Stanniocalcin in the seawater salmon: structure, function, and regulation

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Stanniocalcin (STC) is a homodimeric glycoprotein hormone that has so far been found in fishes and mammals and is likely to be present in all vertebrates. The hormone was first discovered in fish, where gene expression is confined to unique endocrine glands known as the corpuscles of Stannius (CS). In mammals, the gene is much more widely expressed, and mammalian STC probably functions as a paracrine regulator. However, the role of STC in mammals has not yet been resolved, whereas the fish hormone is known to play an integral role in Ca$^{2+}$ transport and renal phosphate excretion to restore normocalcemia. In this report, we have examined STC in seawater salmon. We have studied the distribution of STC protein and mRNA in marine Atlantic salmon CS cells, the responsiveness of these cells to Ca$^{2+}$, and some physical properties of the hormone. Our results demonstrated that all Atlantic salmon CS cells expressed the STC gene. Furthermore, these cells exhibited a Ca$^{2+}$ sensitivity that was remarkably similar to those in freshwater salmon in terms of its ability to stimulate STC secretion and gene expression. When Atlantic salmon glands were fractionated by concanavalin A (ConA)-Sepharose chromatography, two distinct forms of the hormone were identified, both of which were recognized by sockeye salmon STC antiserum, and designated as STC1 and STC2. STC1 was a glycosylated, 42-kDa disulfide-linked dimer, with a high affinity for ConA. STC2 did not bind to ConA, was 44 kDa in size, and had a different subunit structure. STC2 was also a less effective inhibitor of gill Ca$^{2+}$ transport in fish. Collectively, the results suggest that there is a second form of STC in salmon.

Atlantic salmon; calcium; regulation; mRNA

MATERIALS AND METHODS

Tissue Source

Fresh, marine Atlantic salmon CS tissue was obtained from adult, ranched salmon (4–10 kg) on arrival at the Brown’s Bay Fish Packing Plant (Campbell River, BC, Canada). Tissue was either frozen directly on dry ice or fixed overnight in 4% paraformaldehyde containing 0.15 M NaCl and buffered to pH 7.4 with 0.01 M sodium phosphate. Fixed tissue was embedded in paraffin, and sections were cut at a thickness of 4 μm and mounted on microscope slides. Some CS tissue was also collected under sterile conditions into ice-cold tissue culture medium and transported by air back to the laboratory for primary culture.

Histological Characterization of Atlantic Salmon CS Glands

Immunocytochemistry. Immunocytochemistry was conducted as previously described using a well-characterized antibody to sockeye salmon STC (29). Tissue sections were dewaxed, hydrated, and equilibrated in antibody diluent buffer (0.05 M Tris, pH 7.5, containing 0.9% NaCl). The sections were treated with 0.5% H$_2$O$_2$ in diluent buffer for 30 min to reduce endogenous peroxidase activity, washed, and incubated overnight at 4°C in a 1:1,000 dilution of STC antiserum. The slides were washed in diluent buffer and incubated in a 1:100 dilution of peroxidase-coupled goat anti-rabbit IgG. Counterstaining was performed with hematoxylin or Fast Green FCF.
anti-rabbit immunoglobulin G for 30 min. After a further wash in diluent buffer as before, the sites of antibody binding were visualized in a solution of 0.01% 3’, 3-diaminobenzidine and 0.01% H2O2. Tissue sections were counterstained in hematoxylin, dehydrated, and mounted. Controls included the application of nonimmune rabbit serum and primary antiserum preabsorbed with STC (30 µg/ml) in lieu of primary antiserum alone.

