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Effects of Stannius Corpuscle Extract on Calcium Influx Assayed by Perfusion of Isolated Gills of Rainbow Trout *Salmo gairdneri*

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Abstract

The effect of extracts of salmon Stannius corpuscles (CS) on the branchial calcium influx was examined by perfusion of isolated rainbow trout gills under high calcium gradients. The CS extract was added to perfusion fluid or injected intraperitoneally. The intraperitoneal injection of CS extract (50 μgCS/g BW) reduced branchial calcium influx. Addition of the CS extract to perfusion fluid produced a significant reduction in the branchial calcium influx at doses more than 0.1 mgCS/ml. The rate of calcium influx inhibition was dose dependent in a range from 0.04 to 1 mgCS/ml. The results indicated that the method can be a useful bioassay system for CS.

Introduction

The corpuscles of Stannius (CS) are small endocrine glands located in or on the kidney of holostean and teleostean fishes. Numerous experiments have indicated that the CS contain a hypocalcemic factor or factors; removal of the glands causes hypercalcemia probably by inhibiting branchial calcium uptake (Butler, 1969; Chester-Jones et al., 1965; Fenwick, 1974, 1976; Fenwick and So, 1974; Fontaine, 1964, 1967; Fontaine et al., 1972; Lafeber et al., 1988; Ma and Copp, 1982; Ogawa, 1968; Pang, 1971, 1973; Pang and Pang, 1974; Pang et al., 1973, 1974, 1975; So and Fenwick, 1977, 1979).

Although several candidates for the CS hypocalcemic factor have been proposed, their precise nature still remains unclear. Ma and Copp (1978) isolated from the CS of Pacific salmon a 3 kDa glycopeptide, which was hypocalcemic in American eel, and named it "teleocalcin". Angiotensin-like substances generated by incubating CS with homologous plasma have also been shown to be hypocalcemic in carp and *Lophius litulon* (Pang et al., 1981), and Japanese eel (Ogawa and Sokabe, 1972). The CS of American eel contain an acid-stable hypocalcemic factor (So and Fenwick, 1982); the primary anti-hypercalcemic factor was determined to be a protein with a molecular weight greater than 10,000 (Fenwick, 1982). Furthermore, there is some immunological evidence suggesting that the hypocalcemic factor of the CS is related to parathyroid hormone (PTH); Milet et al. (1980, 1982) reported that the hypocalcemic activity of the European eel CS was associated with a molecule resembling mammalian PTH, and named it "parathyrin of the corpuscles of Stannius (PCS)".

Such a confusion in specifying effective substance(s) of the CS seems to be due

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to absence of an adequate bioassay system, which has high sensitivity and consistency. The purpose of this study is to develop a simple method of measuring branchial calcium influx to be used for bioassay of the CS hypocalcemic factor.

Materials and methods

Cultured rainbow trout, *Salmo gairdneri*, weighing 150–200 g, were purchased from a fish farm. They were kept in tap water and fed a commercial diet once a day until use.

The branchial calcium influx was measured by constant flow perfusion of isolated gill arches. The second or third gill arch was isolated and polyethylene catheters (PE 10) were inserted into both the afferent and efferent gill arteries and tied. The cannulated gill preparation was then placed in a bathing medium and flashed with physiological saline containing 1.5 mM calcium for about 10 min until the gill was blanched. The gill arch was then perfused with the physiological saline with or without CS extract. The perfusion rate was 0.1 ml/min and perfusate was collected into a graduated pipett every 10 min. The recovery rate was recorded to check for any leaks. The calcium concentration of the perfusate was measured by atomic absorption spectrophotometry and the difference as compared to that of perfusion fluid was represented as an index of calcium influx. The effect of the CS extract was determined by the rate of change of calcium concentrations of perfusates with and without CS extract in the same gill arch.

Perfusion experiments were also conducted with gill arches taken from fish which had intraperitoneal injection of CS extract. Blood was collected by caudal section in unheparinized hematocrit capillaries 1, 2 and 4 hours after a single injection of CS extract (50 μgCS/g BW). Blood plasma was collected after being centrifuged at 3,000 rpm for 10 min and the total amount of calcium in the plasma was determined by atomic absorption spectrophotometry.

Perfusion fluid

Composition of physiological saline used for perfusion is shown in Table 1. The calcium concentration was 1.5 mM, the same level as the serum inonic calcium

Table 1. Composition of the standard perfusion fluid (mM) used in the experiments.

