreitol and alkylated with iodoacetic acid in 2 N NaOH according to Hirs (1967). The resulting reduced and carboxymethylated (RCM)-STC was recovered as described above. The RPE-protein was digested with trypsin (T) and endoproteinase Asp-N (AN). The RCM-STC was digested with *Staphylococcus aureus* V8 protease (SP). All fragment peptides were fractionated by rpHPLC on a TSK gel ODS-120T column.

2.4. Amino acid sequence analysis

The amino acid composition of the STC and the fragment peptides was determined using an automated amino acid analyzer (Hitachi, Model L-8500, Tokyo) after hydrolysis with 6 M HCl containing 0.6% phenol at 110 °C (Muramoto *et al.*, 1987).

Cysteine was determined as S-pyridylethyl cysteine. The amino acid sequence of the C-terminal region was determined by measuring the amino acids released after incubating RPE-STC with carboxypeptidase Y (CPY), according to Hayashi *et al.* (1973).

Sequences were determined using a gas-phase automated amino acid sequencer (Shimadzu, Model PSQ-1, Tokyo) and 3-phenyl-2-thiohydantoin (pth)-amino acids were identified by rpHPLC on a Wakosil-pth column eluted with pth-amino acids mobile phase solvent (Wako Pure Chemical, Osaka, Japan).

2.5. Cloning of cDNA encoding STC

DNA was manipulated and transformed into *E. coli* according to Sambrook *et al.* (1989). The PCR primer was synthesized and DNA was sequenced using an automated DNA synthesizer (Applied Biosystems, Model 381A, Foster City, CA) and a DNA sequencer (Applied Biosystems, Model 393A), respectively. To amplify the DNA coding for the STC from the mRNA of CS, two degenerate primers were synthesized (Fig. 2). Primer 1 corresponded to the N-terminal amino acid sequence of the STC and contained an additional ATG, which is the initiation codon for expression in *E. coli*. Primer 2 corresponded to the C-terminal amino acid sequence of STC and contained an additional TAG codon with which to terminate translation.

CS was homogenized and mRNA was obtained using a Clontech kit (Takara, Siga, Japan). The cDNA was synthesized from 500 ng of mRNA using a Gene Amp RNA PCR kit (Takara). DNA was amplified by 35 cycles of amplification (first 5 cycles: 95 °C for 30 s, 35 °C for 2 min, and 70 °C for 1 min, followed by 30 cycles: 95 °C for 30 s, 40 °C for 2 min, and 70 °C for 1 min). Amplified fragments were ligated to the pT7Blue T-vector (Novagen, Madison, WI) and transformed into *E. coli*

JM109 (Takara). Forty transformants were selected and the size of inserted DNA was determined by digestion with restriction endonucleases.

2.6. Synthesis of the N-terminal peptide fragment of STC

A synthetic peptide of N-terminal fragment 1-20 of STC from chum salmon (peptide N) was prepared by solid-phase BOC chemistry (Merrifield, 1985) using an amino acid synthesizer (Applied Biosystems, Model 430A). The peptide was cleaved from the resin with anhydrous hydrogen fluoride and the crude peptide was purified by rpHPLC on a TSK gel ODS-120T column. The homogeneity of the preparation of the purified peptide was confirmed with a gas-phase automated amino acid sequencer.

2.7. Calvarial bone-resorption assay

Pregnant Sprague-Dawley rats were injected subcutaneously with $^{45}CaCl_2$ (Amersham International) at a dose of 3.7 MBq per rat on the 18th day of gestation. On the following day, two calvariae from fetuses were selected randomly and cultured in each well of a 24-well plate in Fitton-Jackson modified BGJb medium (Gibco BRL, Gland Island, NY) containing 2 mg/ml BSA for 24 h. The bones were then transferred to fresh medium that contained vehicle as a control, various concentrations of purified STC or peptide N with or without the N-terminal fragment of human PTH (hPTH1-34; Peninsula Laboratories, Belmont, CA) and incubated for an additional 2-4 days. The extent of bone resorption was calculated as the release of ^{45}Ca, expressed as the percentage of original total radioactivity of the bones (sum of the radioactivity of the medium and the bones), as described (Raisz, 1965).

2.8. Generation of osteoclast-like cells

Our procedure was based on that described Takahashi *et al.* (1988). Marrow cells of the tibiae from 7 week-old ddY mice were collected, washed with minimum essential medium α medium (α-MEM; Gibco BRL) and suspended in α-MEM supplemented with 10% fetal calf serum (FCS; Gibco BRL). The suspension was divided among the wells of 24-well plates and incubated with test samples as described above for 8 days. After culture period, adherent cells were rinsed with phosphate buffered saline (PBS), fixed with ethanol-acetone (50:50, vol/vol) for 1 min, and stained for tartrate-resistant acid phosphatase (TRAP) by incubating the cells in 0.1M sodium acetate buffer (pH 5.0) containing naphthol AS MX phosphate (Sigma) and red violet LB salt (Sigma) in the presence of 10 mM sodium tartrate. TRAP positive cells containing three or more nuclei and having a podozome-like structure at the peripheral edge of the cytoplasm were counted under a microscope.

2.9. Production of cyclic AMP in osteoblast-like cells

ROS17/2.8-5 cells derived from a rat osteosarcoma (Riken Cell Bank, Ibaraki, Japan) were grown in Ham's F-12 medium (Gibco BRL) supplemented with 10% FCS. The cells were subcultured at weekly intervals, then plated in 48-well plates. When the cells were reached confluence, they were washed and incubated with the same medium containing 0.3% BSA and 0.1 mM isobutylmethylxanthine (Sigma) as described (Nakatani *et al.*, 1984). Test samples (except for STC) were added to the cells and incubated for a further 5 min. The reaction was stopped by aspirating the medium then immediately adding ice-cold 65% ethanol. The plates were placed in a ultrasonic bath to release cyclic AMP from the cells. The cyclic AMP was quantified by a cyclic AMP-specific enzyme immunoassay system (Amersham International).

2.10. Synthesis of collagen in calvarial bone

The synthesis of bone collagen was assayed as described (Dietrich *et al.*, 1976). Calvariae obtained from 4-day-old ddY mice were cultured in modified BGJb medium supplemented with 4 mg/ml BSA for 3 h. Three pieces of bone per flask were then transferred randomly to flasks of fresh medium containing vehicle, peptide N or hPTH1-34 and incubated on a shaker (60 oscillations/min) for 4 days. The medium containing test samples was changed every day. The bones were pulse-labeled with L-[2, 3-^{3}H]proline at 0.37 MBq/ml (999 MBq/mmol; Amersham International) during the last 2 h of culture. At the end of culture period, the bones were extracted, dried, weighed and homogenized. An aliquot of the homogenate was incubated with highly purified bacterial collagenase (form III from *Clostridium histolyticum*; Advance Biofactures, Lynbrook, NY). The amounts of radio labeled proline that had been incorporated into collagenase-digestible (CDP) and non-collagen protein (NCP) were determined by the method of Peterkofsky and Diegelmann (1971). The data are expressed as dpm per microgram of dry weight of bones. The percent collagen synthesis was calculated after multiplying NCP by 5.4 to correct for the relative abundance of proline in collagen and non-collagen protein (Peterkofsky, 1972).

2.11. Statistical analysis

Statistical evaluations were determined by analysis of variance (ANOVA) with multiple comparisons by the method of Fisher's protected least significant difference. The significance of differences was accepted at $P<0.05$.

3. RESULTS

3.1. Isolation and characterization of STC

Figure 3 shows ion exchange chromatography of an ethanol extract of the CS from chum salmon on a DE52 cellulose column. Figure 4 shows gel filtration of the DE6 fraction obtained from the DE52 column on a Superdex 75. The yields obtained from each step in the extraction of 60g of the CS were as follows: DE-52 column, fraction DE6, 184.1 mg; Superdex 75 column of fraction DE6, fraction G4, 2 mg. When fraction G4 from Superdex 75 was applied to rpHPLC on TSK-gel ODS 120T columns, one protein peak was eluted (Fig. 5).

In SDS-PAGE under nonreducing conditions, STC migrated with a molecular weight of 46.3 kDa (Fig. 6). In the presence of a reducing agent, the protein peak migrated with a molecular weight of 23.4 kDa (Fig. 6).

The effects of STC on J^{Ca}, calculated over 60-min periods before and during perfusion, are presented in Figure 7. Perfusion without STC resulted in no deference from the basal flux, whereas the protein decreased J^{Ca} (2.0 nM STC, 0.89 $\pm$ 0.24 μmol vs. control, 1.02 $\pm$ 0.23 μmol; 10.0 nM STC, 0.97 $\pm$ 0.23 vs. control, 1.28 $\pm$ 0.38 μmol Ca^{2+}/h/kg body weight). In the inset figure (Fig. 7), the J^{Ca} from the three perfusion periods (third hour of perfusion) are normalized and expressed as a percentage of basal flux (second hour of perfusion) in each treatment group. This revealed a dose-related effect of the protein on the intestinal Ca^{2+} influx, in which 2.0 and 10 nM STC decreased the influx by 12.7 and 24.2%, respectively.

3.2. Amino acid and cDNA sequence of STC

RPE-STC eluted as a single peak on rpHPLC following the reduction and pyridylethylation (RPE) of intact STC and it had a single amino acid sequence that allowed the assignment of 24 residues. These and SDS-PAGE results described above, indicate that the protein is a disulfide-linked homodimer.

To determine the complete amino acid sequence, peptide fragments were generated with proteases (T, AN and SP). Tryptic digestion of RPE-STC yielded 14 fragments (T1-T13) by rpHPLC and rechromatography of T13 (Fig. 8). The amino acid composition and yield of the pth-amino acids of the intact protein and fragments are listed in Tables 1 and 2, respectively. Three AN fragments (AN33, AN26, and AN30) and one of SP (SP44) are shown in these tables, although all the fragments were analyzed. T12 overlapped residues of 12-14 of the protein and extended 16 additional residues up to position 40 with the exception of cycle 18 (residue 29). AN33 overlapped residues 39-40 of the protein and thus 47 additional residues were determined up to position 87. T4, T2, T3, T9 and T7 confirmed the sequence of 57-60, 61-64, 65-73, 74-79 and 81-86, respectively. T8 overlapped residue 87 (methionine) of the protein and thus 11 additional residues were determined up to position 98. SP44 overlapped residues 95-98 of the protein and extended 16 additional residues up to position 114. AN26 overlapped residues 100-114 of the protein and extended 23 additional residues up to position 137. T10 and T11 confirmed the sequences of 99-106 and 107-125, respectively. T13B overlapped residues 126-137 of the protein and extended 8 additional residues up to position 145. AN30 overlapped residues 138-145 of the protein and extended 34 additional residues up to position 179. T5, T13A and T1 confirmed the sequences of 147-152, 153-167 and 168-179, respectively. T1 provided the carboxyl-terminal sequence because it contained no lysine and arginine. Kinetic studies of CPY digestion confirmed that the carboxy-terminus of the intact molecule was serine. The monomer consisted of 179 amino acids

including 11 half-Cys residues and one consensus N-glycosylation site at Asn-29 (Fig. 9). This Asn residue was confirmed by detecting Asp in the hydrolysate of the pth-amino acid obtained at the 18th cycle of T12. Based on the sequence analysis, the calculated molecular weight of the polypeptide was 19,327 which is lower than that estimated by SDS-PAGE (23.4 kDa). This discrepancy can be accounted for by the carbohydrate moiety of the molecule.

An analysis of forty transformants showed that one plasmid was suitable for inserting DNA, deduced from the amino acid sequence. The plasmid pKDST1 had a 55-base pair insertion that encoded 179 amino acids, except for an additional first Met and the primer region (Fig. 10). A comparison of the amino acid sequence deduced from the cDNA with that of the protein revealed that total number of amino acids and the positions of all the half-Cys residues were identical. However, the amino acid sequence deduced from the cDNA differed from that of the protein at two positions: Leu for Pro-160 and Pro for Ser-170.

3.3. Synthesis of N-terminal peptide fragment of STC

The synthetic peptide (peptide N); (Fig. 11) was eluted as a single peak after rpHPLC, and the correct anticipated sequence of amino acid was determined by direct sequencing of the amino acids.

3.4. Effects of STC and peptide N on the calvarial bone resorption

Because the reported PTH-like effects of STC are discrepant, we initially examined the bone-resorptive activity of native form of STC and a synthetic peptide, peptide N, from chum salmon on 4-day-cultured calvariae of fetal rats labeled with ^{45}Ca. The effects of the samples from chum salmon were compared with those of hPTH1-34. Although hPTH1-34 (10^{-7}-10^{-10} M) increased the release of ^{45}Ca into the

medium from calvariae in a dose-dependent manner (Fig. 12A), STC had no effect at the concentrations we applied (10^{-9}-10^{-13} M) (Fig. 12B). However, peptide N inhibited the rate of release of ^{45}Ca and the effect was significant at 10^{-11}-10^{-12} M (Fig. 12B).

