Plasma calcium and calcitonin levels at food intake in eels and goldfish

ZOOLOGICAL SCIENCE 13: 731–735 (1996)

Plasma Calcium and Calcitonin Levels at Food Intake in Eels and Goldfish

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ABSTRACT—In order to examine whether calcitonin plays an important role in Ca homeostasis of teleosts, such as suppressing hypercalcemia at food intake, we compared the plasma Ca levels and calcitonin levels in eels fed normally with eels starved for one week (Experiment I), and in goldfish administered with a high Ca-consomme solution into the digestive tract with goldfish given physiological saline solution (Experiment II). In Experiment I, the plasma Ca levels and calcitonin levels in the fed eels were significantly higher than those in the starved eels after one week. In Experiment II, the plasma Ca levels in the high Ca-treated goldfish were significantly higher than those in the saline-treated goldfish after 1 hr and 3 hr. The number of goldfish showing over 500 pg/ml of plasma calcitonin was significantly higher in the high Ca-treated group than in the saline-treated group. From the results of both experiments, we conclude that in these two species, Ca and/or nutriment absorbed *via* the digestive tract may affect plasma calcitonin levels. However, more experiments are needed to directly demonstrate that calcitonin suppresses hypercalcemia at food intake.

INTRODUCTION

In mammals, calcitonin is a hormone that protects bones from demineralization by suppressing the activity of osteoclasts, and has been adopted as a treatment for human osteoporosis. Furthermore, in mammals, calcitonin suppresses the occurrence of hypercalcemia at food intake (Care *et al.*, 1971; Sethi *et al.*, 1989). In teleosts, however, the roles of calcitonin are not clear, since calcitonin administrations have both positive and negative effects (Chan *et al.*, 1968; Hayslett *et al.*, 1971; Pang, 1971; Dacke, 1979). In some cases, calcitonin administrations produce even hypercalcemia (Glowacki *et al.*, 1985; Fouchereau-Peron *et al.*, 1987; Oughterson *et al.*, 1995). The reason why the effects of calcitonin are inconsistent in teleosts seems to be that calcitonin is administered to intact fish, not to fish with calcitonin completely depleted, such as ultimobranchialectomized fish, as the ultimobranchial gland is the source of calcitonin in fish. However, this operation is almost impossible in fish because of the topography and morphology of the ultimobranchial glands. On the other hand, in salmon, rainbow trout and eels, plasma calcitonin levels increase with gonadal maturation, especially in females (Watts *et al.*, 1975; Yamauchi *et al.*, 1978; Björnsson *et al.*, 1986, 1989). Therefore, Björnsson *et al.* (1986, 1989) suggested that in teleosts, calcitonin is a hormone related to reproductive physiology. However, in the chum salmon, production of calcitonin begins in the embryo at three days before hatching, regardless of sex (Sasayama *et al.*, 1989). This suggests that also in fish, calcitonin plays a role indispensable to everyday life, in addition to its role in reproduction.

On the other hand, as teleosts have the ability to absorb a large part of the necessary Ca from environmental water (Urist, 1976), Ca in the diet has not been noticed from the viewpoint of Ca metabolism in fish. In some species of teleosts, however, Ca contained in the diet is physiologically important (Lovell, 1978). Furthermore, juvenile fish growing rapidly seem to demand a large amount of Ca. In this case, Ca contained in the diet may become more important. In this study, therefore, two experiments using eels and goldfish were made to obtain fundamental data on the role of calcitonin on Ca absorbed from the diet.

MATERIALS AND METHODS

Experiment I: Comparison of plasma Ca and calcitonin levels in fed and starved eels

Eels, *Anguilla japonica*, were obtained from the Eel Culture Association of Hamanako, Shizuoka Pref. in 1994. They were cultivated at 28–30°C and fed standard artificial food. In total, ninety individuals were examined. The smallest eel was 22.6 g in body weight and 28.2 cm in total body length, and the largest was 180 g and 50.5 cm long. The eels were captured just at feeding, and were divided into two groups: one group (n=42) was immediately killed, and the other group (n=48) was sampled after starvation for one week. After anesthesia with ethyl 4-amino-benzoate, blood was taken directly from

the dorsal aorta with heparinized syringes to avoid intermingling with tissue fluid. Blood was immediately centrifuged at 3,500 rpm for 10 min at 6°C, and the plasma was stored at -80°C until assay.

