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Calcitonin-like Substance in Plasma of the Hagfish, *Eptatretus burgeri* (Cyclostomata)

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ABSTRACT—Cyclostomes have been regarded as having no ultimobranchial gland. However, cells producing immunoreactive calcitonin (CT) were recently found in cyclostome brains. In the present study, we examined using biochemical and biological methods whether there is a CT-like substance in the plasma of the hagfish, *Eptatretus burgeri*. Hagfish plasma was first subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) and then separated into twenty fractions. The presence of immunoreactive CT in each fraction was then investigated by Western blotting with salmon CT antiserum. Two fractions (36–39% CH₃CN; 39–42% CH₃CN) showed positive immunoreactivity. Hypocalcemic and hypophosphatemic activities were detected by a rat bioassay only in the former fraction (36–39% CH₃CN). The molecular weight (MW) of the CT-like substance in this fraction was 3.5 kDa, which is equal to that of genuine CT. Furthermore, when hagfish plasma was examined by enzyme-linked immunosorbent assay using anti-salmon CT, the dilution curve of the plasma paralleled the standard curve of salmon CT. The CT-like substance detected here was present at a high concentration (14 ng/ml) in the plasma. From the results of the present study, the CT-like substance present in hagfish plasma appears to be very similar to salmon CT.

INTRODUCTION

Calcitonin (CT) is a 32 amino acid peptide hormone which functions to suppress osteoclastic resorption of bones followed by decreases in serum Ca level in mammals [8]. This hormone is secreted from C-cells of the thyroid gland in mammals and from the ultimobranchial gland (UBG) in non-mammalian vertebrates [2].

Watzka [16] reported from morphological observations that there is no UBG in cyclostomes. Administration of the extract from the branchial region of hagfish to rats did not induce significant hypocalcemia [2]. It has been concluded that there is no UBG in Cyclostoma, as judged from the results of morphological observations and pharmacological studies. On the other hand, CT immunoreactivity was found in the brain of one species of hagfish by radioimmunoassay using human CT antiserum [4]. Immunoreactive CT-positive cells were also detected in the brain of two species of hagfish and two species of lamprey by an immunohistological method using salmon CT antiserum [11]. It is possible that these cells present in the brain of cyclostomes secrete a CT-like substance into the blood.

The objective of the present investigation was to examine by biochemical and biological methods whether a CT-like substance is present in the plasma of hagfish, *Eptatretus burgeri*.

MATERIALS AND METHODS

Experimental Animals

Eight brown hagfish, *Eptatretus burgeri*, caught with a trap in

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water 10 to 50 meters deep near Misaki Marine Biological Station, University of Tokyo, on the coast of Sagami bay in November 1993 were used. Fish were anesthetized with 1/3000 tricaine methanesulfonate. Thereafter, blood samples were collected from the heart with heparinized syringes, and centrifuged at 25,000×g for 10 min at 4°C. The plasma was immediately frozen and kept at –50°C until use.

Separation of plasma by reverse-phase high-performance liquid chromatography

The plasma was acidified with glacial acetic acid to a final concentration of 1 M, vortexed and centrifuged. The high molecular weight proteins and lipids were then removed from the supernatant with 67% and 99% cold acetone, respectively. The supernatant was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on an ODS-120T column (4.6×250 mm, Tosoh, Tokyo) with a linear gradient from 20 to 80% CH₃CN in 0.1% trifluoroacetic acid (pH 2.0).

Electrophoresis and Western blotting

After RP-HPLC, 300 μl of each fraction was lyophilized, and solubilized in lysis buffer (4% SDS, 4% mercaptoethanol, 8 M urea and 10 mM Tris buffer adjusted with HCl to pH 6.8). These samples were subjected to electrophoresis using the method of Laemmli [6] on a linear gradient gel from 10% to 20% polyacrylamide with bromophenol blue as a tracking dye. Synthetic salmon CT was used as a positive control for CT.

The samples separated by electrophoresis were transferred onto a polyvinylidene difluoride membrane (Clear blot membrane-p, Atto Co. Ltd., Tokyo) at room temperature for 2.5 hr at 16 V/cm according to the method of Anderson [1]. The membrane was then washed three times in phosphate buffered saline containing 0.05% Tween 20 (PBST). PBST containing 1% normal swine serum was used to block nonspecific binding against the membrane for 15 min at room temperature followed by washing three times in PBST. The membrane was then incubated with polyclonal salmon CT antiserum

(1/40000 dilution) for 12–15 hr at room temperature. The unbound antiserum was removed by washing three times in PBST. Finally, the membrane was immunostained with a labeled streptavidin biotin kit (Dako Co. Ltd., Tokyo).