Figure 13 shows the time course of the effects of intact STC and peptide N on the calvariae from fetal rats. In the control, the rate of release of ^{45}Ca into the medium increased with increase duration of culture, from the second to the fourth day. When the bones were cultured with 10^{-11}M peptide N, the rate of release was significantly reduced on the fourth day (peptide N, 13.6 $\pm$ 1.6% vs. control, 19.1 $\pm$ 1.7% of ^{45}Ca released). There was no effect in the intact STC during the culture period.

Next, we examined the effects of intact STC and peptide N on PTH-stimulated bone resorption. The rate of ^{45}Ca release from calvariae cultured for 2 days in the presence of hPTH1-34 (10^{-8} M) was not affected by intact STC (Fig. 14). However, peptide N inhibited the increased release of ^{45}Ca in cultures exposed to hPTH1-34 at dose that was effective in the absence of the latter (Fig. 14). Peptide N inhibited hPTH1-34-stimulated bone resorption by about 30.7% (hPTH1-34 plus peptide N, 14.1% $\pm$ 1.9% vs. hPTH1-34 alone, 22.4 $\pm$ 3.4% of ^{45}Ca released).

3.5. Effects of STC and peptide N on generation of osteoclast-like cells

The effect of peptide N on the formation of osteoclast-like cells is shown in Figure 15. These were few osteoclast-like cells in the original control culture after 8 days (Figs. 15A, B). An incubation with hPTH1-34 (10^{-7}-10^{-10} M) dose-dependently increased the number of the cells (Figs. 15A, 16). The effect of peptide N was apparent when cultures incubated in the presence and in the absence of hPTH1-34 were compared. No effect of peptide N was detected at the tested concentrations (10^{-9}-

10^{-13} M) in the absence of hPTH1-34 (Fig. 15B), whereas the synthetic peptide inhibited hPTH1-34-dependent increases in the number of osteoclast-like cells. This increase was significantly reduced by peptide N (10^{-10}-10^{-12} M) and the maximum response was detected at 10^{-11} M (hPTH1-34 plus peptide N, 12.8 $\pm$ 1.5 cells vs. hPTH1-34 alone, 21.0 $\pm$ 2.5 cells per well); (Fig. 15C). Intact STC had no effect upon these systems with or without hPTH1-34 (Data not shown).

3.6. Effects of peptide N on the production of cyclic AMP in osteoblast-like cells

Several lines of evidence indicate that one criterion of PTH potency is to stimulate cyclic AMP formation. The effect of peptide N on the formation of cyclic AMP in osteoblastic ROS17/2.8-5 cells was compared with that of hPTH1-34. Because there was no effect of intact STC in PTH-sensitive systems, we examined the effects of peptide N on this system. The addition of hPTH1-34 (10^{-7}-10^{-10} M) to the cells increased cyclic AMP levels in a dose-dependent manner (Fig. 17A), whereas peptide N (10^{-9}-10^{-13} M) did not affect the levels of cyclic AMP (Fig. 17B). When peptide N at various concentrations was added with 10^{-8} M hPTH1-34, the accumulation of cyclic AMP in response to hPTH1-34 was significantly inhibited at 10^{-11}-10^{-13} M (Fig. 17C). The maximum effect was seen at 10^{-11}M (hPTH1-34 plus peptide N, 1.11 $\pm$ 0.06 pmol vs. hPTH1-34 alone, 2.84 $\pm$ 0.49 pmol cyclic AMP per well).

3.7. Effects of peptide N on the synthesis of calvarial bone collagen

Table 3 summarizes the effects of hPTH1-34 and peptide N on the synthesis of collagen in calvariae of newborn mice. Human PTH1-34 (10^{-8} M) caused a significant decrease in the rate of incorporation of [^{3}H]proline into CDP, while labeling of NCP

was increased (but not significantly). In bones incubated with peptide N, NCP labeling by proline was slightly increased (but not significantly). By contrast, the labeling of CDP by proline was significantly increased by peptide N (10^{-11}-10^{-13} M), so that the relative level of collagen synthesis in the presence of the peptide appeared to be greater than that in the control. The effect of peptide N on the rate of incorporation of [^{3}H]proline into CDP was maximal at 10^{-12} M (control, 12.01 $\pm$ 0.57 dpm vs. peptide N, 17.41 $\pm$ 1.31 dpm/μg dry weight).

4. DISCUSSION

4.1. Chemical properties of chum salmon STC

Intact chum salmon STC migrated as one band on SDS-PAGE with a molecular weight of 46.3 kDa. This is comparable to reported STCs ranging from 39 kDa in sockeye salmon (Wagner *et al.*, 1986) to about 54 kDa in the rainbow trout (Lafeber *et al.*, 1988). After reduction with β-mercaptoethanol, STC migrated as one 23.4 kDa-band, suggesting that STC is a homodimer. Further support for this notion was obtained from rpHPLC after the reduction of intact STC. Thus, chum salmon STC is likely consist of two identical polypeptide chains, as also proposed for coho salmon STC (Wagner *et al.*, 1988) and rainbow trout STC (Lafeber *et al.*, 1988 and Filk *et al.*, 1989, 1990). The purified STC from chum salmon represents a biological activity in the intestine, which is proposed as one target organ of STC in marine teleosts (Sundell *et al.*, 1992).

Chum salmon STC monomer, prepared from the intact STC by reduction and alkylation, consisted of 179 amino acid residues with 11 half Cys residues and one N-linked glycosylation site at position 29. The complete amino acid sequence of chum salmon STC was compared with those deduced from the cDNA of coho salmon (Wagner *et al.*, 1992), Australian eel STC (Butkus *et al.*, 1987) and human (Chang *et al.*, 1995); (Fig. 11). From residues 1-179, chum salmon STC was identical with coho salmon STC except for the 3rd and 18th residues. It also had high sequence identity with Australian eel STC (78%) and human (62%). Moreover, all half-Cys residues and an N-linked Asn of chum salmon STC were located in positions homologous to those in the three deduced sequences.

The cDNA was cloned by RT-PCR by using two primers corresponding to the N- and C-terminal sequences of the protein. However, the amino acid sequence deduced from the cDNA differed from that of protein at two residues (positions 160 and 170). Although it is possible that the cDNA is a mutant produced during the PCR process, this might be due to the occurrence of two genes encoding STC in chum salmon, according to the tetraploid hypothesis of salmonid fishes. Although the cDNA obtained here may have been a variant which is rarely or never expressed, the nucleotide sequence of the cDNA was comparable with that of coho salmon STC cDNA (Fig. 10). Thus STC is well conserved in these salmonid fishes, at the gene and protein levels .

It is notable that the chum salmon STC was 44, 52 and 35 residues shorter at the C-terminal region than the sequences deduced from coho salmon, eel and human STC cDNAs, respectively. The C-terminal residue of chum salmon STC is Ser, which is not generally cleaved by proteolytic enzymes. There were also two dibasic pairs (Arg79-Arg80, and Lys167-His168) in the sequence of chum salmon STC, which were much more subject to enzymatic cleavage by endoproteinases than the Ser residue. Thus, the possibility that the chum salmon STC obtained here was a product of proteolysis during purification, was negated.

The C-terminal region (180-223) of the deduced sequence of coho salmon STC, which is absent in that of chum salmon STC has low sequence identity with Australian eel and human STC , although the N-terminal to residue 179 has high identity . The full length cDNA of chum salmon CS is required to clarify whether the STC is a primary transitional product or a further post-transitional processing product.

4.2. Effects of STC and peptide N on the metabolism of mammalian bone

The PTH-like effects of STC have been mainly determined by bone resorption assays in extracts of silver eel CS (Milet *et al.*, 1979), rainbow trout CS (Lafeber *et al.*, 1986) and rainbow trout STC (Lafeber *et al.*, 1989). However, coho (Stern *et al.*, 1991) and chum salmon STC (this study) had no effect in that system. Moreover, peptide N, which had high sequence identity among those bony fishes inhibited hPTH1-34-stimulated bone resorption. The assay of formation of osteoclasts-like cells from bone marrow cells seemed to support the lack of STC effects and the inhibition by peptide N in the bone resorption assay. Evidence from a wide variety of studies suggests that osteoclasts are derived from hemopoietic progenitor cells (Nijeweide *et al.*, 1986 and Mundy and Roodman, 1987). Osteoclast-like TRAP positive multinucleated cells can be generated from mouse bone marrow cells in culture by stimulation with PTH (Takahashi *et al.*, 1988). TRAP positive multinucleated cells formed in response to osteotropic hormones satisfy most of the criteria for osteoclasts. In this study, STC and peptide N did not induce the formation of such cells from murine bone marrow cells, whereas peptide N alone inhibited the response to hPTH1-34 in a number of the cells. It is suggested that the differentiation of osteoclasts is regulated by stromal cells and various humoral factors (Takahashi *et al.*, 1988 and Hattersley and Chambers, 1989), although the details remain uncertain. The understanding the cell linage of osteoclasts may be an important clue to elucidating the effect of STC1-20 on the formation of osteoclast-like cells.

Peptide N also decreased bone resorption in the absence of hPTH1-34, but an obvious effect was detected after a long culture period (4 days). This delay might have been due to the influence of non-PTH factors, such as endogenous prostaglandins (PGs; Katz *et al.*, 1981 and Chambers *et al.*, 1985). As Chambers *et al.* (1985) pointed out, PGs also exert bone-resorbing activities via increasing cyclic AMP levels

in osteoblasts, as does PTH. It seems that the effects of peptide N on the PTH-sensitive systems, described above, represent an indirect, rather than a specific action at the PTH receptor because the peptide did not have a pronounced effect on hPTH1-34-dependent bone resorption and had only a slight effect on PTH-independent, PG-related bone resorption (Katz *et al.*, 1981 and Chambers *et al.*, 1985).

It is apparent that cyclic AMP in osteoblasts is an important second messenger for PTH (*e.g.* Nakatani *et al.*, 1984 and Lerner *et al.*, 1989) in the PTH-dependent activation of osteoclasts. PTH stimulates the accumulation of cyclic AMP in osteoblast cell lines and osteoblasts from rat calvariae (*e.g.* Nakatani *et al.*, 1984 and Lerner *et al.*, 1989). Lafeber *et al.* (1989) reported that rainbow trout STC resembled PTH in its bone-resorbing effect, even though it did not stimulate the production of cyclic AMP. They suggested that rainbow trout STC lacks a cyclic AMP-stimulating segment, since fragments of PTH can induce bone resorption without increasing cyclic AMP levels. In this study, peptide N did not stimulate the production of cyclic AMP in ROS 17/2.8-5 cells, as seen with native STC from trout (Lafeber *et al.*, 1989) and coho salmon (Stern *et al.*, 1991). However, peptide N reduced the production of cyclic AMP in response to hPTH1-34 in ROS 17/2.8-5 cells. It is possible that the activities of peptide N on the metabolism of mammalian bone are at least partly related to the response of cyclic AMP-mediated signals.

PTH has bone-catabolic action and it may also have an anabolic effect (Dietrich *et al.*, 1976, Linkhart *et al.*, 1989 and Canalis *et al.*, 1989). Since the effects of STC on bone formation had not previously been reported, we examined the direct effects of peptide N on the synthesis of bone collagen. Peptide N enhanced synthesis of bone collagen specifically, whereas hPTH1-34 inhibited such synthesis, as reported (Dietrich *et al.* 1976). Thus, we found that peptide N and hPTH1-34 have dissimilar effects on the synthesis of bone collagen, as well as on bone resorption.

These effects of peptide N were apparently similar to those of CT (Holtrop *et al.*, 1974, Feldman *et al.*, 1980, Baron and Vignery, 1981, Takahashi *et al.*, 1988 and Farley *et al.*, 1988). It is unlikely that peptide N binds to the CT receptor, because there is no constitutional homology recognized between STC and CT (Butkus *et al.*, 1987). STC decreases calcium levels in blood, although CT seems of minor importance as a calcium-regulating factor in fish (Feinblatt, 1986). Moreover, the STC receptor has not been cloned, even in fish. We will continue to examine this issue in detail. In addition, peptide N elicited biphasic concentration-dependence in our assay systems. PGE_2 also biphasically affects bone formation (Raisz *et al.*, 1990), which might have been due to the presence of two cell types. Different targets may respond to peptide N, since it has diverse effects on mammalian systems.