Standard perfusion fluid	
NaCl	156
KCl	5.0
$CaCl_2$	1.5
$MgCl_2$	1.5
NaH_2PO_4	0.3
Na_2HPO_4	1.2
Heparin	50 I.U./ml
Adrenaline	10 μM
pH	7.2

concentration of rainbow trout. Just prior to perfusion, 50 I.U. of heparin and 10 μl of 1 mM adrenalin were added to each milliliter of perfusion fluid and the fluid was filtered with a Millipore filter to avoid contamination by insoluble micro-substances.

Bathing media

The bathing media used were $CaCl_2$ solutions of different concentrations, mostly 10 mM. In an experiment to see the effect of external osmolality, gill preparations were bathed in $CaCl_2$ solution, physiological saline, or artificial seawater, all contained 10 mM Ca. The bathing was performed at 14 ± 1°C.

Preparation of the CS extract

CS tissue was collected from mature dog salmon, *Oncorhynchus keta*, captured in a river near Moheji, Southern Hokkaido. One hundred mg of the CS tissue were homogenized in a glass homogenizer with 10 ml of the same physiological saline as that used for perfusion fluid and centrifuged at 3,000 rpm for 10 min. The supernatant was filtered with a 0.2 μm Millipore filter, divided into 0.2 ml aliquots and

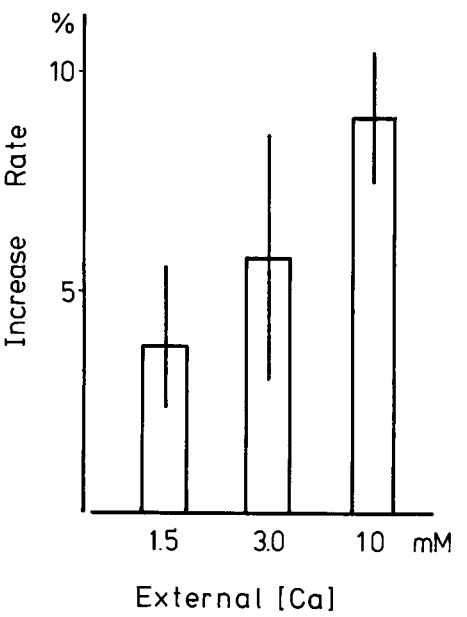

Fig. 1. Effect of calcium concentrations of the bathing medium on the branchial calcium influx in perfused gills of rainbow trout. Values represent mean$\pm$ S.E.M., n=6.

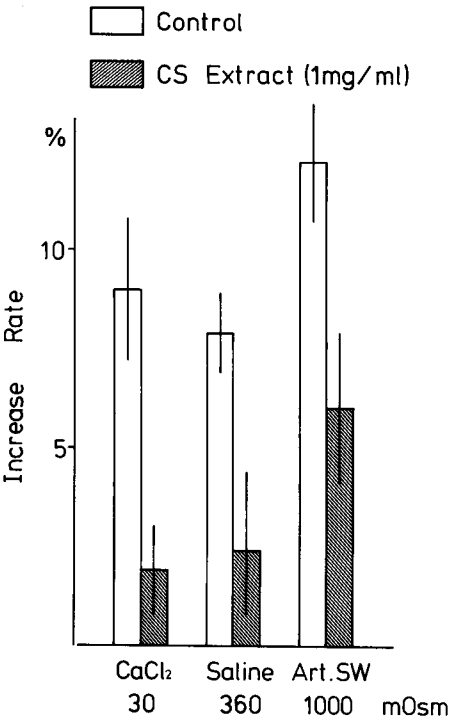

Fig. 2. Branchial calcium influx in different osmolalities of bathing media and the effect of CS extracts in perfusion fluid (1 mgCS/ml) on branchial calcium influx. Values represent mean$\pm$S.E.M., n=6.

stored at −20°C until use. Just before use it was thawed and diluted with perfusion fluid to an appropriate concentration. The dosage of the CS in the perfusion fluid was 1 mgCS/ml, except for assays for the effect of different does (0.04-4 mgCS/ml).

Results

Changes of calcium influx into gill arch preparations bathed in solutions of different $CaCl_2$ concentrations (1.5, 3.0 and 10 mM) are shown in Fig. 1. The branchial calcium influx increased with calcium concentrations of the bathing media.

Calcium influxes in gill arches when bathed in $CaCl_2$ solution, physiological saline and artificial sea water, all contained 10 mM calcium but differed in osmolality, the calcium concentration of the perfusate increased 8.9, 7.9 and 12.1%, respectively, compared to the perfusion fluid (Fig. 2). There were no significant differences among the bathing media. When CS extract was added to the perfusion fluid (1 mgCS/ml), increases of the calcium concentration of perfusates were only 2.6, 2.8 and 6.0%, respectively, showing the inhibitory effect of the CS extract on the branchial calcium influx regardless of osmolalities of the bathing media (Fig. 2).