Rat bioassay

Aliquots of the fraction which showed positive immunoreactivity on Western blotting (each 300 μ l) administered to 7 rats as described by Uchiyama *et al.* [14]. Briefly, 300 μ l of each immunoreactive CT fraction was concentrated to about 10 to 20 μ l under vacuum, and made up to a final volume of 400 μ l by adding vehicle (0.9% saline containing 0.1% bovine serum albumin, pH 4.6) prior to administration. The immunonegative fraction was adopted as a control and administered to rats in the same way. Blood was sampled just before (zero) and at 0.5, 1, 2, and 3 hr after administration. Serum Ca, inorganic phosphate (Pi) and Na levels were monitored. Serum Ca and Na levels were determined with an atomic absorption spectrophotometer (180–70 type, Hitachi-Zeeman, Hitachi Co. Ltd., Tokyo). Serum Pi was measured with a spectrophotometer (100–20 type, Hitachi Co. Ltd., Tokyo) by a modification of the method of Fiske and Subbarow [3]. Student's *t*-test was applied for statistical evaluation of numerical data.

Measurement of CT-like substance in plasma

CT-like substance present in the plasma was quantified by competitive inhibition enzyme-linked immunosorbent assay (ELISA) using salmon CT antiserum, according to the procedure described by Robertson [9, 10]. Furthermore, an ELISA system with increased sensitivity for salmon CT was developed using the streptavidin-biotin system in the present study. In this ELISA system, the detection limit was 10–30 pg/ml. The specificity of anti-salmon CT antiserum was checked using synthetic peptide hormones and tissue extraction.

RESULTS

Purification of CT-like substance

The plasma was separated into twenty fractions by RP-HPLC (Fig. 1). Aliquots of 300 μ l of each fraction were lyophilized and subjected to Western blotting. Two fractions (Fraction 9 eluted with 36–39% CH₃CN and fraction 10 eluted with 39–42% CH₃CN) showed positive immunoreactivity (Fig. 2). In fraction 9, the molecular weights of the two immunopositive substances were estimated as 3.5 and 4.7 kDa. In fraction 10, the molecular weight (MW) of the positive substance was 4.7 kDa.

Hypocalcemic activity of CT-like substance

Changes in serum Ca and Pi levels in rats are shown in Figures 3 and 4, respectively. Administration of fraction 9 evoked significant hypocalcemia at 0.5 and 1 hr (both $P < 0.05$). Fraction 10 also induced hypocalcemia at 0.5 hr after administration ($P < 0.005$). Only fraction 9 provoked significant hypophosphatemia at 0.5, 1 and 2 hr ($P < 0.001$, $P < 0.005$ and $P < 0.05$, respectively). Serum Na levels did not change, remaining at 330 mg/100 ml following administration of either the immunopositive or the control fraction to rats (data not shown).

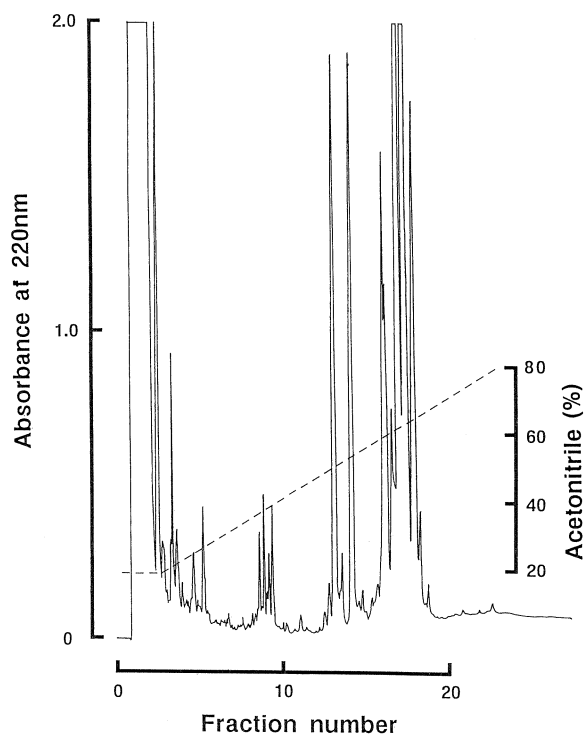


Fig. 1. Reverse-phase HPLC on an ODS-120T column. Sample: Hagfish plasma; flow rate, 1 ml/min; fraction size, 3 ml/tube. Solvent system: linear-gradient elution from 20% to 80% CH₃CN in 0.1% TFA for 60 min.