It seems that STC and material from the CS show marked similarity, in terms of bioactivity, to mammalian PTH in both mammalian (Milet *et al.*, 1979, Lafeber *et al.*, 1986 and Lafeber *et al.*, 1989) and teleostean assay systems (Wendelaar Bonga *et al.*, 1988). However, there is still some controversy as to whether STC is identical to a PTH-like substance of the CS. In fact, PTH does not cross-react with antiserum against STC secreted from primary cultures of rainbow trout CS cells (Gellersen *et al.*, 1988). Two types of glandular cells have been described in the CS of several teleosts (Lopez, 1969, Krishnamurthy and Bern, 1969, Wendelaar Bonga and Greven, 1975, Wendelaar Bonga *et al.*, 1976 and Olivereau and Olivereau, 1978). Type I cells are supposed to produce a proteinaceous hypocalcemic hormone, while type II cells may be engaged in the synthesis or metabolism of a hormone involved in the regulation of sodium and potassium levels (Wendelaar Bonga *et al.*, 1976). Stern *et al.* (1991) have reported that purified STC from coho salmon has no effect on several types of fetal bone and by the same token, our observations indicated that chum salmon STC did not affect the PTH-sensitive systems. On the other hand, calcium and phosphate release, as well as lactate production in embryonic mouse calvariae are stimulated in a dose-

dependent manner by purified STC from rainbow trout (Lafeber *et al.*, 1989). One explanation for these differences is that the tertiary structure of the STC molecule reacts to PTH-sensitive systems. Milliken *et al.* (1990) have reported that the C-terminal fragment of Australian eel STC (peptide W) enhances whole-body calcium uptake in the trout fly. The work of Fenwick and Verbost (1993) suggests that the effect of peptide W on calcium uptake in the trout and eel lies in the origin of the peptide. It is indeed clear that the C-terminal end of STC differs among Australian eel (*Butkus et al.*, 1987), coho salmon (Wagner *et al.*, 1992), human (Chang *et al.*, 1995) and chum salmon (this study). In our experiments moreover, the N-terminal peptide fragment of STC did not have PTH-like activities in mammalian systems. Thus, the expression of PTH-like activities of STC might be dependent on the amino acid sequence at the C-terminal end. One explanation for the various bioactivities of STC in mammals as well as in teleosts, is that there are several active sites in the entire STC molecule. There is evidence that STC is modified prior to or just after entering the blood circulation. Several modified immunoreactive forms of the STC have been identified in trout (Wagner *et al.*, 1991) and salmon (Wagner *et al.*, 1992) plasma. Interestingly, eel, salmon and human STC molecule (Butkus *et al.*, 1987, Wagner *et al.*, 1992, Chang *et al.*, 1995 and this study) possess a dibasic amino acid pair, Arg-Arg, which could be a key site for endopeptidase activity. Additional di- and tri-basic sequence domains exit in STCs, that could account for the different truncated forms of STCs. Studies of the other peptide fragments of STC will clarify this issue.

For a long time, it had been widely assumed that STC is a specific hormone in bony fishes. However, Chang *et al.* (1995) was successful in cloning a human cDNA coding for a protein highly homologous to STC. Northern blot analysis was carried out on a variety of human tissues and the mRNA is expressed in the ovary, prostate, thyroid and many of other tissues. The results would be more consistent with a paracrine than an endocrine role and suggest that STC has important physiological

functions in several organs including bone in human. It would be expected that this study is available for elucidation of functions of STC in human and that a peptide fragment from STC applys to developing agents which may be useful in the disease of bone loss and so on.

5. SUMMARY

Stanniocalcin (STC) from chum salmon (*Oncorhynchus keta*) was isolated by extracting the corpuscles of Stannius (CS) with ethanol-ammonium, followed by ion-exchange chromatography, gel filtration and reversed phase high performance liquid chromatography. STC migrated as a 46-kDa product under nonreducing conditions and as a 23-kDa product under reducing conditions on sodium dodecylsulphate-polyacrylamide gel electrophoresis. The protein is likely to be a homodimer composed of two subunits of 23 kDa each. Purified STC decreased the intestinal calcium uptake in a dose related manner in the Atlantic cod (*Gadus morhua*). A cDNA was cloned from cDNAs of the CS by means of PCR using two primers corresponding to the N- and C-terminal amino acid sequence of STC. Sequence analysis of the protein and the cDNA revealed that chum salmon STC is a homodimer, and that the monomer consists of 179 amino acids including 11 half-Cys residues and one N-linked glycosylation site, which is 44, 52 and 35 residues smaller at the C-terminal region than the sequences deduced from coho salmon, Australian eel and human STC cDNA, respectively.

A synthetic peptide (peptide N) corresponding to the N-terminal amino acid residues which had a high amino acid sequence identity with coho salmon and Australian eel STC, was prepared from chum salmon. Its effects were compared with those of intact STC on mammalian bone metabolism *in vitro*. Peptide N (10^{-10}-10^{-11} M) slightly decreased the rate of loss of radioactivity from fetal rat calvariae labeled with ^{45}Ca, both with and without stimulation by N-terminal peptide fragment of human parathyroid hormone (hPTH1-34). Intact STC had no effect on the release of ^{45}Ca from the calvariae. Peptide N (10^{-10}-10^{-12} M) also inhibited increases in the

number of tartrate-resistant acid phosphatase-positive multinucleated cells promoted by hPTH1-34 in cultures of murine hemopoietic cells, although intact STC had no effect on the number of the cells. The accumulation of cyclic AMP induced by hPTH1-34 in ROS 17/2.8-5 cells was suppressed by peptide N (10^{-10}-10^{-12} M). Peptide N (10^{-11}-10^{-13} M) increased the rate of incorporation of [^{3}H]proline into the collagenase-digestible protein of calvariae in newborn mice. These results indicated that the highly conserved amino-terminal region of STC from teleosts has diverse effects on the metabolism of mammalian bone, causing a biphasic response. Such effects have not been observed with materials from the CS and intact STC and they also differ from the effects of hPTH1-34.

6. REFERENCES

Baron, R. and Vignery, A. (1981) Behavior of osteoclasts during a rapid change in their number induced by high doses of parathyroid hormone or calcitonin in intact rats. Metab. Bone Dis. Relat. Res. 2: 339-346.

Butkus, A., Roche, P.J., Fernley, R.T., Haralambidis, J., Penschow, J.D., Ryan, G.B., Trahair, J.E., Tregear, G.W. and Coghlan, J.P. (1987) Purification and cloning of a corpuscles of Stannius protein from *Anguilla australis*. Mol. Cell. Endocrinol. 54: 123-133.

Butkus, A., Yates, N.A., Copp, D.H., Milliken, C., McDougall, J.G., Roche, P.J., Tregear, G.W. and Coghlan, J.P. (1989) Processing and bioactivity of the corpuscles of Stannius protein of the Australian eel. Fish Physiol. Biochem. 7: 359-365.

Canalis, E., Centrella, M., Burch, W. and McCarthy, T.L. (1989) Insulin-like growth factor I mediates selective anabolic effects of parathyroid hormone in bone cultures. J. Clin. Invest. 83: 60-65.

Chambers, T.J. and Moore, A. (1983) The sensitivity of isolated osteoclasts to morphological transformation by calcitonin. J. Clin. Endocrinol. Metab. 57: 819-824.

Chambers, T.J., Chambers, J.C., Symonds, J. and Darby, J.A. (1986) The effect of human calcitonin on the cytoplasmic spreading of rat osteoclasts. J. Clin. Endocrinol. Metab. 63: 1080-1085.

Chambers, T.J., McSheehy, P.M.J., Thomson, B.M. and Fuller, K. (1985) The effect of calcium-regulating hormones and prostaglandins on bone resorption by osteoclasts disaggregated from neonatal rabbit bones. Endocrinology 116: 234-

239.

Chang, A.C.M, Janosi, J., Hulsbeek, M., De Jong, D., Jeffrey, K.J., Noble, J.R. and Reddel, R.R. (1995) A novel human cDNA highly homologous to the fish hormone stanniocalcin. Mol. Cell. Endocrinol. 112: 241-247.

Dietrich, J.W., Canalis, E.M., Maina, D.M. and Raisz, L.G. (1976) Hormonal control of bone collagen synthesis *in vitro*: effects of parathyroid hormone and calcitonin. Endocrinology 98: 943-949.

Farley, J.R., Tarbaux, N.M., Hall, S.L., Linkhart, T.A. and Baylink, D.J. (1988) The anti-bone-resorptive agent calcitonin also acts *in vitro* to directly increase bone formation and bone cell proliferation. Endocrinology 123: 159-167.

Feinblatt, J.D. (1986) The comparative physiology of calcium regulation in submammalian vertebrates. In: Löwenstein, O. (ed.) Advances in comparative physiology and biochemistry, Vol. 8, Academic Press, New York, pp. 74-97.

Feldman, R.S., Krieger, N.S. and Tashjian A.H.Jr. (1980) Effects of parathyroid hormone and calcitonin on osteoclast formation *in vitro*. Endocrinology 107: 1137-1143.

Fenwick, J. C. (1989) Calcium exchange across fish gills. In: Pang PKT, Schreibman M.P. (eds.) Vertebrate endocrinology: fundamentals and biomedical implications, vol. 3. Academic Press, San Diego, pp. 319-341.

Fenwick, J.C. and Verbost, P. (1993) A C-terminal fragment of the hormone stanniocalcin is bioactive in eels. Gen. Comp. Endocrinol. 91: 337-343.

Filk, G., Labedz, T., Lafeber, F.P.J.G., Wendelaar Bonga, S.E.and Pang, P.K.T. (1989) Studies on teleost corpuscles of Stannius: physiological and biochemical aspects of synthesis and release of hypocalcin in trout, goldfish and eel. Fish Physiol. Biochem. 7: 343-349.

Filk, G., Labedz, T., Neelissen, J.A.M., Hanssen, R.G.J.M., Wendelaar Bonga, S.E. and Pang P.K.T. (1990) Rainbow trout corpuscles of Stannius: stanniocalcin

synthesis *in vitro*. Am. J. Physiol. 258: R1157-R1164.

Friedman, M., Krull, L.H. and Cavins, J.F. (1970) The chromatographic determination of cysteine and cysteine residues in proteins as s-beta-(4-pyridyl-ethyl) cysteine. J. Biol. Chem. 245: 3868-3871.

Gellersen, B., Wagner, G.F., Copp, D.H. and Friesen, H.G. (1988) Development of a primary culture system for rainbow trout corpuscles of Stannius and characterization of secreted teleocalcin. Endocrinology 123: 913-921.

Hattereley, G. and Chambers, T.J. (1989) Generation of osteoclasts from hemopoietic cells and a multipotential cell line in vitro. J. Cell. Physiol. 140: 478-482.

Hirano, T. (1989) The corpuscles of Stannius. In; Pang, P.K.T. and Schreibman, M.P. (eds.) Vertebrate endocrinology: fundamentals and biomedical implications, vol. 3. Academic Press, San Diego, pp. 139-169.

Hirs, C.H.W. (1967) Reduction and s-carboxymethylation of proteins. Methods Enzymol. 11: 199-203.

Holtrop, M.E., Raisz, L.G. and Simmons, H.A. (1974) The effects of parathyroid hormone, colchicine, and calcitonin on the ultrastructure and the activity of osteoclasts in organ culture. J. Cell Biol. 60: 346-355.

Hyashi, R., Moore, S. and Stein, W.H. (1973) Carboxypeptidase from yeast. Large scale preparation and the application to COOH-terminal analysis of peptides and proteins. J. Biol. Chem. 248: 2296-2302.

Katz, J. M., Wilson, T., Skinner, S.J.M. and Gray, D.H. (1981) Bone resorption and prostaglandin production by mouse calvaria *in vitro*: response to exogenous prostaglandins and their precursor fatty acids. Prostaglandins 22: 537-551.

Krishnamurthy, V.G. and Bern, H.A. (1969) Correlative histologic study of the corpuscles of Stannius and the juxtaglomerular cells of teleost fishes. Gen. Comp. Endocrinol. 13: 313-335.

Lafeber, F.P.J.G., Hanssen, R.G.J.M., Choy, Y.M., Flik, G., Herrmann-Erlee, M.

P.M., Pang, P.K.T. and Wendelaar Bonga, S.E. (1988) Identification of hypocalcin (teleocalcin) isolated from trout Stannius corpuscles. Gen. Comp. Endocrinol. 69: 19-30.

Lafeber, F.P.J.G., Herrmann-Erlee, M.P.M., Frisk, G. and Wendelaar Bonga, S.E. (1989) Rainbow trout hypocalcin stimulates bone resorption in embryonic mouse calvaria in a PTH-like fashion. J. Exp. Biol. 143: 165-175.

Lafeber, F.P.J.G., Schaefer, H.I.M.B., Herrmann-Erlee, M.P.M. and Wendelaar Bonga, S.E. (1986) Parathyroid hormone-like effects of rainbow trout Stannius products on bone resorption of embryonic mouse calvaria *in vitro*. Endocrinology 119: 2249-2255.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227: 680-684.

Lerner, U.H., Ransjö, M., Sahlberg, K., Ljunggren, Ö. and Fredholm, B.B. (1989) Forskolin sensitizes parathyroid hormone-induced cyclic AMP response, but not the bone resorptive effect, in mouse calvarial bone. Bone and Mineral 5: 169-181.