The effect of an intraperitoneal injection of the CS extract (50 μgCS/g BW) on the plasma calcium level is presented in Fig. 3. The injection tended to lower the plasma calcium concentration for 4 hours, but showed no significant differences. The gill calcium influx was measured by perfusion of gills removed after the injection (Fig. 4). A significant decrease of the gill calcium influx was shown 2 hours after the injection.

The dose effect of CS extract added to perfusion fluid was examined using 10

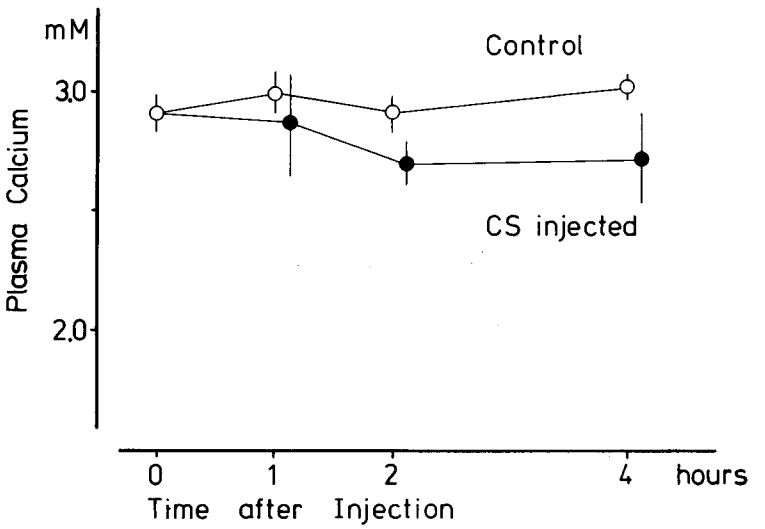

Fig. 3. Changes of plasma calcium concentration after intraperitoneal injection of CS extract (50 μgCS/g BW) or saline (control). Values represent mean±S.E.M., n=6.

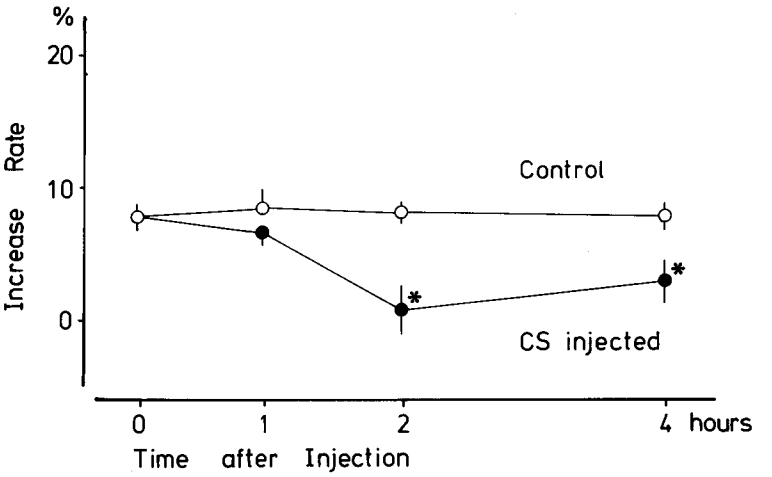

Fig. 4. Changes of branchial calcium influx in the gills taken at different times after intraperitoneal injection of CS extract (50 μgCS/g BW). Values represent mean$\pm$S.E.M., n=6.