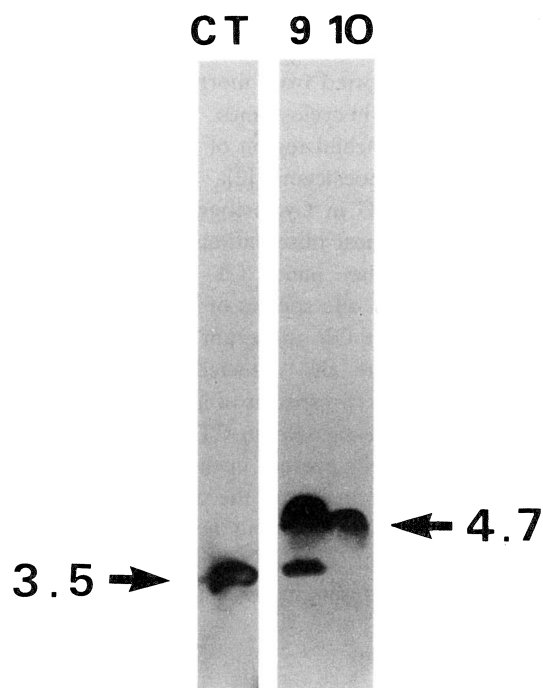


Fig. 2. Western blotting of immunoreactive calcitonin (CT) in hagfish plasma. The HPLC fractions 9 and 10 (see Fig. 1) and synthetic salmon calcitonin (sCT) as a positive control are shown. The arrows show the positions of sCT and immunoreactive CT with respective molecular mass in kDa.

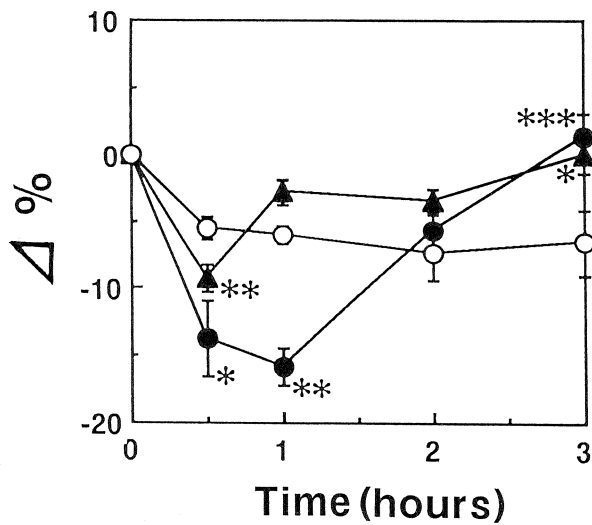


FIG. 3. Changes in serum Ca levels in rats after administration of fraction 9 (●), fraction 10 (▲) and fraction 11 (○) after HPLC of hagfish plasma (see Fig. 1). Vertical bars show \pm SEM. The numbers of rats used were 7 for fraction 9, 7 for fraction 10 and 5 for fraction 11. * P <0.05, ** P <0.005, *** P <0.001, compared with the values of fraction 11.