Linkhart, T.A. and Mohan, S. (1989) Parathyroid hormone stimulates release of insulin-like growth factor-I (IGF-I) and IGF-II from neonatal mouse calvaria in organ culture. Endocrinology 125: 1484-1491.

Lopez, E, Tisserrand-Jochem, E.M., Eyquem, A., Milet, C., Hillyard, C., Lallier, F., Vidal, B. and MacIntyre, I. (1984) Immunocytochemical detection in eel corpuscles of Stannius of mammalian parathyroid-like hormone. Gen. Comp. Endocrinol. 53: 28-36.

Lopez, E. (1969) Histophysiological study of the corpuscles of Stannius in *salmo salar* L. during various stages of its life cycle. Gen. Comp. Endocrinol. 12: 339-349.

Lu, M. and Wagner, G.F. (1994) Stanniocalcin stimulates phosphate reabsorption by flounder renal proximal tubule in primary culture. Am. J. Physiol. 267: R1356-R1362.

Ma, S.W.Y. and Copp, D.H. (1982) Role of the corpuscles of Stannius and teleocalcin in calcium regulation in the teleost. In: Oguro, C. and Pang, P.K.T. (eds.) Comparative endocrinology of calcium regulation, Japan Scientific Societies Press, Tokyo, pp. 173-179.

Merrifield, R. G. (1985) Solid phase synthesis. Angew. Chem. Int. Ed. Engl. 24: 799-892.

Milet, C., Hillyard, C.J., Martelly, E., Chartier, M.M., Girgis, G., MacIntyre, I. and Lopez, E. (1982) A parathyroid-like hormone from eel corpuscles of Stannius which exhibits hypocalcemic action. In: Oguro, C. and Pang, P.K.T. (eds.) Comparative endocrinology of calcium regulation, Japan Scientific Societies, Tokyo, pp. 181-186.

Milet, C., Lopez, E., Chartier, M.M., Martelly, E., Lallier, F. and Vadil, B. (1979) A new calcium regulating hormone from the corpuscles of Stannius. In: MacIntyre, I. and Szelke, M. (eds.) Molecular endocrinology, Elsevier Science Publishers, Amsterdam, pp. 341-348.

Milet, C., Peignoux-Deville, J. and Martelly, E. (1979) Gill calcium fluxes in the eel, *Anguilla anguilla* (L.). Effects of Stannius corpuscles and ultimobranchial body. Comp. Biochem. Physiol. 63A: 63-70.

Milliken, C. E., Fargher, R.C., Butkus, A., McDnald, M. and Copp, D.H. (1990) Effects of synthetic peptide fragments of teleocalcin (hypocalcin) on calcium uptake in juvenile rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 77: 416-422.

Mundy, G.R. and Roodman, G.D. (1987) Osteoclast ontogeny and function. In: Peck, W. A. (ed.) Bone and mineral research. Elsevier Science Publishers, Amsterdam, Vol. 5, pp. 209-279.

Muramoto, K., Sunahara, S. and Kamiya, H. (1987) Measurement of tryptophan in peptides by acid hydrolysis in the presence of phenol and its application to the

amino acid sequence of a sea anemone toxin. Agric. Biol. Chem. 51: 1607-1616.

Nakatani; Y., Tsunoi, M., Kurihara, N, Fujita, K. and Kumegawa, M. (1984) Effects of parathyroid hormone on cAMP production and alkaline phosphatase activity in osteoblastic clone MC3T3-E1 cells. Biochem. Biophys. Res. Comn. 123: 894-898.

Nijweide, P.J., Burger, E.H. and Feyen, J.H.M. (1986) Cells of bone: proliferation, differentiation, and hormonal regulation. Physiol. Rev. 66: 855-886.

Olivereau, M. and Olivereau, J. (1978) Prolactin, hypercalcemia and corpuscles of Stannius in seawater eels. Cell Tiss. Res. 186: 81-96.

Peterkofsky, B. (1972) The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. Arch. Biochem. Biophys. 152: 318-328.

Peterkofsky, B. and Diegelmann, R. (1971) Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry 10: 988-994.

Raisz, L.G. (1965) Bone resorption in tissue culture. Factors influencing the response to parathyroid hormone. J. Clin. Invest. 44: 103-116.

Raisz, L.G. and Fall, P.M. (1990) Biphasic effects of prostaglandin E_2 on bone formation in cultured fetal rat calvariae: interaction with cortisol. Endocrinology 126: 1654-1659.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in molecular cloning, a laboratory manual, 2nd edn., Cold Spring Harbor Laboratory Press, New York.

So, Y.P. and Fenwick, J.C. (1977) Relationship between net ^{45}calcium influx across a perfused isolated gill and the development of post-stanniectomy hypercalcemia. J. Exp. Zool. 200: 259-264.

So, Y.P. and Fenwick, J.C. (1979) *In vivo* and *in vitro* effects of Stannius corpuscle extract on the branchial uptake of ^{45}Ca in stanniectomized North American eel

(*Anguilla rostrata*). Gen. Comp. Endocrinol. 37: 143-149.

Stannius, H. (1839) Ueber Nebennieren bei Knochenfischen. Müller's Arch.: 97-101.

Stern, P.H., Schankar, G., Fangher, R.C., Copp, D.H., Milliken, C. E., Sato, K.J., Goltzman, D. and Herrmann-Erlee, M.P.M. (1991) Salmon stanniocalcin and bovine parathyroid hormone have dissimilar actions on mammalian bone. J. Bone Mineral Res. 6: 1153-1159.

Sundell, K. and Björnsson, B.T. (1990) Effects of vitamin D_3, 25(OH) vitamin D_3, 24, 25 $(OH)_2$ vitamin D_3, and 1, 25 $(OH)_2$ vitamin D_3 on the in vitro intestinal calcium absorption in the marine teleost, Atrantic cod (*Gadus morhua*). Gen. Comp. Endocrinol. 78: 74-79.

Sundell, K., Björnsson, B.T., Itoh, H and Kawauchi, H. (1992) Chum salmon (*Oncorhynchus keta*) stanniocalcin inhibits *in vitro* intestinal calcium uptake in Atlantic cod (*Gadus morhua*). J. Comp. Physiol. 162B: 489-495.

Takahashi, N., Yamana, H., Yoshiki, Y., Roodman, G.D., Mundy, G.R., Jones, S.J., Boyde, A. and Suda, T. (1988) Osteoclast-like cell formation and its regulation by osteotrophic hormones in mouse bone marrow cultures. Endocrinology 122: 1373-1382.

Verbost, P.M., Butkus, A., Atsma, W., Willems, P., Flik, G. and Wendelaar Bonga, S.E. (1993) Studies on stanniocalcin: characterization of bioactive and antigenic domains of the hormone. Mol. Cell. Endocrinol. 93: 11-16.

Verbost, P.M. and Fenwick, J.C. (1995) N-terminal and C-terminal fragments of the hormone stanniocalcin show differential effects in eels. Gen. Comp. Endocrinol. 98: 185-192.

Wagner, G.F., Dimattia, G.E., Davie, J.R., Copp, D.H. and Friesen, H.G. (1992) Molecular cloning and cDNA sequence analysis of coho salmon stanniocalcin. Mol. Cell. Endocrinol. 90: 7-15.

Wagner, G.F., Fenwick, J.C., Park, C.M., Milliken, C., Copp, D.H. and Friesen,

H.G. (1988) Comparative biochemistry and physiology of teleocalcin from sockeye and coho salmon. Gen. Comp. Endocrinol. 72: 237-246.

Wagner, G.F., Guirandon, C.C., Milliken, C. and Copp, D.H. (1995) Immunological and biological evidence for stanniocalcin-like hormone in human kidney. Proc. Natl. Acad. Sci. U.S.A. 92: 1871-1875.

Wagner, G.F., Hampong, M., Park, C.M. and Copp, D.H. (1986) Purification, characterization, and bioassay of teleocalcin, a glycoprotein from salmon corpuscles of Stannius. Gen. Comp. Endocrinol. 63: 481-491.

Wagner, G.F., Milliken, C., Friesen, H.G. and Copp, D.H. (1991) Studies on the regulation and characterization of plasma stanniocalcin in rainbow trout. Mol. Cell. Endocrinol. 79: 129-138.

Wendelaar Bonga, S.E. and Greven, J.A.A. (1975) A second cell type in Stannius bodies of two euryhaline teleost species. Cell Tiss. Res. 159: 287-290.

Wendelaar Bonga, S.E., Greven, J.A.A. and Weenhuis, M. (1976) The relation between ionic composition of the environment and secretary activity of the endocrine cell types of Stannius. Cell Tiss. Res. 175: 297-312.

Wendelaar Bonga, S.E., Pang, R.K. and Pang, P.K.T. (1986) Hypocalcemic effects of bovine PTH(1-34) and Stannius corpuscle homogenates in teleost fish adapted to low calcium water. J. Exp. Zool. 240, 363-367.

$$J^{Ca} = \frac{\sum_{t=n}^{t=n+60} ({}^{45}Ca_f \cdot V)}{{}^{45}Ca_{sp}} \cdot \frac{1000}{X}$$

Fig. 1. The formula for the unidirectional intestinal Ca^{2+} influx (J^{Ca}) of the Atlantic cod. J^{Ca} was calculated as the amount of ^{45}Ca appearing in each fraction of the vascular perfusate divided by the amount of perfused ^{45}Ca (sp. act. 0.85 $\pm$ 0.06 MBq/μmol, $^{45}Ca_{sp}$) J^{Ca} was expressed for each fraction as well as for 60-min periods with the flow linearly correlated with body weight. The amount of ^{45}Ca (dpm; $^{45}Ca_f$) in 1 ml of each fraction collected was assessed using a liquid scintillation counter, and V was assessed by weighing. X is the body weight in grams.

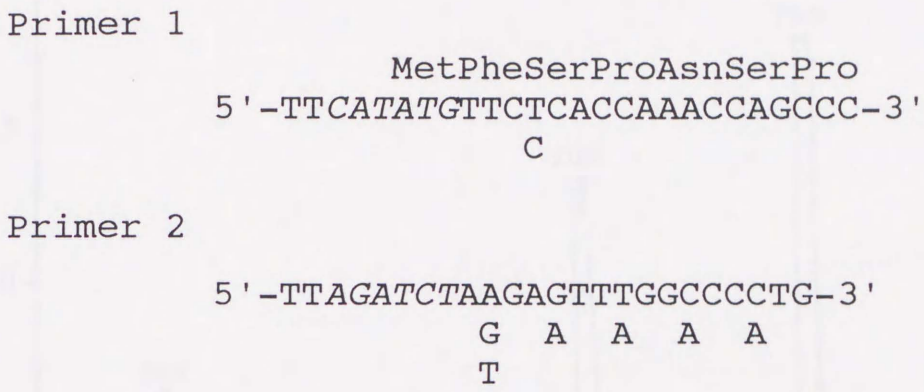

Fig. 2. Nucleotide sequence of the synthetic primers. Primer 1 corresponds to the N-terminal amino acids of STC and contains an additional ATG initiation codon as well as an *Nde*1 site at its 5'-end. Primer 2 corresponds to the C-terminal amino acids of STC and contains an additional TAG termination codon as well as a *Bgl*II site at its 5'-end. *Nde*1 and *Bgl*II sites are shwon in italic.

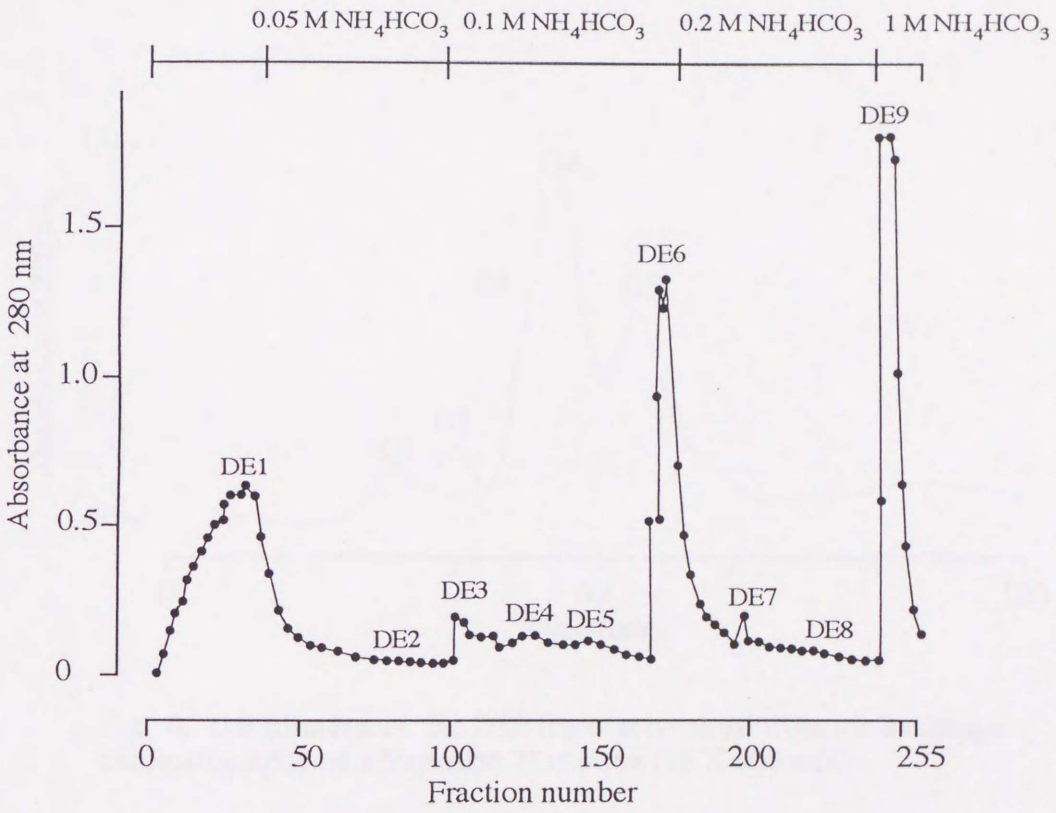

Fig. 3. Ion exchange chromatography of an ethanol extract from the CS of chum salmon on a DE52 cellulose column (10 X 300 mm).

