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# *In vitro* Measurements of Calcium Influx into Isolated Goldfish Scales in Reference to the Effects of Putative Fish Calcemic Hormones

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**ABSTRACT**—Calcium influx into the goldfish scale was measured by incubating individual scales in fish saline containing $^{45}Ca$. The influx was proportional to the calcium concentration of the incubation medium and was not affected by the presence of 0.1 mM KCN. The influx into the scale taken from fish acclimatized to low temperature (8°C) was significantly lower than that into scale taken from fish acclimatized to 25°C. The influx into the scales of starved fish was not significantly different from that in normally fed fish. On the other hand, when fish were starved in deionized water after removal of all scales from one side of the body, the influx into the remaining scales was significantly greater than that into the scales of the intact fish starved in tap water. Extract of the corpuscles of Stannius from mature chum salmon, salmon prolactin or eel calcitonin did not affect the influx when added to the incubation medium. Thus, calcium influx into the isolated scale seems to be a passive process affected by the condition of the fish.

## INTRODUCTION

Teleost fish scales, containing a large quantity of calcium, serve as an important internal calcium reservoir together with the bone [1, 2]. It is well known that some teleosts can survive under acalcic conditions; European eels survive and maintain the plasma calcium concentration in deionized water without food for several weeks [3–6]. Goldfish reared in deionized water and fed on a diet deficient in calcium and phosphorus can survive for at least 4 weeks with even higher plasma calcium levels than are seen in control fish [7]. In order to maintain plasma calcium concentration at a constant level, these fish must mobilize calcium from internal sources such as bones, scales or soft tissues, since passive loss of calcium from the body surface is inevitable.

Compared with bones, scales seem to have more labile calcium. Goldfish scales show greater turnover rate of $^{45}Ca$ than the bone [8]. Estrogen injection to goldfish and killifish induces mobilization of scale calcium but not bone calcium into plasma [9]. Scales are resorbed when goldfish are starved [10, 11], or when precocious male parr of masu salmon reach sexual maturity [12].

In the present study, calcium influx into the scales of goldfish (*Carassius auratus*) was measured by incubating individual scales in fish saline containing $^{45}Ca$, and effects of rearing conditions and of some putative calcemic hormones were examined.

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## MATERIALS AND METHODS

### *Fish*

Immature goldfish (*Carassius auratus*), weighing 5–20 gm, were obtained from a commercial dealer or from a laboratory stock at Faculty of Fisheries of Hokkaido University in Hakodate. They were kept in tap water (Ca: 0.1 mM) at 25°C and fed a commercial carp or trout chow (Nihon Haigo Shiryo) at a rate of 3% of body weight per day, unless otherwise indicated.

*Measurement of calcium influx into scales*

Scales were removed from the fish using a fine forceps. They were individually incubated in 0.1 ml fish saline containing 0.2 $\mu$Ci/ml $^{45}Ca$, by shaking in a water bath at 25°C for up to 4 hr. Fish saline was composed of 135 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 2.0 mM $KH_2PO_4$, 1.0 mM $MgSO_4$, 10 mM $NaHCO_3$, 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and 5 mM glucose. In some experiments, concentrations of $CaCl_2$ and $KH_2PO_4$ were modified from 1.5 mM to 0.5 or 3.5 mM and from 1.0 mM to 0.5 or 4.0 mM, respectively. The pH was adjusted to 7.4 with NaOH. After incubation, scales were briefly rinsed in saline and their outlines were sketched under a light microscope using a drawing device. They were then dried at 90°C for 24 hr and solubilized in a mixture of perchloric acid and hydrogen peroxide (1:1) or in "Soluen" (Packard Instrument), and their radioactivity was counted using a liquid scintillation counter (Aloka LSC-673 or Packard Tri-carb 300). Calcium influx into the scale was expressed as mean±S.E.M. (nmol/cm$^2$ or nmol/mg dry weight). Concentrations of calcium in the fish saline and in the aquarium water were measured by atomic absorption photometry (Hitachi 518 or Hitachi 180–50).

*Experiments*

In the first series of experiments, effects of rearing conditions on calcium influx into the scale were examined. For each group, 5–8 fish were used, and one scale per fish was taken from the midportion of the row under the lateral line. All scales were incubated in a standard saline at 25°C. The first experiment examined the effect of rearing temperature. Fish were reared in tap water at 8 or 25°C for 14 days. Although all fish were given a diet at a rate of 3% of body weight per day, the fish kept at 8°C ingested only 0.6% of their body weight. In the second experiment, fish were starved for 30 days in tap water at 25°C. Control fish were fed at a rate of 3% of body weight. In the third experiment, fish were subjected to calcium deficiency in both diet and environment. Experimental fish reared in deionized water were separated into two groups: one group remained intact and the other group had one side of the body descaled. Control fish were reared in tap water. Both control and experimental fish were starved during the 15-day experiment.