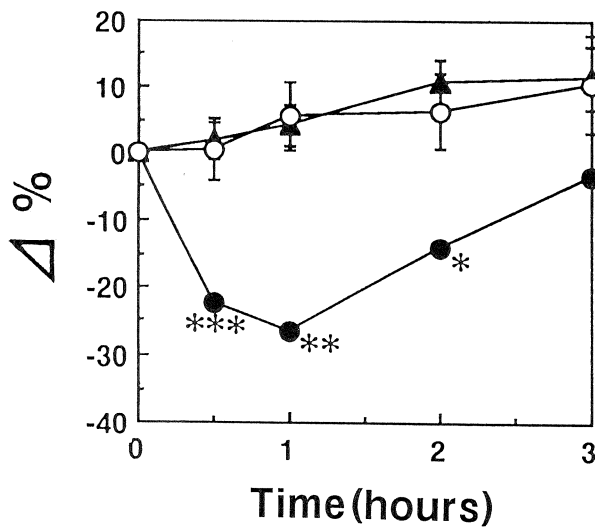


FIG. 4. Changes in serum Pi levels in rats after administration of fraction 9 (●), fraction 10 (▲) and fraction 11 (○) after HPLC of hagfish plasma (see Fig. 1). Vertical bars show \pm SEM. The numbers of rats used were 7 for fraction 9, 7 for fraction 10 and 5 for fraction 11. * P <0.05, ** P <0.005, *** P <0.001, compared with the values of fraction 11.

Measurement of CT-like substance in plasma

The level of an iCT-like substance present in the hagfish plasma was measured in an ELISA system by reactivity to anti-salmon CT serum and was calculated to be about 14 ng/ml (Fig. 5).

DISCUSSION

In the rat bioassay, hypocalcemic activity was recognized

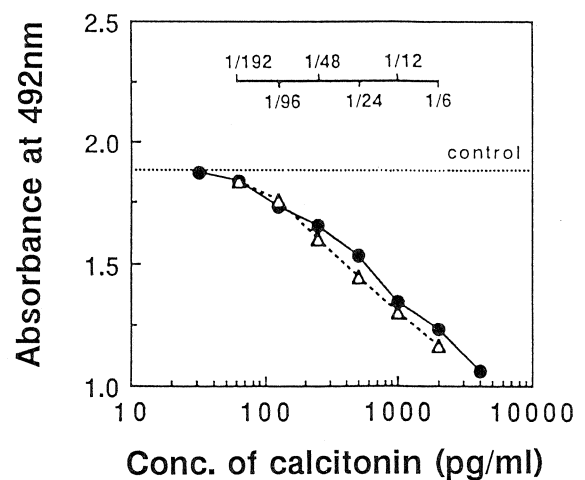


FIG. 5. Competitive inhibition curve for salmon calcitonin (sCT):● and dilution curve of hagfish plasma (△) in the ELISA for sCT. The dilution curve paralleled the standard curve of sCT. The dotted line shows control with only antibody. The sample values of pg/ml plasma were determined by multiplication of the dilution factor.

in 2 fractions of separated hagfish plasma. However, only fraction 9 showed hypophosphatemic activity. Fraction 9 contained two immunopositive substances with MW of 3.5 and 4.7 kDa, while in fraction 10, only one CT-like substance of 4.7 kDa was detected. However, the quantity of the 4.7 kDa CT-like substance in fraction 10 was less than that in fraction 9 according to the results of Western blotting (Fig. 2). Therefore, it is possible that the quantity of 4.7 kDa was proportional to the strength of hypocalcemic activity in both fractions. On the other hand, it has been reported as a characteristic of rat bioassay that the response of serum Pi level to CT is larger than that of Ca [12]. Therefore, if the 4.7 kDa molecule included in fraction 10 is genuine CT, the change in serum Pi level should be greater than that in Ca level on administration of fraction 10 to rats. However, the serum Pi level did not change. This suggests that the CT-like substance with MW of 3.5 kDa possessed genuine CT-like activity. Furthermore, the concentration of CT-like substance in the hagfish plasma was estimated to be about 14 ng/ml. In teleost fish, plasma CT levels determined to date are 10–700 pg/ml in salmon [15], 2000 pg/ml in eel [17] and 1500–2000 pg/ml in bass [5]. The level of CT-like substance in the plasma of hagfish was much higher than in these other fish. However, it has been reported that there are very few CT-producing cells present in the brain of hagfish [11], and it is unlikely that the CT-producing cells in the brain of hagfish can release such a large amount into the blood. Therefore, there are probably other CT-producing tissues in the hagfish in addition to the brain.

The hagfish has the highest bile Ca concentration determined to date among vertebrates, the level of which corresponds to 12 times that in the plasma [7]. It is known that in rats [18] and cartilaginous fish [13], CT acts on Ca excretion *via* bile. Therefore, the abundance of CT-like substance in

the hagfish plasma detected here may be related to this phenomenon.

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