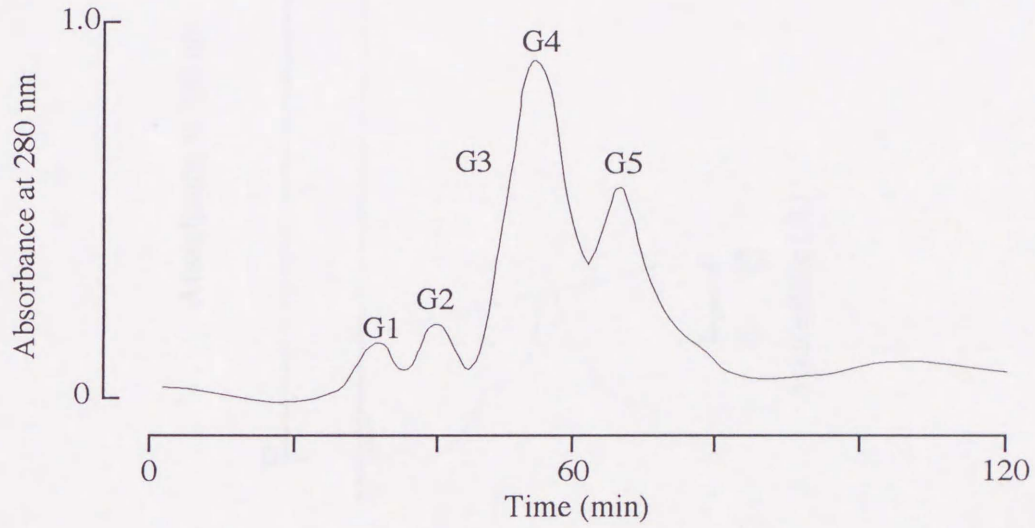

Fig. 4. Gel filtration of the DE6 fraction obtained from ion exchange chromatography on a Superdex 75 column (16 X 600 mm).

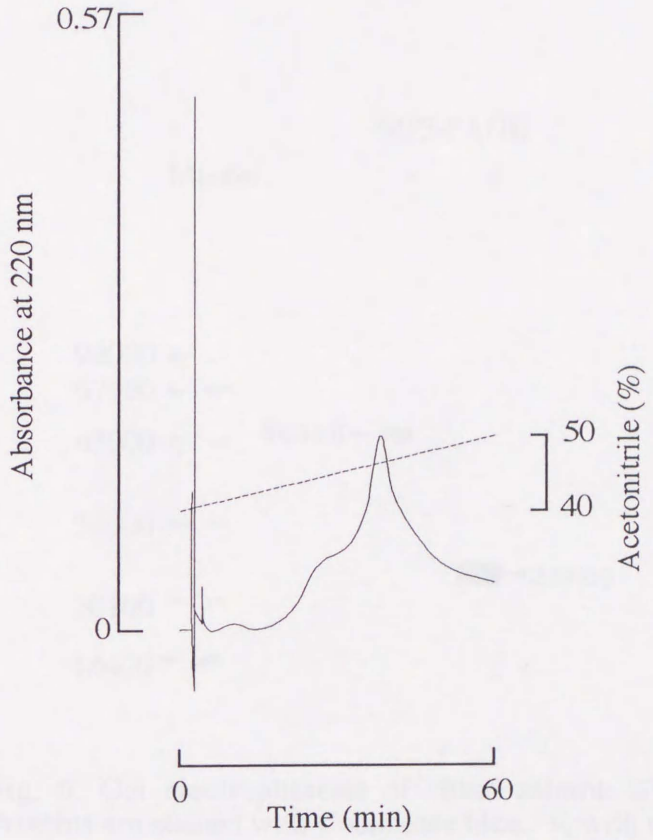

Fig. 5. rpHPLC of the G4 fraction obtained from gel filtration on a TSK-Gel ODS-120T column (0.46 X 25 cm) with a linear gradient of acetonitrile in 0.1% TFA (dotted line).

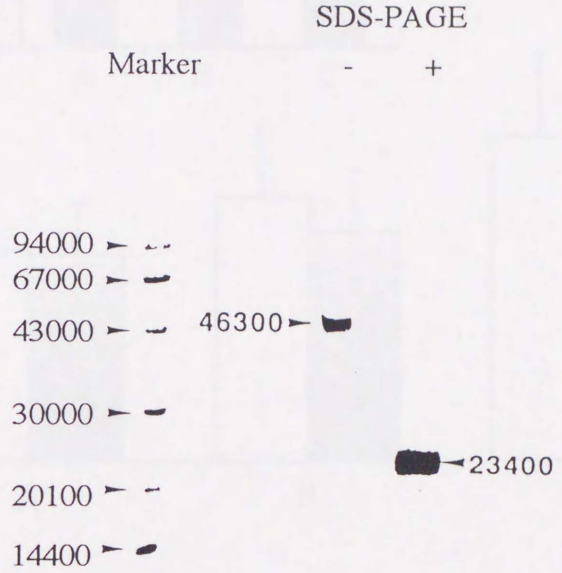

Fig. 6. Gel electrophoresis of chum salmon STC. Proteins are stained with Coomassie blue. +, with and -, without reduction by β-mercaptoethanol.

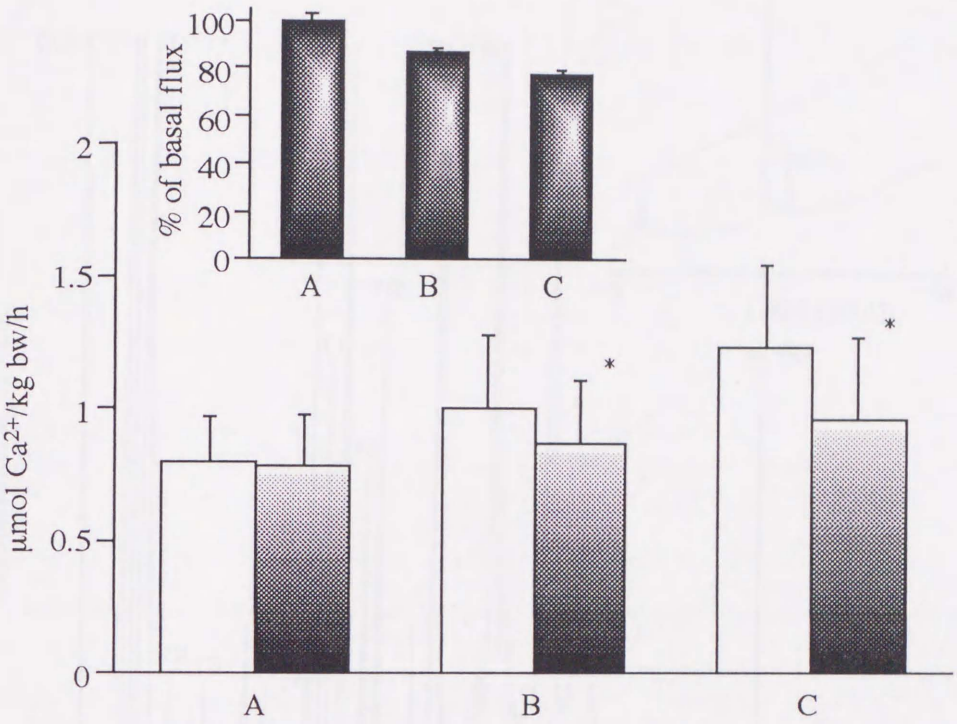

Fig. 7. Calcium uptake across the intestinal mucosa of in vitro-perfused intestinal preparations from the Atlantic cod. For each group of fish the basal influx was measured for 60 min (unfilled bars), followed by influx measurements during 60 min of treatment (filled bars). The treatment groups are: A, perfusion with cod Ringer's solution only; B, perfusion with 2 nM STC and C, perfusion with 10 nM STC. The inset figure presents the data expressed as a percentage of the mean basal flux of each procedure. Each value indicates the mean $\pm$ SE of results from 10 fishes. Significantly different from the basal Ca^{2+} influx within group: *P< 0.05.

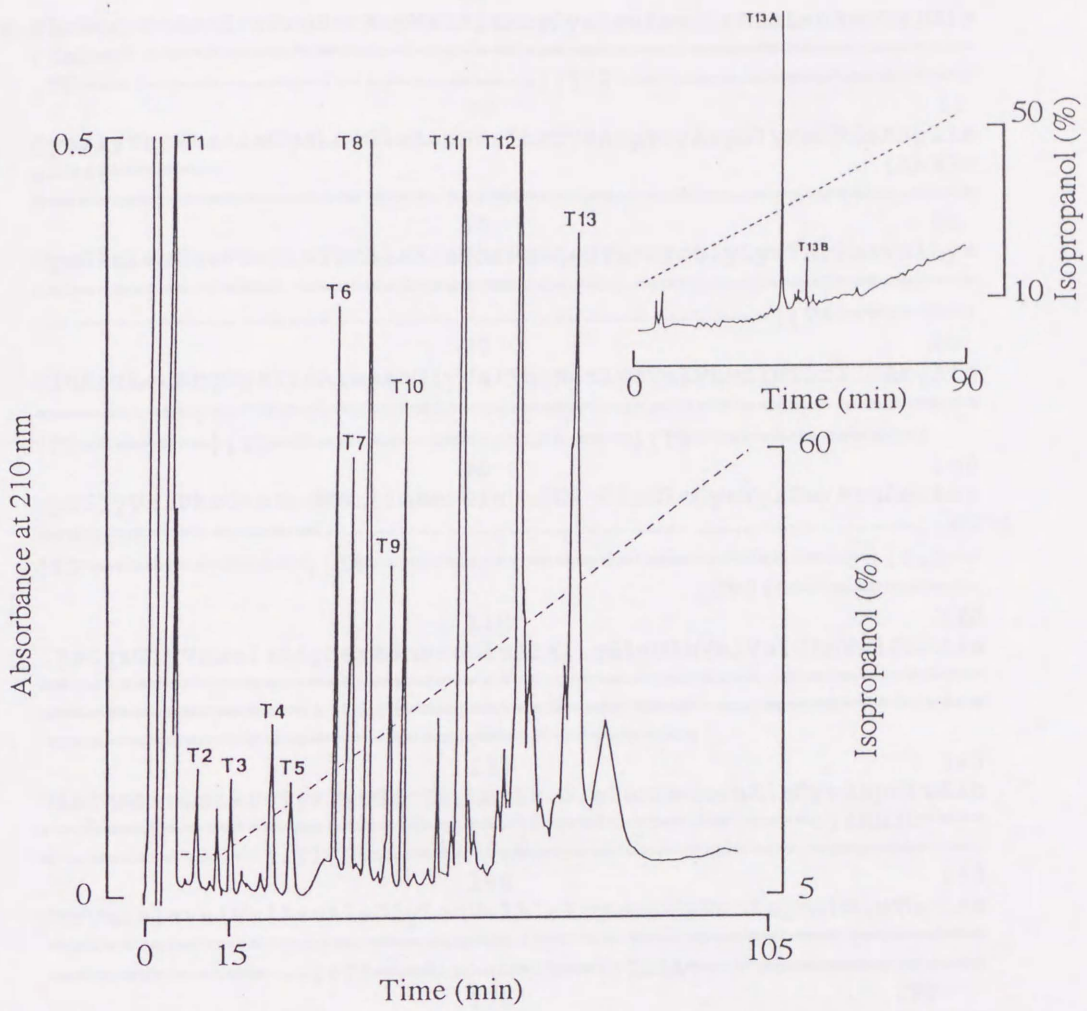

Fig. 8. rpHPLC of a trypsin digest of RPE-STC on a TSK gel ODS-120T column (0.46 X 25 cm) with a linear gradient of isopropanol in 0.1% TFA (dotted line). Inset: rechromatography of T13 on a TSK gel ODS-120T column with a linear gradient of isopropanol in 0.01 M ammonium acetate (pH 6.0).