In situ hybridization. To localize STC mRNA at the cellular level, in situ hybridization was carried out on fixed tissue sections. In previous studies, we have used 35S-labeled riboprobes to localize STC mRNA in salmon CS glands (24). In the present study, we used 32P-labeled, sense and antisense oligonucleotides as probes instead. Probes were synthesized based on nucleotides 473–489 of the published cDNA sequence of coho salmon STC (5’ C AGT AGA CTG GAC ATC T 3’) encoding amino acids 97–101 of the mature hormone (30). The oligos were labeled on the 5’-end with [32P]ATP (7,000 Ci/mmol, ICN). In a 30-µl reaction, 1.5 µl of T4 polynucleotide kinase (Pharmacia) and 1.25 µl of labeled ATP were used to label 300 ng of DNA at 34.5°C. This hybridization temperature was calculated to be 15°C below the melting temperature (Tm) according to the following formula, 

\[ T_m = 16.6 \log [M] + 0.41 \log (P_{GC}) + 81.5 - P_M - [B/A] - 65[P_F] \]

where M is NaCl concentration, PGC is percent GC content (53%), PM is percent base-pair mismatch, B is a constant equal to 675, L is probe length, and PF is percent formamide (7). The percent mismatch between the oligonucleotide probe and the corresponding nucleotide sequence of Atlantic salmon STC mRNA was assumed to be 6%, or 1 bp. Formamide was omitted from the hybridization solution. After the prehybridization period, 80 µl of hybridization solution containing 104 counts/min (cpm)/µl of sense (control) or antisense (experimental) probe were added to each slide. The sections were covered with silanized coverslips and incubated at 34.5°C overnight.

The next day, slides were washed in four changes of 5x SSC and two changes of 1x SSC for 10 min at room temperature and twice for 30 min under high-stringency conditions according to the formula above in 0.15x SSC at 37°C. Slides were then dehydrated in graded ethanol containing 0.3 M ammonium acetate, dipped in nuclear track emulsion (diluted 1:1 and containing 0.3 M ammonium acetate), and stored in light-tight boxes under desiccant at 4°C. Slides were developed in Kodak D19 developer after 7–14 days, counterstained with hematoxylin and eosin, and mounted.

Effects of Ca2+ on STC Secretion and mRNA Levels in Cultured CS Cells

The preparation, culture, and experimental use of salmon CS cells has already been described in detail (9, 36). Briefly, Atlantic salmon glands were transported on ice to the laboratory, where residual fat and renal tissue were removed under a dissecting scope. The glands were then teased apart with fine forceps and digested overnight at 4°C in Leibovitz medium (L-15) (18) containing antibiotics and 0.5% trypsin. Freshly prepared cells were seeded in L-15 containing 10% fetal bovine serum plus antibiotics (100 U/ml each of penicillin and streptomycin) at a density of 0.5 x 10^6 cells/ml in 24-well plates. Cell viability was estimated to be 89% by trypan blue exclusion. The cells were maintained in a normal atmosphere at 15°C and allowed to attach for 3 days. Immediately before experimental use, the cells were washed twice in serum-free L-15 containing 0.1% BSA to remove traces of fetal bovine serum and maintained in the same medium for the duration of the experiment. CaCl2 was then added to the cells from 100-fold concentrates to achieve the final desired concentrations (0.7–2.7 mM CaCl2). The actual levels of ionic Ca2+ in the final media were not measured. However, in earlier work, when ionic Ca2+ levels were measured after Ca2+ additions, we have found that they agreed quite well (9, 36). Each concentration of Ca2+ was tested on triplicate wells of cells for periods of 1 and 3 days.

Tissue culture medium was washed and stored at −70°C for subsequent analysis of STC content by radiomunnoassay (37). Total RNA was isolated from each well of cells according to Chomczynski and Sacchi (6) and resolved on 1% agarose-formaldehyde gels. The resolved RNA was transferred to nylon membrane (Hybond N, Amersham) by capillary action and cross-linked by ultraviolet irradiation. After a 2-h prehybridization period, the membrane was hybridized overnight sequentially to random-primed, 32P-labeled salmon STC and 18S ribosomal RNA cDNA probes as previously described (9, 36) under conditions of high stringency (50% formamide, 6x SSC, 125x Denhardt’s solution, 100 µg/ml salmon sperm DNA, and 0.1% SDS at 42°C). Blots were washed four times for 15 min in 2x SSC-0.1% SDS, followed by twice for 30 min in 0.1x SSC-0.1% SDS at 65°C. After exposure to X-ray film, individual STC and 18S RNA bands were quantified by scanning densitometry and expressed as STC-to-18S mRNA ratios. These ratios were expressed as percentages for graphical display, using message levels in the control cells (1.1 mM CaCl2) as the 100% baseline value. For statistical testing, the raw data were subjected to ANOVA followed by Dunnett’s test, again using cells in 1.1 mM Ca2+ as the controls. Groups were considered to be significantly different from controls at P < 0.05.