In this study, we designed a sandwich enzyme-linked immunosorbent assay (ELISA) method with two kinds of polyclonal antibody. Anti-salmon calcitonin antibodies were used as a solid-phase antibody (dilution rate: 10,000 times) on ELISA plates (Corning Co. Ltd.). These antibodies were absorbed on to the plates at 4°C for 24 hr. Then, the plates were blocked with 0.5% milk casein (skimmed milk: Yuki-jirushi Co. Ltd.) to prevent non-specific immunoreactions, on a shaker for 1 hr at room temperature. Eel calcitonin standards or blood samples were incubated on the plates at 4°C for 24 hr. Anti-eel calcitonin antibodies were then added as a liquid phase antibody to sandwich the eel calcitonin at 4°C overnight. Following this treatment, a peroxidase labeled-streptavidin biotin kit (Dako Co. Ltd.) was applied to visualize the calcitonin-anticalcitonin antibody immunoreaction (dilution rate of biotin-conjugated anti-guinea pig antibodies: 100,000 times). Fresh peroxidase substrate (ortho-ethylene-diamine · 2HCl) was added and the color intensified for about 10 min. Sulfuric acid was added to stop the reaction, and the color of the immunoreaction products was quantified by absorbance at 492 nm. In our assay system, the correlation coefficient between the theoretical values and the actual values was 0.99, and the minimum detection level was about 10 pg/ml. The intra-assay standard deviation was always under 10%.

The plasma Ca levels were determined by routine atomic absorption spectrophotometry.

Experiment II: Comparison of plasma Ca and calcitonin levels in goldfish administered with high Ca solution and goldfish given saline solution

Goldfish, *Carassius auratus*, were purchased from a commercial source. The sizes of the goldfish were about 10 g body weight and about 7 cm in total body length. They were acclimatized at 22°C for 3 days until used. In total, 43 goldfish were used irrespective of sex. They were divided into two groups. One group was slightly anesthetized and was administered with high Ca solution directly into the digestive tract through the oral cavity by a plastic tube. The Ca solution consisted of a consomme cube and $CaCl_2$, and the final Ca concentration was 1 g Ca/100 ml. Usually, artificial fish food contains rather volume of fish meal (2.5–65%) with fish bones (Nomura *et al.*, 1986). Therefore, the Ca level of the solution seemed to be within physiological range. The solution was administered at a rate of 10 µl/g body weight 100 µg Ca/g body weight). The other group was given 0.6% NaCl solution in the same way. Just before administration and at 1 and 3 hr after the treatments, the goldfish were killed, and blood was taken through the heart by heparinized syringes. Plasma was separated by centrifuge (4°C, 10,000 rpm, 5 min). In this experiment, the plasma Ca concentration was determined using a microplate reader with a modified Gitelman's method (1967). In this method, each 1 µl of standard Ca solution (Ca: 0–30 mg/100 ml) or of plasma samples was put to the wells of a microplate. Then, 100 µl of 0.88 M mono-ethanol-amine buffer (pH 11.0) was added. To colorize the solution according to the Ca concentration, 10 µl each of 0.63 mM ortho-cresolphthalein complex (OCPC) and 0.69 mM 8-kinole detergents was added. At 10–15 min later, color intensity of each solution in the wells was determined at 550 nm absorbance by a microplate reader. The correlation coeffecient between the theoretical value and the actual Ca concentration was 0.998. The advantage of this method is that the plasma volume of the sample is saved as only 1 µl. Therefore, this method to determine the Ca is recommended for experiments using small animals.

The plasma calcitonin levels were measured by the sandwich method of ELISA used in Experiment I, although goldfish calcitonin antibodies were used as the liquid phase antibody to sandwich the goldfish calcitonin.