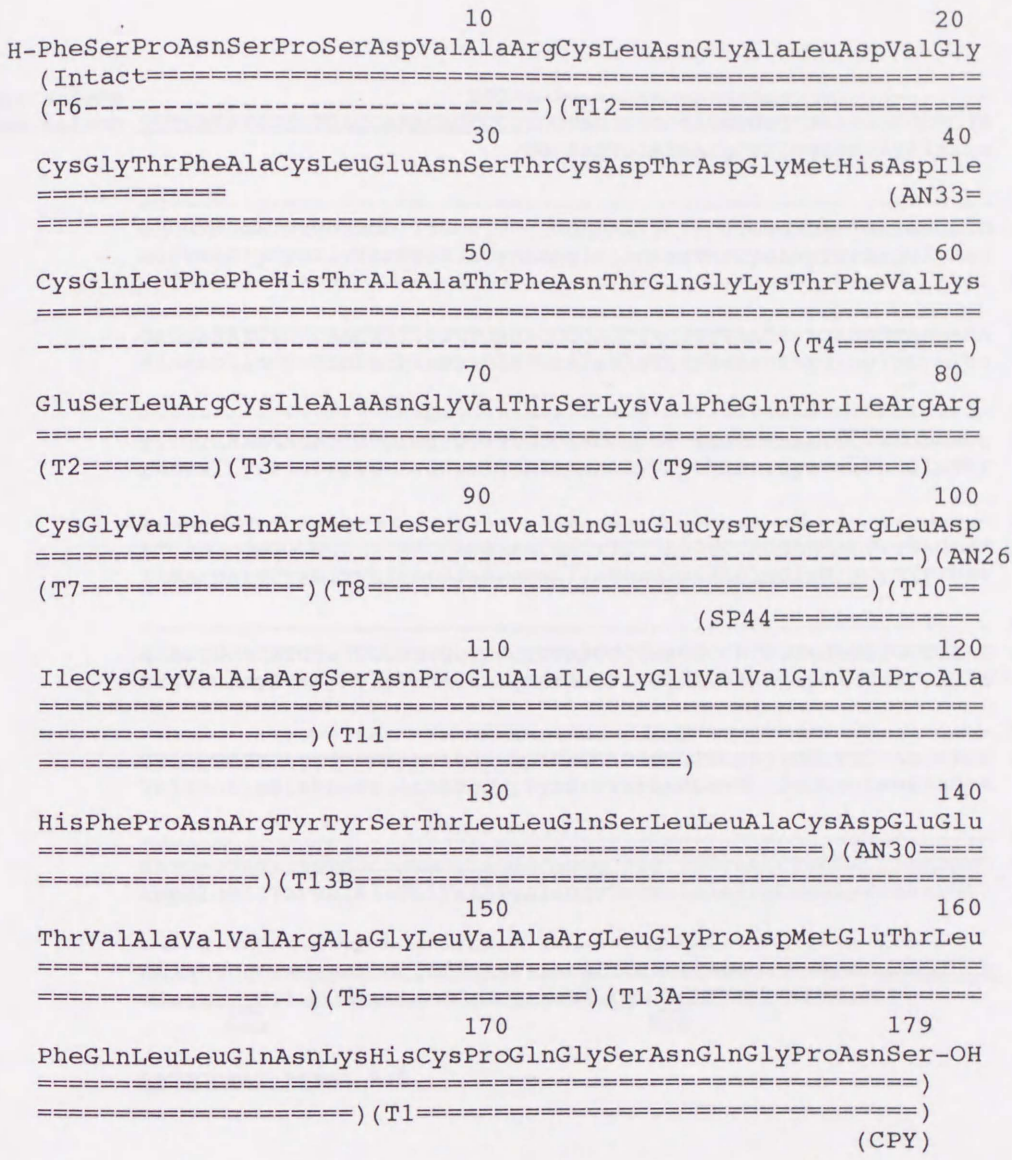

Fig. 9. Proposed amino acid sequence of chum salmon STC monomer. The double broken line represents residues that were determined by amino acid sequence analysis. T, AN, SP and CPY indicate fragment peptides prepared by digestion with trypsin, endoproteinase ASp-N, *Staphylococcus aureus* V8 proteinase and carboxypeptidase Y, respectively. Asn29 is N-glycosylated.

```
Coho salmon                          203---------------------------------- 235
Chum salmon TTTCATATGTTCTCACCAAACAGCCCCTCGGATGTGGCTAGGTGTTTGAATGGCGCTCTA  60
                                  7SerAspValAlaArgCysLeuAsnGlyAlaLeu

            -C------------------------------------------------------------ 295
            GACGTGGGATGTGGTACGTTTGCCTGCCTGGAGAATTCTACCTGTGACACTGATGGCATG 120
            AspValGlyCysGlyThrPheAlaCysLeuGluAsnSerThrCysAspThrAspGlyMet
            --T------------------------------------------------------------ 355

            CACGATATCTGTCAACTGTTCTTTCACACCGCAGCTACCTTTAACACACAGGGTAAGACA 180
            HisAspIleCysGlnLeuPhePheHisThrAlaAlaThrPheAsnThrGlnGlyLysThr

            ---------------------G--------------T------------------------ 415
            TTTGTAAAGGAGAGTCTGAGATGTATTGCCAACGGCGTCACGTCTAAAGTCTTTCAGACC 240
            PheValLysGluSerLeuArgCysIleAlaAsnGlyValThrSerLysValPheGlnThr

            ---------------A---------------------------------------------- 475
            ATCAGGCGCTGTGGCGTCTTCCAGAGAATGATTTCTGAGGTCCAGGAGGAGTGTTACAGT 300
            IleArgArgCysGlyValPheGlnArgMetIleSerGluValGlnGluGluCysTyrSer

            ------------------------------------------------------------- 535
            AGACTGGACATCTGTGGTGTGGCTCGCTCTAACCCTGAGGCCATTGGAGAGGTGGTGCAG 360
            ArgLeuAspIleCysGlyValAlaArgSerAsnProGluAlaIleGlyGluValValGln

            ------------------------------------------------------------- 595
            GTCCCTGCACACTTCCCCAACAGGTACTACAGCACTCTGCTCCAGTCCCTGCTAGCCTGT 420
            ValProAlaHisPheProAsnArgTyrTyrSerThrLeuLeuGlnSerLeuLeuAlaCys

            ----------------------------------------------------G--------- 655
            GATGAGGAGACAGTGGCTGTGGTCAGGGCAGGGCTTGTTGCTAGGCTGGGACCAGACATG 480
            AspGluGluThrValAlaValValArgAlaGlyLeuValAlaArgLeuGlyProAspMet

            --------T------T---------------------C ---------------536
            GAAACTCCCAACCAGCTGCTGCAGAACAAACACTGCACCCAGGGTTCTAACCAGGGTCCA  540
            GluThrProPheGlnLeuLeuGlnAspLysHisCysSerGlnGlySerAsn174
                  Leu                             Pro

            AATTCCTAGATCTAA 535
```

Fig. 10. Nucleotide and deduced amino acid sequences of the chum salmon STC cDNA clone pKDST1 and comparison with coho salmon STC cDNA. Underlines show the positions of synthetic oligonucleotide primers for PCR. Double underlines show the positions of substitution compared with the protein. The nucleotide sequence of coho salmon was taken from Wagner *et al.* (1992). (-) represents bases identical with those of chum salmon STC.

```
                                10        20        30        40
1) Chum salmon                  FSPNSPSDVARCLNGALDVGCGTFACLENSTCDTDGMHDI
2) Coho salmon                  --S--------------A----------------------
3) Australian eel               --AS-------------Q---SA----D----N-----E-
4) Human                        VAAQNSAE-V----S--Q----A--------------Y--

          50        60        70        80        90        100
1)  CQLFFHTAATFNTQGKTFVKESLRCIANGVTSKVFQTIRRCGVFQRMISEVQEECYSRLD
2)  ------------------------------------------------------------
3)  -RS-L-G--K-D-----------K-----I-----L-----SS--K-----------K--
4)  -KS-LYS--K-D----A------K-----------LA----ST-----A--------K-N

          110       120       130       140       150       160
1)  ICGVARSNPEAIGEVVQVPAHFPNRYYSTLLQSLLACDEETVAVVRAGLVARLGPDMETL
2)  ------------------------------------------------------------
3)  L-S--Q-----M---A---SQ--------------T---D--EQ------S--E-E-GV-
4)  V-GI-KR-----T----L-NH-S----NR-VR---E---D--STI-DS-MEKIG-N-AS-

          170       180       190       200       210       220
1)  FQLLQNKHCPQGSNQGPNS
2)  ------------------APAGWRWPMGSPPSFKIQPSMRGRDPTHLFARKRSVEALER
3)  -----T-A--PSAAG-TGPVGAGGSWRCPWGPPCSRSSPTCAPGTPPTSLLRNARPAPNY
4)  -HI--TD--AQTHPRADFNRRRTNEPQKLKVLLRNLRGEEDSPSHIKRTSHESA

          230
2)  VME
3)  HPPRLALMDCP
```

Fig. 11. Comparison of the amino acid sequences of STCs. The amino acid sequences are taken from the Australian eel (Butkus *et al.*,1987), coho salmon (Wagner *et al.*, 1992) and human (Chang *et al.*,1995). (-) represents residues identical with those of chum salmon STC. The synthetic peptide based on STC from chum salmon is underlined.

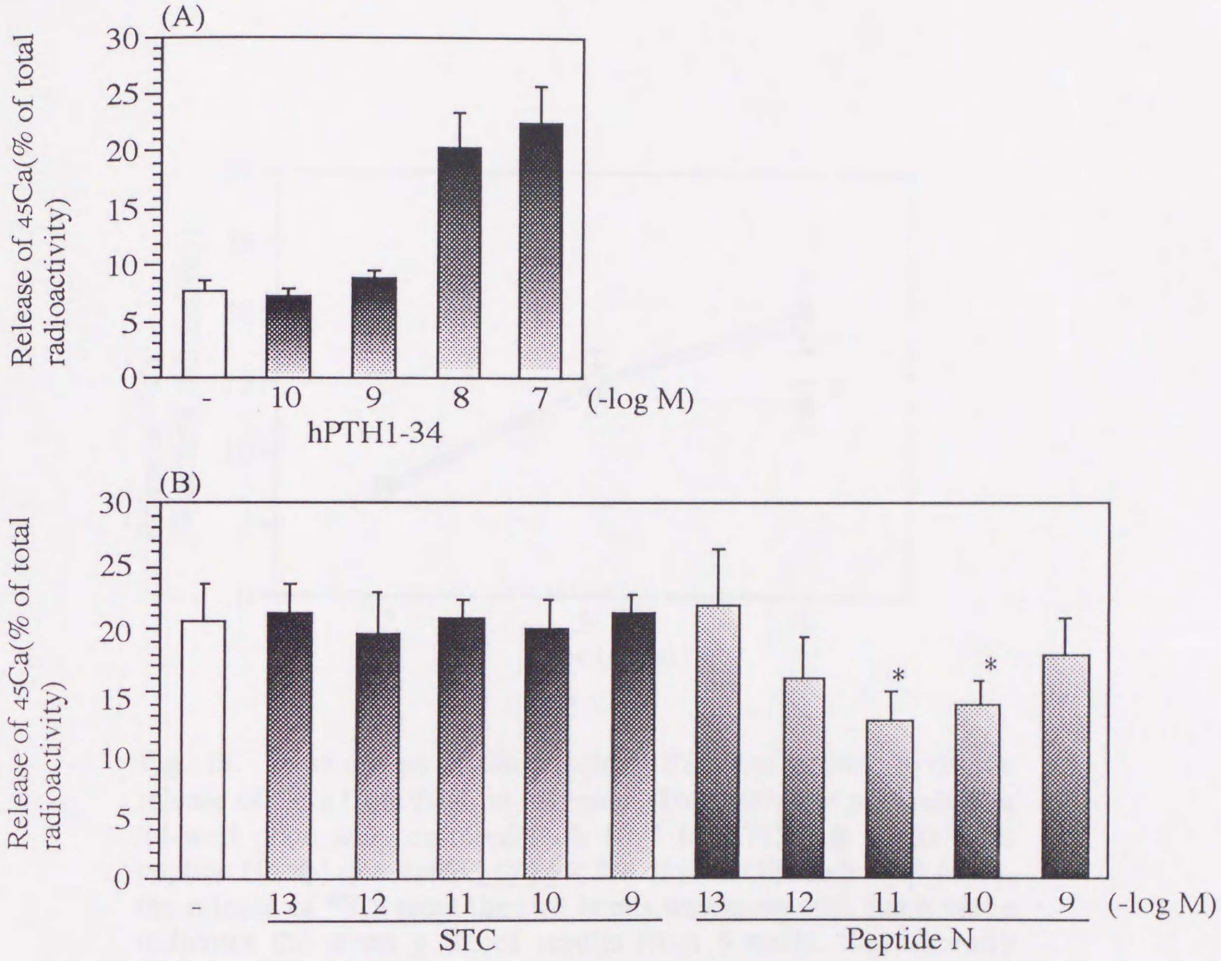

Fig. 12. Effects of hPTH1-34, STC and peptide N on the release of ^{45}Ca from fetal rat calvariae. Two calvariae per well of a 24-well plate were cultured with various concentrations of hPTH1-34 (A) for 2 days and STC or peptide N (B) for 4 days. Each value indicates the mean $\pm$ SE of results from 5 wells. Statistical analysis was performed in (B). Significantly different from control (-). *$P<0.05$.