Fractionation and Characterization of Atlantic Salmon CS Glands

Frozen CS tissue was extracted exactly as described for the isolation of sockeye and coho salmon STC (32, 35). Briefly, frozen glands (4.5 g) were extracted in 10 vol of ice-cold 0.05 M acetic acid and centrifuged at 50,000 g for 1 h, and the supernatant was lyophilized. The resulting powder was dissolved in concanavalin A (ConA)-Sepharose column buffer (0.05 M Tris·HCl, pH 7.5, containing 0.5 M NaCl and 1 mM CaCl2) and recycled three times through a 10 cm column of ConA equilibrated in the same buffer. The protein fraction that did not bind to the column was saved (ConA void). After an extensive buffer wash, the bound fraction was eluted with 0.5 M methyl-α-mannoside in column buffer (ConA bound). The bound and void fractions were dialyzed and lyophilized. We attempted to purify both fractions further by cationic-exchange chromatography on CM Sepharose, again as previously described (32, 35). ConA-bound and void protein powders were each dissolved in cationic-exchange buffer (0.05 M sodium acetate, pH 4.7), centrifuged to remove undissolved material, and applied to a 1 × 20 cm column of CM Sepharose equilibrated in the same buffer. The column was washed with at least 2 vol of starting buffer and then developed with a 0–0.5 M linear gradient of NaCl in column buffer (100 ml/side) to elute bound proteins.
The major protein peaks eluting from the column were pooled, dialyzed, and lyophilized. Amino acid and NH\textsubscript{2}-terminal analyses were performed on the CM Sepharose-purified material using an Applied Biosystems amino acid analyzer (model 119 CL) and an Applied Biosystems gas-phase automatic Sequenator (model 470).

Protein fractions were subjected to SDS-PAGE and Western blot analysis using buffers and washing conditions as previously described (29) and a 1:1,000 dilution of the same salmon STC antiserum. An alkaline phosphatase detection system was used to reveal STC immunoreactive proteins. As a staining control, identical blots were treated with preimmune rabbit serum from the same rabbit used to prepare the antiserum.

An established STC bioassay was used to monitor the effects of the CM Sepharose-purified, bound and void fractions on gill Ca\textsuperscript{2+} transport in rainbow trout. The bioassay monitors the inhibitory effects of STC on whole body \textsuperscript{45}Ca uptake, which is considered to be a reliable indicator of GCAT in fish (38). For an experiment, groups of 10 fingerling trout were given single intraperitoneal injections of the bound and void fractions (2-10 mg/kg body wt). The control group in each experiment received solvent alone. After injection, each group of fish was placed in individual tanks of \textsuperscript{45}Ca water. Two hours later, the fish were killed in a 0.25% solution of benzocaine, washed in 0.1 N HCl for 10 min to remove externally bound isotope, weighed, and ashed overnight in crucibles at 600°C. The total isotope content of each fish was determined by scintillation counting of the dissolved ash. The rate of GCAT for each fish was calculated on the basis of the isotope content of the fish and the specific activity of the water and was expressed as micromoles of Ca\textsuperscript{2+} per kilogram of body weight per hour (38). The data were subjected to ANOVA followed by Dunnett’s test. Groups were considered to be significantly different from solvent-injected controls at P < 0.05.