In Experiments I and II, Student's *t*-test and Fisher's exact probability test were used to evaluate the numerical data.

RESULTS

Experiment I: Comparison of plasma Ca and calcitonin levels in fed and starved eels

The body weights of the fed group, 86.3±6.13 g, tended to be larger ($0.05<p<0.1$) than those of the starved group, 71.9±5.39 g, although these differences were not significant in statistics. In body lengths, there were no significant differences (39.6±0.83 cm and 38.9±0.73 cm, respectively). Body sizes showed no correlation with the plasma Ca or calcitonin levels in either group. However, the plasma Ca levels of the fed group, 15.4±0.27 mg/100 ml, were significantly higher ($p<0.001$) than those of the starved gorup, 12.4±0.20 mg/100 ml (Fig. 1). The plasma calcitonin levels of the fed group, 888.4±137.79 pg/ml, were also significantly higher ($p<0.001$) than those of the starved group, 356.5±51.52 pg/ml (Fig. 1).

Experiment II: Comparison of plasma Ca and calcitonin levels in goldfish administered with high Ca solution and goldfish given saline solution

The plasma Ca concentrations of the goldfish before the experiments were 7.2±0.4 mg/100 ml (n=8). In the goldfish given saline solution, the plasma Ca levels were 7.3±0.5 (n=7) and 7.8±0.3 mg/100 ml (n=8) at 1 and 3 hr, respectively, after the treatment, and were not statistically different from the initial levels (Fig. 2). In contrast, in the goldfish administered with high Ca solution, the plasma Ca levels were 18.0±1.3 (n=9) and 17.8±0.8 mg/100 ml (n=11) at 1 and 3 hr, respectively, after the treatments, and they were significantly higher ($p<0.001$) than the initial levels (Fig. 2).

The initial plasma calcitonin concentrations were 236±126 pg/ml (n=8). In the goldfish given saline solution, the plasma calcitonin levels were 221±51 pg/100 ml (n=7) and 256±115 pg/100 ml (n=8) at 1 and 3 hr, respectively, and were not

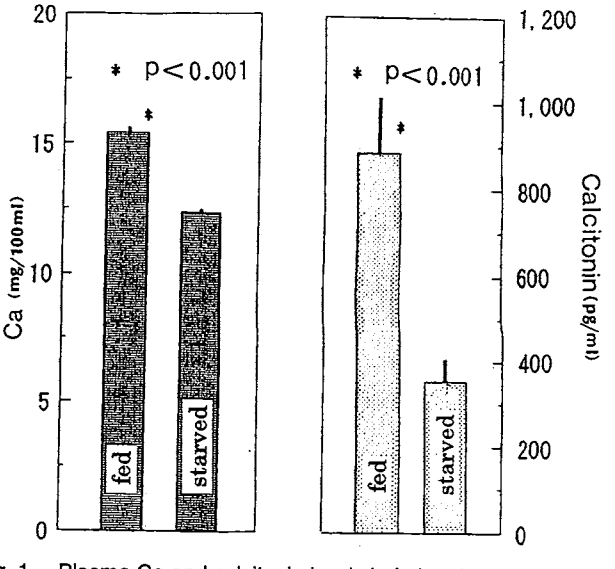

Fig. 1. Plasma Ca and calcitonin levels in fed and starved eels.

statistically different from the initial levels (Fig. 3). In the goldfish administered with high Ca solution, the plasma calcitonin concentrations were 371±106 pg/100 ml (n=9) and 381±94 pg/ml (n=11) at 1 and 3 hr, respectively (Fig. 3). The two groups showed no significant differences each time when compared using the Student's *t*-test. In the goldfish given saline solution, the number of individuals with a high level (over 500 pg/ml: this value corresponds to about the third quartiles in statistics) of plasma calcitonin was only two among 15 goldfish (13%) until 3 hr after the treatment (Fig. 4). In contrast, in the goldfish