In the next series of experiments, *in vitro* effects of an extract of the corpuscles of Stannius from mature female chum salmon (sCS extract), chum salmon prolactin and eel calcitonin were examined. The sCS extract was prepared as follows. Frozen tissue was homogenized in 0.9% NaCl (100 mg/ml). After centrifugation at 20000 rpm for 10 min, the supernatant was stored at −20°C until use. An extract of the body kidney was similarly prepared. The sCS extract or kidney extract was added to the incubation medium at a final concentration of 1 mg/ml. The chum salmon prolactin (sPRL), from Prof. Hiroshi Kawauchi, Kitasato University, was added to the incubation medium at a concentration of 1 $\mu$g/ml. Eel calcitonin (Elcatonin, Toyo Jozo) was added to the medium at a concentration of 0.1 U/ml. In this series of experiments, all control and appropriate experimental scales were taken from same fish, which were reared in tap water at 25°C and fed a diet at a rate of 3% of their body weight per day.

## RESULTS

The calcium influx into the scale incubated in standard fish saline was almost linear over a period of 4 hr (Fig. 1). The influx during the first 2 hr

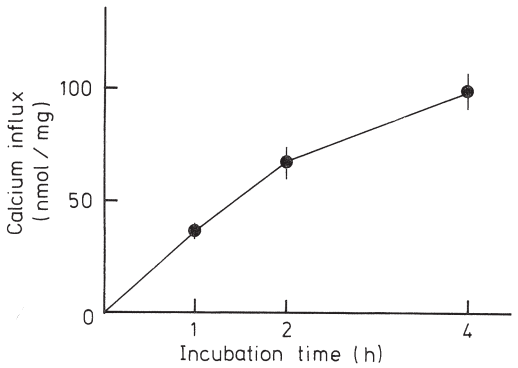
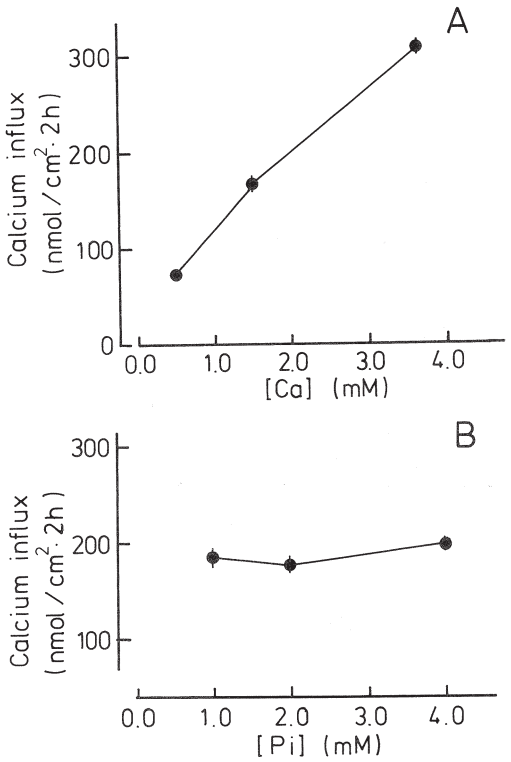



FIG. 1. Cumulative influx of calcium as measured by $^{45}Ca$ accumulation in scales incubated in standard fish saline at 25°C. Scales were removed from the fed fish reared in tapwater at 25°C. Each point is mean of 5 scales; vertical bars represent S.E.M.

($171.3 \pm 4.5$ nmol/cm$^2$, n=7) was not affected by the addition of 0.1 mM KCN in the incubation medium ($182.6 \pm 9.4$ nmol/cm$^2$, n=8).




The calcium influx during 2 hr was proportional to the calcium concentration of the medium, when the inorganic phosphate concentration was kept at a constant level of 2.0 mM (Fig. 2A). On the other hand, the influx was not affected by changes of the inorganic phosphate concentration at a constant calcium concentration of 1.5 mM (Fig. 2B).

Table 1 summarizes the calcium influx during 2 hr into the scale taken from fish reared under various conditions. The calcium influx was significantly lower in the scales taken from fish reared in tap water at 8°C for 14 days (about 85%) than in those from fish acclimatized to 25°C. When the fish were starved for 30 days in tap water, the calcium influx was not significantly different from the control value in fed fish. On the other hand, when the fish were starved in deionized water for 15 days after removal of all the scales from one side of the body, the calcium influx into the remaining scales was significantly greater than that

FIG. 2. Calcium influx as a function of calcium (A) or phosphate (B) concentration of the incubation medium. Phosphate concentration in experiment A was kept at 2.0 mM and calcium concentration in experiment B at 1.5 mM. Scales were removed from the fed fish reared in tapwater at 25°C, and incubated at 25°C. Each point is mean of 8 scales; vertical bars represent S.E.M.