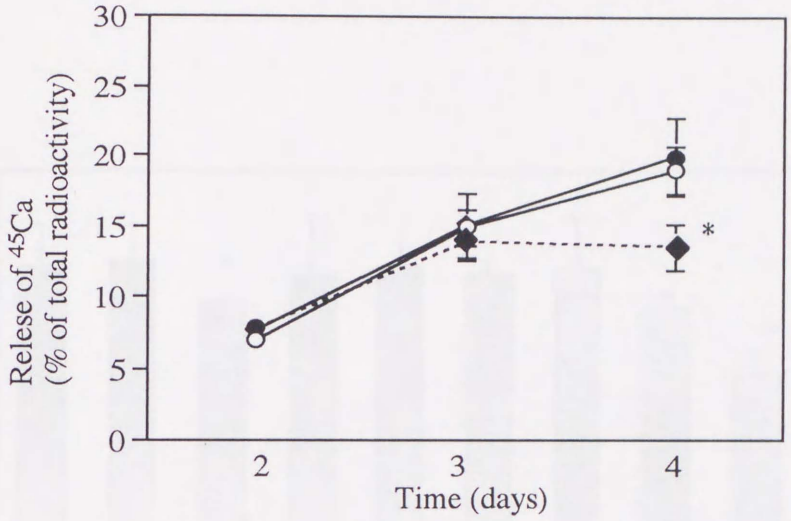

Fig. 13. Time course of the effects of STC and peptide N on the release of ^{45}Ca from fetal rat calvariae. Two calvariae per well of a 24-well plate were cultured with 10^{-11} M STC (●), 10^{-11} M peptide N (◆) or vehicle (○) for 2-4 days. At the indicated times, the release of ^{45}Ca from the two bones was measured. Each value indicates the mean ± SE of results from 5 wells. Significantly different from control (vehicle) of the same time. *$P<0.05$.

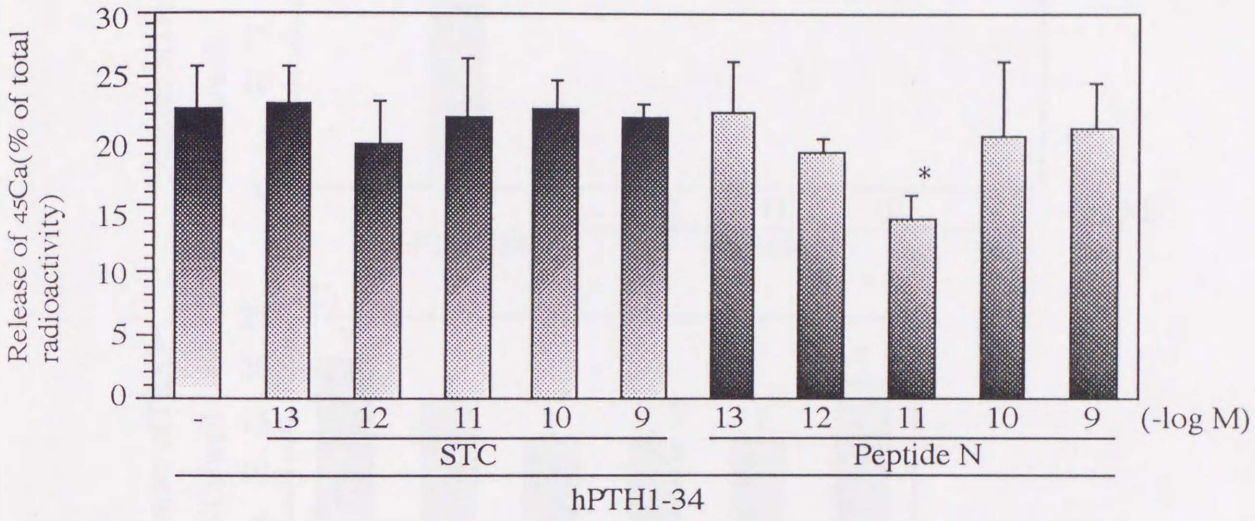

Fig. 14. Effects of STC and peptide N in the presence of hPTH1-34 on the release of ^{45}Ca from fetal rat calvariae. Two calvariae per well of a 24-well plate were cultured with various concentrations of STC or Peptide N with 10^{-8} M hPTH1-34 for 2 days. Each value indicates the mean $\pm$ SE of results from 5 wells. Significantly different from hPTH1-34 only. *$P<0.05$.

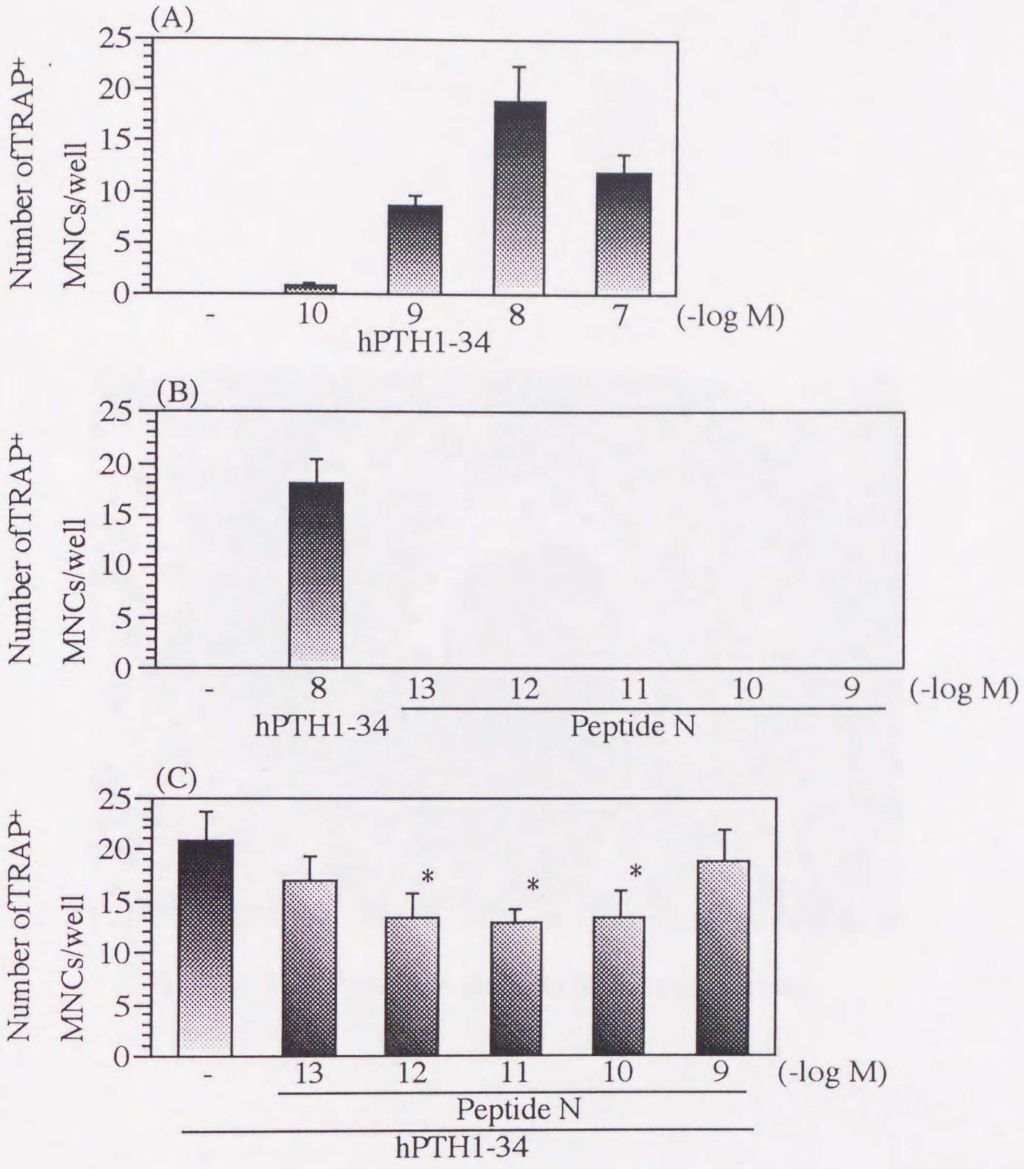

Fig. 15. Effects of hPTH1-34 and/or peptide N on the formation of TRAP positive multinucleated cells (TRAP$^+$MNCs) from mouse bone marrow cells. Bone marrow cells (7 X 10^5 per well of a 24-well plate) were cultured with various concentrations of hPTH1-34 (A), peptide N (B) or peptide N plus hPTH1-34 at 10^{-8} M (C). After culture for 8 days, TRAP$^+$MNCs were counted. Each value indicates the mean $\pm$ SE of results from 5 wells. Statistical analysis was performed in (B) and (C). Significantly different from hPTH1-34 only. *$P<0.05$.

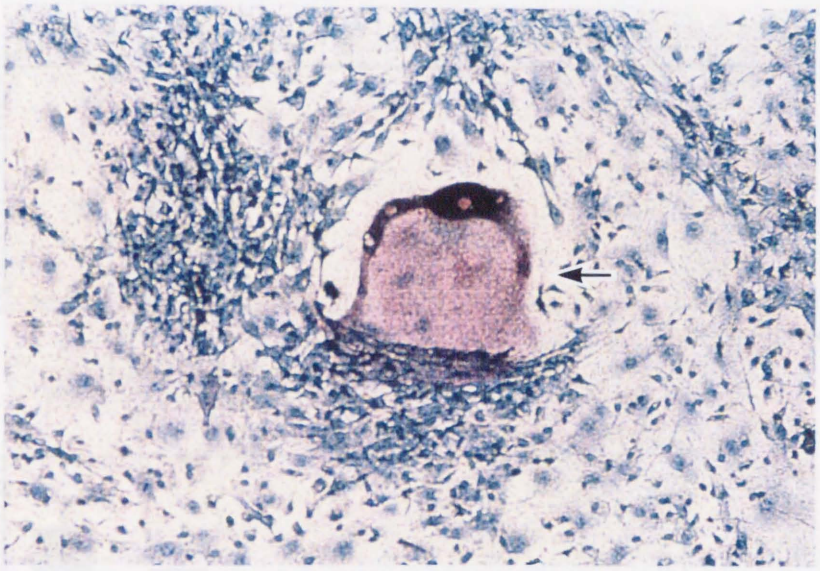

Fig. 16. TRAP positive multinucleated cell (arrow).