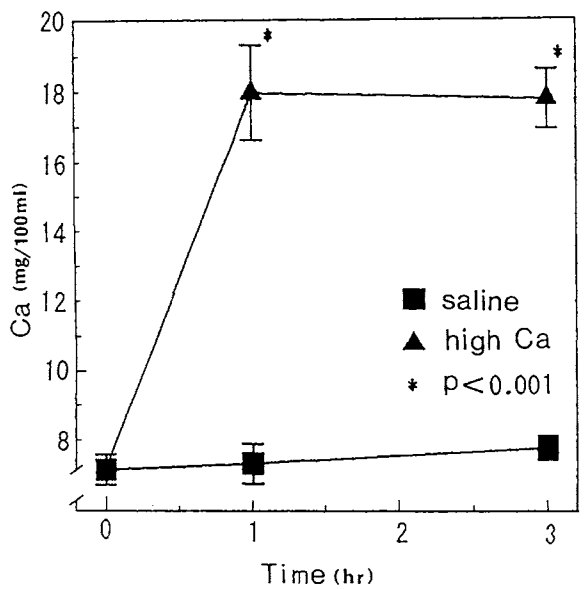

Fig. 2. Changes in the plasma Ca levels in goldfish administered with high Ca solution (high Ca) and goldfish given saline solution at 0, 1 and 3 hr after the treatments.

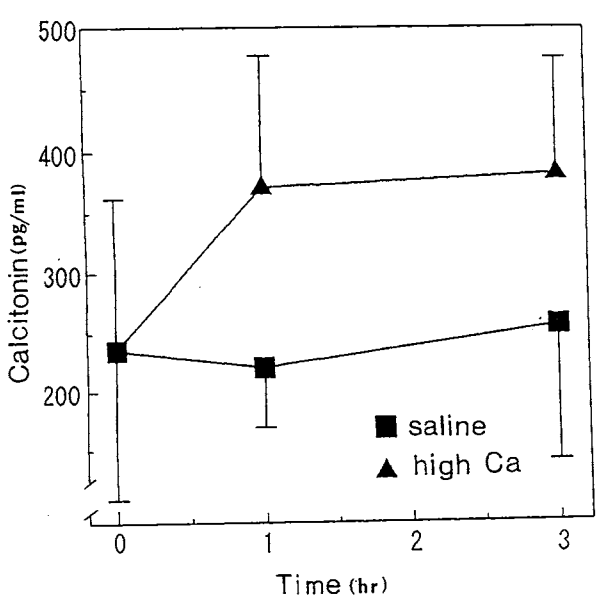

Fig. 3. Changes in the plasma calcitonin levels in goldfish administered with high Ca solution (high Ca) and goldfish given saline solution at 0, 1 and 3 hr after the treatments.

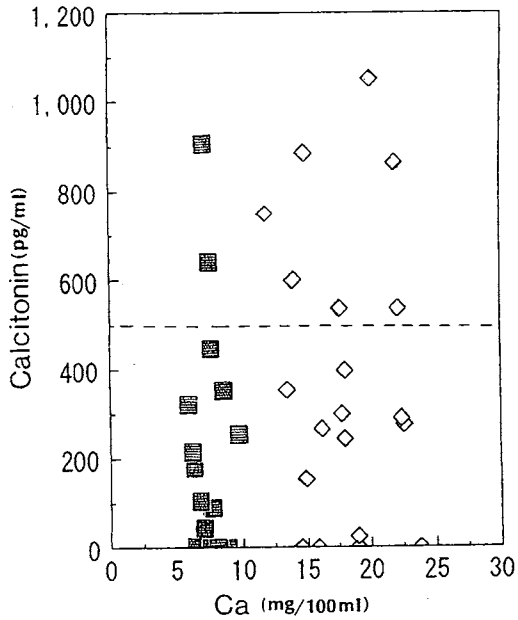

Fig. 4. Plasma Ca and calcitonin concentrations of goldfish given saline solution (▩) and goldfish administered with high Ca solution (◇).

administered with high Ca solution, the number of individuals with a high level of plasma calcitonin was seven among 20 goldfish (35%) until 3 hr. The two groups showed a significant difference ($p<0.001$) in the number of individuals with a plasma calcitonin level over 500 pg/ml according to the Fisher's exact probability test.