TABLE 1. Calcium influx into scales taken from fish reared under various conditions

| Condition | Period of rearing (days) | Influx (nmol/cm$^2$·2 hr) |
|---|---|---|
| Control[a)] | 14 | $190.8 \pm 5.4$ (6) |
| Low temperature (8°C) | 14 | $164.9 \pm 6.7$* (7) |
| Control[a)] | 30 | $161.9 \pm 6.6$ (7) |
| Starved | 30 | $143.9 \pm 7.0$ (7) |
| TW, intact[b)] | 15 | $166.4 \pm 5.7$ (5) |
| DW, intact[b)] | 15 | $192.3 \pm 10.1$ (7) |
| DW, descaled[b)] | 15 | $207.0 \pm 3.8$**(6) |

Mean±S.E.M. (number of fish)

* Significantly different ($P<0.05$) from the control group.

** Significantly different ($P<0.01$) from TW, intact group.

a) Control fish were reared in tap water at 25°C and fed at a rate of 3% of the body weight per day.

b) Fish were reared at 25°C and starved during the experiment.

TW, tap water; DW, deionized water.

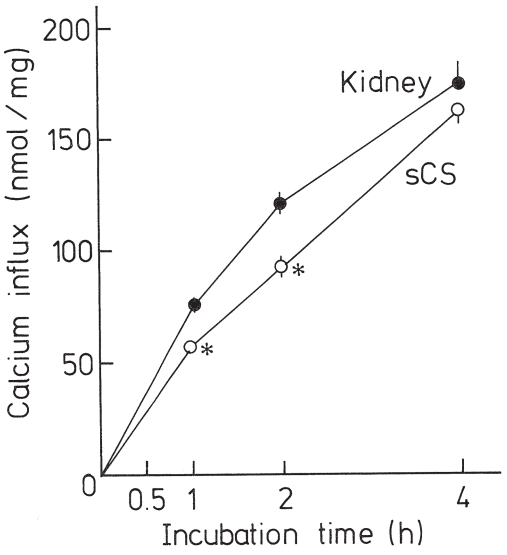



FIG. 3. Cumulative influx of calcium as measured by $^{45}Ca$ accumulation in scales incubated in standard fish saline at 25°C, in the presence of extract of salmon corpuscles of Stannius (sCS, 1 mg/ml) or of kidney extract (1 mg/ml). Scales were removed from the fed fish reared in tap water at 25°C. Each point is mean of 5 scales; vertical bars represent S.E.M. *Significantly ($P<0.01$) different from kidney extract group.

TABLE 2. *In vitro* effect of extract of salmon corpuscles of Stannius (sCS) on calcium influx into scales incubated for 4 hr

| Medium Ca | Tissue extract[a)] | Influx (nmol/mg·4 hr) |
|---|---|---|
| 0.5 mM | kidney | 79.7±4.4 |
| | sCS | 79.8±7.7 |
| 4.0 mM | kidney | 156.1±17.6 |
| | sCS | 153.1±3.6 |

Mean±S.E.M. (n=5)

a) Extract of the body kidney of chum salmon or corpuscles of Stannius (sCS) was added to the medium at a concentration of 1 mg/ml. Scales were removed from fed fish reared in tap water at 25°C.

TABLE 3. *In vitro* effects of salmon prolactin (sPRL) and eel calcitonin (eCT) on calcium influx into scales incubated for 1 hr

| Medium Ca | Hormone[a)] | Influx (nmol/mg·hr) |
|---|---|---|
| 0.5 mM | Control | 23.2±1.2 |
| | sPRL | 19.7±1.4 |
| | eCT | 25.0±1.5 |
| 1.5 mM | Control | 37.3±1.2 |
| | sPRL | 46.0±2.5* |
| | eCT | 48.4±5.6 |
| 4.0 mM | Control | 68.9±7.0 |
| | sPRL | 79.0±8.8 |
| | eCT | 69.8±7.3 |

Mean±S.E.M. (n=5)

* Significantly different from control ($P<0.05$).

a) 1 μg/ml sPRL or 0.1 U/ml eCT was added to the medium. Scales were removed from fed fish reared in tap water at 25°C.

TABLE 4. *In vitro* effect of salmon prolactin (sPRL) on calcium influx into the scales incubated for 4 hr

| Medium Ca | Group | Influx (nmol/mg·4 hr) |
|---|---|---|
| 0.5 mM | Control | 70.1±5.8 |
| | sPRL[a)] | 64.0±4.5 |
| 1.5 mM | Control | 80.6±3.8 |
| | sPRL | 99.1±8.2 |
| 4.0 mM | Control | 128.0±10.1 |
| | sPRL | 112.2±3.0 |

Mean±S.E.M. (n=5)

a) 1 μg/ml sPRL was added to the medium.
Scales were removed from fed fish reared in tap water at 25°C.

to the scales taken from the intact fish starved in tap water. The influx into the scales taken from the intact fish starved in deionized water tended to be greater than that in intact fish in tap water, but the difference was not significant.