**RESULTS**

**Histological Characterization of Atlantic Salmon CS Glands**

The CS glands in adult Atlantic salmon ranged in size from 0.2 to 1.0 cm in diameter, and their histological features are illustrated in Fig. 1, A–H. In most instances, individual glands were made up of a single body or corpuscle (Fig. 1C). However, some of the larger glands were made up of a number of smaller corpuscles that had coalesced together into a single gland (Fig. 1, B and H). Some glands had one face exposed on the surface of the kidney (Fig. 1B), whereas others were buried entirely in kidney tissue (Fig. 1, C and F). At higher magnification, it was apparent that the glands were composed of sheets of cells that were columnar in shape and arranged in rows bordering the capillaries (Fig. 1D).

Immunocytochemical staining of the glands with salmon STC antiserum is featured in Fig. 1, A–D. The use of preimmune serum or primary antiserum preabsorbed with salmon STC produced no staining of the CS or surrounding kidney tissue (Fig. 1A), whereas antiserum alone yielded strong, specific staining of CS parenchymal cells (Fig. 1, B–D). There was no evidence of staining in kidney tissue. Within the CS cells, the immunoreactive protein was generally concentrated apically, adjacent to the capillaries in apparent readiness for release (Fig. 1D). Most, if not all, CS parenchymal cells were positively stained by the antiserum.

The results of the in situ hybridization with \textsuperscript{32}P-labeled oligonucleotide probes are shown in Fig. 1, E–H. Figure 1E shows a CS gland surrounded by kidney tissue after hybridization with a sense probe and in which there is no specific staining of kidney or CS tissue. Figure 1F contains the section adjacent to that in Fig. 1E after hybridization with antisense probe and shows strong, specific staining of the CS gland. The silver grains are concentrated heavily over the CS cells and are virtually absent over surrounding kidney tissue. It was also apparent that the cells on the outer edges of the gland in Fig. 1F did not express the gene at the same high level of intensity as those in the center of the gland. However, regional disparity of gene expression was not always evident (see Fig. 1G). Some glands were made up of a heterogeneous collection of variable-sized corpuscles, all of which expressed the gene at different levels of intensity (Fig. 1H). The virtual absence of any gaps in the hybridization signal over the CS tissue suggested that most, if not all, cells expressed the STC gene.

**Effects of Ca\textsuperscript{2+} on STC Secretion and STC mRNA Levels in Cultured CS Cells**

When we examined the effects of Ca\textsuperscript{2+} on cultured Atlantic salmon CS cells, the cation had concentration-dependent effects on both STC secretion and gene expression. The effects of a 1-day exposure to Ca\textsuperscript{2+} on secretion are shown in Fig. 2. Ca\textsuperscript{2+} had stimulatory effects on secretion between 1.1 and 2.3 mM and caused a maximal twofold increase in hormone output. The effects were statistically significant for cells maintained in 1.9 (P < 0.05), 2.3, and 2.7 mM Ca\textsuperscript{2+} (both P < 0.01) compared with control cells in 1.1 mM Ca\textsuperscript{2+}. The response to Ca\textsuperscript{2+} plateaued between 2.3 and 2.7 mM Ca\textsuperscript{2+}.

As in the case of other salmonids (28), Atlantic salmon CS glands contained a single STC transcript that was roughly 2 kb in length (not shown). Furthermore, Ca\textsuperscript{2+} had dose-related, stimulatory effects on STC mRNA levels in cultured CS cells (Fig. 3). A 1-day exposure to Ca\textsuperscript{2+} produced modest, stepwise increases in mRNA levels that achieved statistical significance in cells exposed to 1.9 (P < 0.05), 2.3, and 2.7 mM Ca\textsuperscript{2+} (both P < 0.01). One-day exposures produced a maximal 1.8-fold rise in message levels compared with cells in 1.1 mM Ca\textsuperscript{2+}. A 3-day Ca\textsuperscript{2+} exposure produced a maximal 3.5-fold rise in message levels compared with controls in 1.1 mM Ca\textsuperscript{2+} (Fig. 3). The effects of a 3-day Ca\textsuperscript{2+} exposure were also dose related and statistically significant for cells maintained in 1.9, 2.3, and 2.7 mM Ca\textsuperscript{2+} (P < 0.05–0.01).