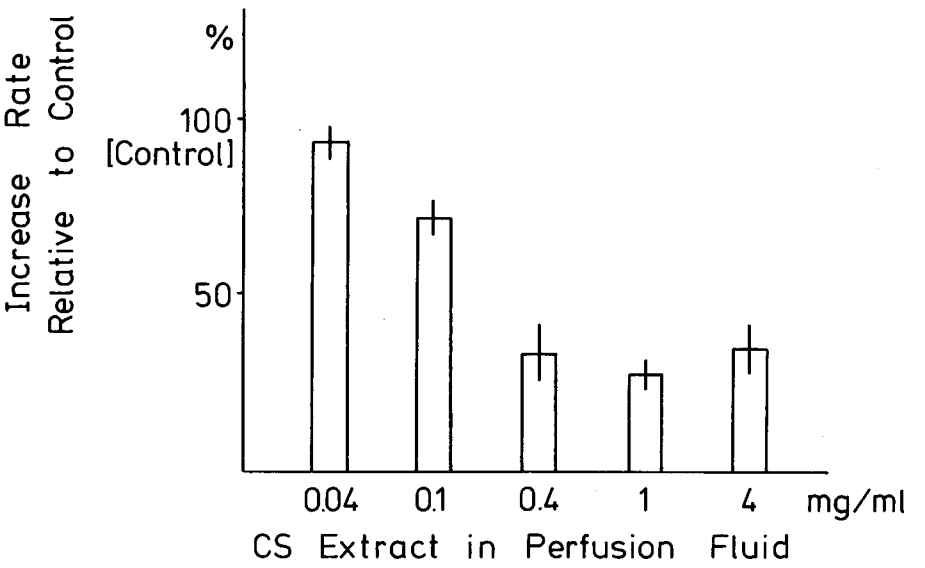

Fig. 5. Effect of different doses of CS extract in perfusion fluid on the branchial calcium influx rate relative to control. Values represent mean$\pm$S.E.M., n=6.

mM $CaCl_2$ solution as the bathing medium (Fig. 5). The calcium influx was inhibited significantly when the dose was more than 0.1 mgCS/ml; the rate of calcium influx inhibition in the same gill arches was found to be dose dependent in a range from 0.04 to 1 mgCS/ml.

Discussion

Hypocalcemic effects of the corpuscles of Stannius have been assayed by changes of plasma calcium concentration or uptake of ^{45}Ca in fish to which CS extracts were administered. Pang et al. (1974) used killifish adapted to calcium deficient seawater and fed a low-calcium diet for 6 weeks and observed serum calcium levels. This bioassay was feasibly sensitive, and linear log-dose-related responses were obtained with CS extracts from killifish and cod. However, it requires four daily injections, is time-consuming, and above all, a handy supply of killifish is essential. Fenwick (1982) used a bioassay based on the effect of CS principles to prevent the development of poststanniectomy hypercalcemia. In this bioassay, daily intraperitoneal injections of CS preparation, equivalent to 1 CS (1 mg), into stanniectomized eels in fresh water for 2 weeks were found to be effective.

In the present study, a significant decrease in gill calcium influx was observed in perfused gills of rainbow trout after intraperitoneal injection of salmon CS extract (50 μgCS/g BW), despite the fact that there was no significant changes in plasma calcium concentration. It is, therefore, considered that the calcium influx in isolated gills is more sensitive to the CS than plasma calcium concentration.

In fresh water, calcium influx through the gills takes place against an uphill gradient (0.1–0.5 mM vs 2.5 mM). Calcium-45 has been usually used for measurement of isolated gill calcium uptake. Milet et al. (1979) studied the effects of CS extract on ^{45}Ca influx and outflux through the gills. Using isolated perfused gills of eel, Fenwick and So (1974) and So and Fenwick (1977) have shown a hypercalcemia and an increase of ^{45}Ca influx after stanniectomy. So and Fenwick (1979, 1982) reported that injection of CS extract or its addition to the perfusion fluid reduced ^{45}Ca influx in the stanniectomized eel, but dose dependence effects were not presented. Wagner et al. (1986) isolated "teleocalcin" based on its inhibitory effect on ^{45}Ca uptake *in toto* in juvenile rainbow trout. The problem in this bioassay was that the ^{45}Ca uptake rate was not consistent in controls. They reported that the inhibitory effect of teleocalcin was only observed at high rates of Ca uptake, that varied possibly due to rhythmic secretion of teleocalcin. In the present study, the calcium influx rate measured in bathing medium containing 10 mM Ca was very high compared with that of Milet et al. (1979) because of a high calcium gradient. It is expected that the calcium influx rate under such a high gradient could not be affected by physiological conditions of material fish.

The calcium influx rate increased with the calcium concentration of the bathing medium. However, the measured influx through the gills may represent not only a diffusional process but also a positive calcium uptake mechanism. The increased influx was reduced about 70% by addition of CS extract in the perfusion fluid. This effect is in agreement with the result of ^{45}Ca uptake in isolated gills by So and Fenwick (1979) and in juvenile rainbow trout by Wagner et al. (1986). In the present study, a dose of 0.1 mg salmon CS/ml showed a significant decrease of gill calcium influx and a maximal inhibitory effect was obtained at doses more than 0.4 mg/ml. Wagner et al. (1986) observed a dose of teleocalcin that significantly decreased calcium influx was only 0.02 μg/g BW. Teleocalcin separated in their experiment was 0.38% wet weight of the CS tissue. This means that 1 mg of CS tissue yields 3.8 μg of teleocalcin. By comparision, the present method is less

sensitive than that used by Wagner et al. (1986). However, in terms of consistency to the effect of CS extract, the present method can be a useful bioassay system. Further studies are needed to validate this method by testing the effects of purified materials.

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