DISCUSSION

Azria (1989) reported that in humans, the plasma calcitonin levels are higher in babies than in adults, although in rats the relationship is reversed. In tadpoles of the frog and in the larvae of the salamander, the serum Ca concentrations or contents of total Ca of the body increase with the body growth (Oguro *et al.*, 1975, 1980). These reports suggest a possibility that also in eels, the plasma Ca levels increase with body growth, and that the plasma calcitonin levels also change with body growth. Therefore, in this study, many eels of various body sizes were examined. In eels, however, the plasma Ca levels of both fed and starved eels did not differ between their various body sizes. In eels, only juveniles such as the leptocephalus stages before metamorphosis may correspond to the larvae of amphibians.

In mammals, the circulating levels of calcitonin increase following food intake, since Ca in the food stimulates calcitonin secretion (Care *et al.*, 1971; Sethi *et al.*, 1983; Azria, 1989). This shows that in mammals, calcitonin regulates precisely the Ca levels in the blood. On the other hand, a pioneer work by Shiraki *et al.* (1982) showed that in eels, Ca infused directly into blood stream does not cause an increase in plasma calcitonin levels, even though the plasma Ca levels increased

significantly by the treatments. Therefore, we thought that the Ca must be loaded *via* physiological routes such as the digestive tract, because some unknown factors may influence the processes of Ca absorption before plasma calcitonin levels increase. In this study, we showed that both plasma Ca and calcitonin levels in fed eels were significantly higher than those in starved eels. From these results, two interpretations are possible. One explanation is that the plasma calcitonin levels decreased in the starved eels, because plasma Ca levels were low due to no Ca supply from the diet. On the other hand, in mammals, it is well known that gastrointestinal hormones secreted at food intake stimulate calcitonin secretion. Therefore, the other explanation is that plasma calcitonin levels decreased due to starvation itself. That the body weights of the starved eels tended to be smaller than those of the fed eels may support the second hypothesis. In eels, however, it is reasonable to conclude now that the increase in the plasma Ca levels and/or feeding may stimulate the secretion of calcitonin.

In goldfish administered with a high Ca solution, the plasma Ca levels were significantly higher than those in goldfish given saline solution. In a preliminary study, we found that absorption of Ca from the digestive tract was largely accerelated if nutriments such as consomme soup were added into the high Ca solution. The average values of the plasma calcitonin levels in the high Ca-treated goldfish were higher than those in the saline-treated goldfish, although these groups showed no significant differences. However, the number of individuals showing a high level of plasma calcitonin was about three times higher in the goldfish administered with high Ca solutions than in the goldfish given the saline solution. These results seem to suggest that calcitonin was secreted corresponding to the increase in plasma Ca levels by administering the high Ca solution. In mammals, however, the quantity of secreted calcitonin depends not only on the Ca, but also on the protein which are contained together with the Ca in the diet (Azria, 1989). Therefore, it is possible that some parts of the increase in the plasma calcitonin levels may be due to the consomme components. Consequently, in Experiment II, a control group in which only consomme solution was administered should be provided, in order to clarify if the dietary protein affects the plasma calcitonin levels in the goldfish.

Also, in this study, some goldfish given only saline solution or had no treatment showed high levels of plasma calcitonin. The reason for this is not clear now. Examination of the physiological background of the individual goldfish may be necessary. Furthermore, the sexes may have to be distinguished in experiments, if possible, as the estrogen affects the plasma Ca levels. In this study, four individuals which showed plasma calcitonin levels from the highest value to the fourth value were female fish. However, those fish were not especially mature compared to other females.

Wendelaar Bonga and Pang (1991) suggested that in fish the essential role of calcitonin is to protect bones from the excess demands of Ca at growth, viterogenesis and reproduction, *etc.* Judging from the results of our two experiments, it is suggested that, in eels and goldfish, Ca and/or nutriment absorbed *via* the digestive tract affect plasma calcitonin levels. However, more experiments are needed to conclude reasonably that calcitonin is secreted every time at food intake to suppress hypercalcemia.

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(Received April 23, 1996 / Accepted July 1, 1996)