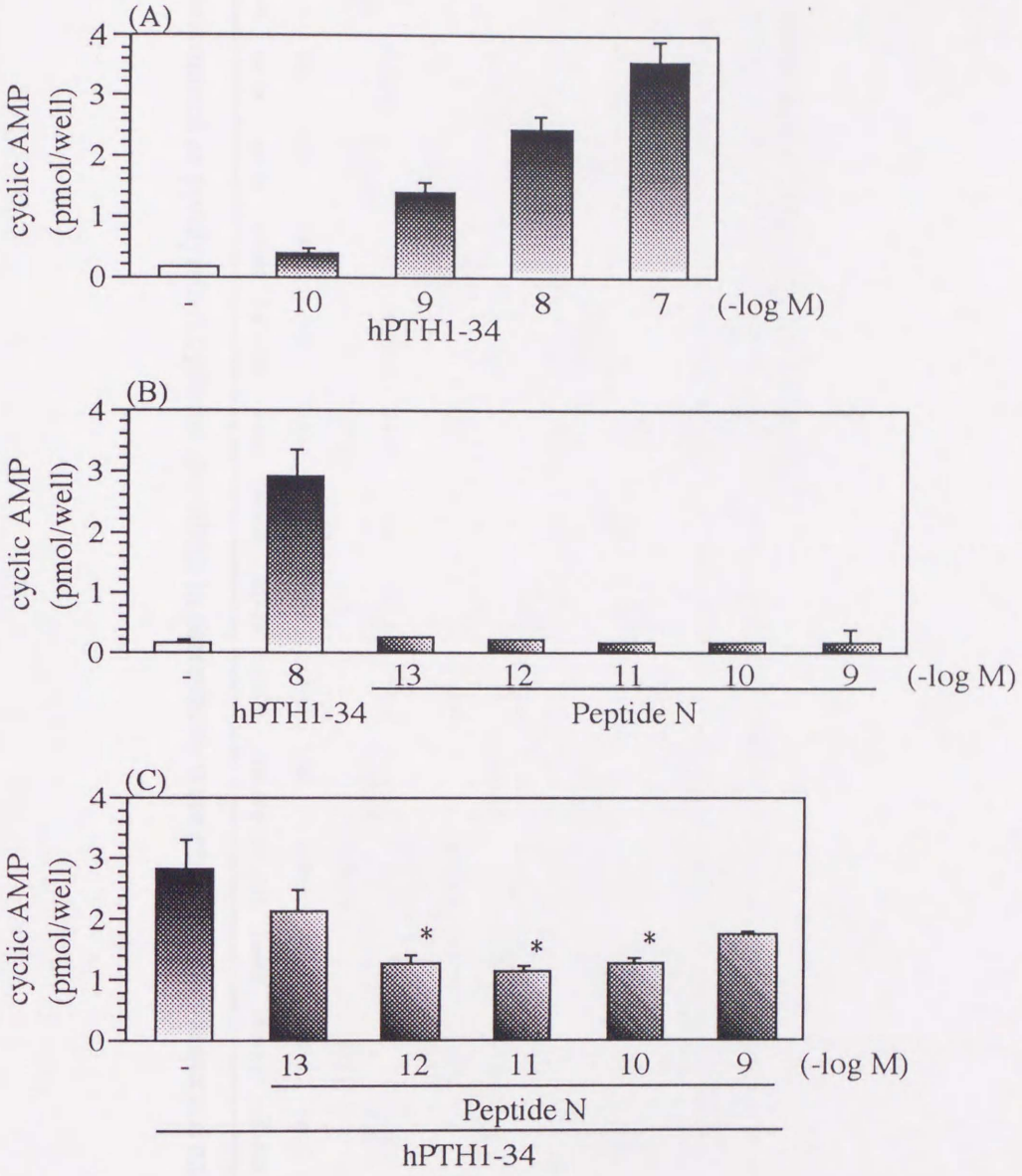

Fig. 17. Effects of hPTH1-34 and/or peptide N on the formation of cyclic AMP in ROS17/2.8-5 cells. ROS17/2.8-5 cells in each well of a 48-well plate were stimulated for 5 min with various concentrations of hPTH1-34 (A), peptide N (B) or peptide N plus hPTH1-34 at 10^{-8} M (C). Levels of cyclic AMP were measured by an enzyme immunoassay. Each value indicates the mean $\pm$ SE of results from 4 wells. Statistical analysis was performed in (B) and (C). Significantly different from hPTH1-34 only. *$P<0.05$.

Table 1.
Amino acid composition of fragments and intact STC.

AA	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13A	T13B	AN26	AN30	AN33	SP44	PE-intact
Asp	2.0(2)		1.2(1)			1.9(2)				1.2(2)	2.1(2)	7.2(7)	2.1(2)	1.5(1)	3.1(3)	4.8(5)	4.0(3)	1.6(2)	18.5(18)
Thr			1.0(1)	1.0(1)					0.9(1)			4.2(6)	1.1(1)	1.9(2)	1.3(1)	2.8(2)	4.7(6)		10.4(12)
Ser	2.2(2)	1.1(1)	1.2(1)			2.6(3)		1.8(2)			1.0(1)	1.9(1)		1.8(2)	2.1(3)	1.9(2)	3.9(4)	1.6(2)	12.7(13)
Glu	1.6(2)	1.2(1)					0.9(1)	4.4(4)	1.6(1)		3.6(3)	3.9(3)	3.8(3)	3.1(3)	4.3(4)	6.7(7)	8.3(9)	2.2(2)	22.7(21)
Gly	2.0(2)		1.4(1)		1.0(1)		0.9(1)			1.3(1)	1.0(1)	5.2(5)	1.0(1)		2.2(2)	4.2(4)	5.3(3)	1.7(2)	13.7(13)
Ala			1.1(1)		2.2(2)	1.3(1)				1.3(1)	2.1(2)	3.5(4)		2.0(2)	3.9(4)	3.6(3)	3.7(3)	1.8(2)	13.5(13)
Val			1.2(1)	1.2(1)	1.3(1)	1.2(1)	1.0(1)	1.3(1)	1.2(1)	1.3(1)	3.0(3)	1.4(1)		2.3(2)	2.8(4)	2.8(4)	4.5(5)	1.0(1)	13.5(13)
Met								0.9(1)				0.8(1)	0.8(1)			0.6(1)	(1)		2.9(3)
Ile			1.0(1)					1.0(1)	1.1(1)	1.1(1)	1.0(1)	1.2(1)			1.7(2)		3.4(4)	1.2(2)s	5.7(6)
Leu		1.2(1)			1.1(1)					1.3(1)		4.2(4)	4.3(4)	3.6(4)	3.7(5)	4.2(5)	3.6(3)	1.0(1)	14.8(15)
Tyr								0.8(1)						1.7(2)	1.2(2)		(1)	0.5(1)	2.5(3)
Phe				0.8(1)		0.8(1)	0.7(1)		0.8(1)		0.9(1)	3.8(4)	1.1(1)		1.3(1)	2.0(1)	4.9(6)	10.2(10)	
Lys			1.0(1)	1.0(1)								1.0(1)	1.0(1)			1.5(1)	2.4(3)		3.9(4)
His	0.5(1)										1.1(1)	2.0(2)			1.1(1)	1.2(1)	2.1(1)		4.0(4)
Arg		1.0(1)			1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)			1.0(1)	2.0(2)	2.5(2)	4.5(5)	1.2(2)	9.8(10)
1/2Cys	1.1(1)		0.6(1)				0.7(1)	0.7(1)		0.6(1)		4.3(5)		0.8(1)	0.9(1)	1.5(1)	3.3(4)	ND(2)	11.3(11)
Pro	1.8(2)					2.1(2)					3.3(3)		1.3(1)		2.0(3)	0.9(3)		0.8(1)	7.9(8)
Total	(12)	(4)	(9)	(4)	(6)	(11)	(6)	(12)	(6)	(8)	(19)	(45)	(15)	(21)	(38)	(42)	(61)	(20)	(179)
Position	168-179	61-64	65-73	57-60	147-152	1-11	81-86	87-98	74-79	99-106	107-125	12-56	153-167	126-146	100-137	138-179	39-99	95-114	1-179

1/2Cys was determined as pyridylethyl cysteine. Numbers in parenthesis were obtained from sequence analysis. ND, not determined.

Table 2.
Amino acid sequence of fragments and intact STC.

Cycle	T1 yield	T2 yield	T3 yield	T4 yield	T5 yield	T7 yield	T8 yield	T9 yield	T10 yield
1	H162.7	E259.3	C7.3	T99.8	A417.1	C72.3	M365.2	V292.7	L793.1
2	C267.5	S37.3	I17.2	F337.8	G154.0	G67.7	I377.7	F289.9	D421.1
3	P282.2	L92.7	A17.9	V246.4	L189.1	V96.0	S64.6	Q219.9	I478.0
4	Q182.9	R6.3	N13.6	K175.0	V120.9	F75.7	E267.6	T40.9	C330.6
5	G191.0		G5.9		A91.7	Q57.6	V269.8	I115.8	G235.6
6	S42.4		V3.8		R14.5	R9.9	Q232.7	R21.1	V356.9
7	N174.6		T0.5				E229.0		A337.8
8	Q141.3		S0.7				E231.2		R84.8
9	G105.7		K0.5				C91.6		
10	P123.3						Y144.9		
11	N96.8						S19.1		
12	14.0						R24.4		
13									
14									
15									
16									
17									
18									
19									
20									
21									
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47									
48									
49									

C, Determined as PE-Cys and CM-Cys. ND, not detected. [a]Not recorded.

Cycle	T11 yield	T12 yield	T13A yield	T13B yield	AN26 yield	AN30 yield	AN33 yield	SP44 yield	Intact yield
1	S131.5	C249.2	L28.9	Y7.1	D255.5	D - [a]	D298.6	C48.24	F22.3
2	N552.1	L274.2	G16.1	Y6.5	I244.1	E327.1	I140.5	Y47.0	S5.3
3	P427.6	N93.7	P21.4	S0.9	C286.9	E327.3	C231.2	S8.4	P14.3
4	E457.1	G74.9	P18.4	T2.0	G121.5	T62.7	Q276.6	R9.5	N16.1
5	A386.4	A113.2	M17.0	L8.1	V224.3	V218.4	L357.6	L32.1	S3.6
6	I394.0	L114.7	E15.9	L6.1	A233.5	A196.2	F309.5	D17.1	P10.3
7	G245.8	D66.7	T5.4	Q3.6	R139.9	V180.1	F339.9	I18.6	S3.0
8	E314.9	V89.8	L18.1	S0.5	S34.3	V178.4	H77.5	C8.1	D7.7
9	V332.2	G44.3	F17.1	L3.4	N133.7	R118.2	T63.7	G10.0	V10.0
10	V342.0	C62.5	Q13.4	L3.5	P99.7	A189.0	A279.3	V22.7	A12.9
11	Q275.6	G21.5	L13.6	A2.0	E125.2	G101.6	A291.4	A22.0	R5.5
12	V220.7	T14.0	L15.5	C1.5	A119.9	L153.4	T41.8	R12.2	C9.2
13	P206.9	F54.7	Q9.3	D1.9	I101.9	V126.6	E136.5	S2.6	L11.3
14	A189.4	A51.9	N11.3	E1.5	G75.0	A141.7	N105.8	N12.3	N3.9
15	H103.6	C45.8	K4.8	E1.1	E89.3	R97.6	T28.8	P10.7	G5.9
16	F89.9	L44.0		T0.4	V93.6	L125.2	Q73.7	E9.7	A6.9
17	F60.9	E34.9		V1.2	V101.4	G67.4	G69.6	A9.9	L5.0
18	N58.7	NND		A0.7	Q90.1	P70.6	K104.1	I7.2	D4.1
19	R19.0	S5.3		V0.8	V62.6	D51.5	T16.9	G6.7	V5.7
20		T5.5		V0.2	P55.6	M92.1	F53.2	Q1.6	G4.8
21		C25.3			A78.8	E59.5	V53.4	C3.9	
22		D11.0			H39.2	T16.4	K66.6	G5.1	
23		T3.8			F66.4	L57.9	E28.4	T0.9	
24		D4.6			P49.0	F58.5	S5.5	F4.9	
25		G5.4			N59.8	Q56.3	L28.0		
26		M9.5			P39.8	L44.2	R19.1		
27		H4.1			Y50.9	L45.6	C16.0		
28		D8.5			Y63.1	Q37.6	I24.4		
29		I7.0			S7.1	N30.9	A29.0		
30					T5.2	K36.9	N14.5		
31					L23.5	H21.3	G14.2		
32					L30.9	C34.1	V15.6		
33					Q14.7	P20.2	T3.1		
34					S2.2	Q28.4	S2.7		
35					L10.8	G10.6	K11.2		
36					L12.4	S2.6	V8.2		
37					A2.5	N14.1	F4.6		
38					C1.4	Q7.2	Q5.2		
39						G5.8	T1.6		
40						P6.7	I3.7		
41						N4.5	R2.9		
42						S1.3	R2.3		
43							C1.8		
44							G1.9		
45							V1.7		
46							F2.7		
47							Q1.8		
48							R1.0		
49							M2.5		

Table 3.
Effects of hPTH1-34 and peptide N on the incorporation of [^{3}H]proline into collagen-digestible (CDP) and non-collagen protein (NCP) in calvariae of newborn mice.

Treatment	-log (M)	Incorporation of [^{3}H]proline (dpm/μg dry wt.)		collagen synthesized (%)[a]
		CDP	NCP	
Control		12.01±0.57	5.81±1.20	27.63±0.93
PTH	8	7.16±0.53***	7.38±0.67	17.14±2.17**
STC1-20	9	14.27±1.72	6.42±0.37	27.60±0.24
	10	13.59±0.96	5.56±0.09	31.05±2.27
	11	14.31±0.60*	5.55±0.36	32.37±1.11*
	12	17.41±1.31**	6.77±0.47	32.20±0.30**
	13	16.09±0.58**	6.44±0.48	31.44±0.76*

Three calvariae per flask were cultured with hPTH1-34 at 10^{-8} M or with various concentrations of peptide N for 4 days, then pulse-labeled with [^{3}H]proline for the last 2 h of culture. Each value indicates the mean ± SE of results from 5 flasks. [a]The percentage of collagen synthesized was calculated correcting for the relative abundance of proline in CDP vs. NCP as described in Materials and Methods. Significantly different from control: *P<0.05, **P<0.01 and ***P<0.001.