Figure 3 shows the effect of sCS extract on calcium influx into the scale taken from the fed fish reared in tap water and incubated in standard saline. The influx was significantly ($P<0.01$) reduced after 1- and 2-hr incubation with sCS extract compared with that seen in scales incubated with kidney extract, whereas no significant effect was observed after 4 hr. When sCS extract was added to either high calcium (4.0 mM) or low calcium (0.5 mM) medium and scales were incubated for 4 hr, no significant difference was observed between the scales incubated with sCS extract and those with kidney extract (Table 2).

One hour incubation with sPRL or eCT added to saline with various calcium concentrations did not affect the calcium influx into the scales taken from the fed fish reared in tap water, except for a significant ($P<0.05$) increase when incubated in the standard saline (Ca: 1.5 mM) with sPRL (Table 3). However, when scales were incubated for 4 hr with sPRL, no significant effect was observed at any calcium concentrations (Table 4).

## DISCUSSION

The presence of diffusible (or labile) and non-diffusible (or stable) calcium has been shown in bones and scales of an acellular boned fish, *Fundulus kansae* [13] and also in goldfish scales [8]. During short-term incubation as was employed in this study, calcium appears to be incorporated into the diffusible pool. The calcium influx was not affected in the presence of KCN but was proportional to the calcium concentration in the incubation medium, suggesting that the calcium influx is a passive process. This is in accord with earlier studies using avian or mammalian bones. Scarpace and Neuman [14, 15] suggested that the calcium influx into the chick calvaria is a passive process; the flux was proportional to the calcium concentration of the incubation medium and to the absolute temperature/viscosity, and ouabain and carbonyl cyanide m-chloro phenyl hydrazine (CCCP), an uncoupler of oxydative phosphorylation, were without effect. Furthermore, Neuman *et al.* [16] showed that calcium influx into rat pup calvaria or to adult mouse calvaria was not directly linked to ATP levels of the bone cells.

Calcium influx into the goldfish scale was affected by rearing conditions; the flux decreased significantly when the fish were reared at low water temperature, whereas it increased significantly when the fish were starved in deionized water after removal of all the scales from one side of the body. The intact fish starved in deionized water showed a similar tendency. Fish acclimatized to low temperature did not feed actively, and little weight gain was seen as compared with the control fish (data not shown), suggesting their low metabolic activity. Both descaled and intact fish starved in deionized water showed a decrease in their scale

calcium content compared with intact fish starved in tap water (unpublished data). This suggests that fish starved in deionized water mobilized scale calcium to maintain plasma calcium concentration or to compensate for the shortage of calcium in the environmental water. These changes in the condition of the fish may affect the size of labile calcium pool of the scale, resulting in modification of calcium influx. Seasonal change in the size of the labile calcium pool of the scales and bones was shown in *F. kansae* by Brehe and Fleming [13].

The corpuscles of Stannius are unique in holostean and teleostean fishes and are considered endocrine glands producing a hypocalcemic hormone [17, 18]. Prolactin is likely to function as a hypercalcemic hormone in teleosts; injection of mammalian prolactin induces hypercalcemia in some species such as killifish, *Fundulus heteroclitus,* [19, 20], tilapia, *Oreochromis mossambicus,* [21, 22] or stickleback, *Gasterosteus aculeatus* [22]. Calcitonin is a major hypocalcemic hormone in mammals, but its effects in fish are inconsistent. Some investigators reported hypocalcemia after injection of calcitonin in some fishes [23–25], while others found no effect on plasma calcium level [26–28]. Little is known about the effects of these possibly calcemic hormones on fish bones or scales. When corpuscles of Stannius were removed from the European eel, the degree of mineralization of the vertebrae increased along with plasma calcium level [29]. Mammalian prolactin increased calcium content of bones and scales in tilapia [21, 22]. Calcitonin increased the degree of mineralization of the bones in European eels [25], although no effect was seen on the calcium and phosphate contents of the bones and scales in tilapia [26]. In the present study, none of these calcemic hormones affected the calcium influx into the goldfish scale *in vitro*. Since influx appears to be passive, it is possible that these calcemic hormones do not control the influx directly but control indirectly through effects on plasma calcium level. However, in the present study, only one dose of each hormone was used, and all scales were taken from fish reared in tap water and fed normally at 25°C. Further studies are required using different doses of the hormones under different experimental conditions to investigate the mechanisms controlling calcium influx into the fish scale.

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