**Fractionation and Characterization of Atlantic Salmon CS Glands**

In all previously reported isolations of salmon STC using ConA-Sepharose chromatography, the hormone
Amino acid analysis revealed only minor differences in exposure (2-fold stimulation). Each bar represents mean ± SE of 3 wells of cells. *P < 0.05; **P < 0.01 (ANOVA and Dunnett's test).

has consistently partitioned into the bound fraction (17, 32, 35). However, the fractionation of Atlantic salmon glands on ConA-Sepharose yielded evidence of two different forms of the hormone, one that bound to ConA (bound STC or STC1) and one that did not (void STC or STC2). Both were characterized as “STC-related proteins” on the basis of their common cross-reactivity to salmon STC antiserum (see below). Both STC1 and STC2 were fractionated further on CM Sepharose in an attempt to achieve a sufficient level of purity for sequence analysis. Each had slightly different elution characteristics on CM Sepharose, presumably due to differences in overall charge. STC1 eluted as one major peak between 0.15 and 0.25 M NaCl (Fig. 4A), whereas STC2 eluted as a broad peak with an asymmetric leading edge (Fig. 4B, 1st run). When the fractions containing STC2 from the first run (fractions 14–22) were pooled and rechromatographed on the column, they eluted as one peak between 0.24 and 0.28 M NaCl (Fig. 4B, 2nd run). However, CM Sepharose chromatography step did not improve substantially on the purity of either preparation, since we were unable to obtain dear NH2-terminal sequence information. Amino acid analysis revealed only minor differences in their relative composition, the most notable being that the lysine content of STC2 was twice as high on a mole percent basis (Table 1), which could explain its tendency to be more strongly retained by the cationic exchanger. From 8.95 g of starting material, we obtained 80 mg (0.9% yield) of partially purified STC1 and 15 mg of partially purified STC2 (0.17% yield) after CM Sepharose chromatography. Hence STC1 was clearly the more abundant of the two forms.

The electrophoretic analysis of STC1 and STC2 after CM Sepharose chromatography is shown in Fig. 5. After SDS-PAGE under nonreducing conditions, STC1 (bound STC) was resolved as one major band of 42 kDa, whereas the principal component of STC2 (void STC) was 44 kDa. STC2 also contained minor contaminants of ~14 kDa (Fig. 5a). The major bands in STC1 and STC2 cross-reacted strongly with antibodies to sockeye salmon STC (Fig. 5b). Additional low-molecular-mass contaminants that cross-reacted with the antiserum were also revealed by Western blot analysis (Fig. 5b). Staining of all bands was abolished when the antiserum was preabsorbed with salmon STC before use (not shown). In addition to differences in overall size, SDS-PAGE analysis revealed differences in subunit structure. After chemical reduction with β-mercaptoethanol, STC1 split into two closely spaced bands of 22 and 24 kDa, whereas STC2 broke up into four to five closely spaced bands ranging in size from 21 to 33 kDa (Fig. 5c).

The common cross-reactivity of STC1 and STC2 to salmon STC antiserum suggested that both fractions contained STC-related proteins and this was borne out in the bioassay results (Fig. 6). Both fractions inhibited GCAT in rainbow trout, although not to the same extent. Greater amounts of STC2 were required to achieve a significant reduction in GCAT, whereas STC1 inhibited GCAT to a greater extent and at much lower dosages (2 mg/kg STC1 vs. 10 mg/kg STC2; Fig. 6).

DISCUSSION

In this report, we have examined the CS glands in seawater-adapted Atlantic salmon and the regulation of STC protein and gene expression by the cells and delineated some physical and chemical properties of the hormone. The study was intended to complement previous work on STC physiology in freshwater salmon and add to our understanding of its comparative function in the two aquatic milieu. The CS glands in freshwater Atlantic salmon were described in detail some 20 years ago in animals during the upstream spawning migration (2, 13), a description that is equally
applicable to the seawater glands examined in the present study. We found that a typical adult salmon had up to 10 individual glands varying in size from 1 to 10 mm distributed throughout the central mesonephros. At the light-microscopic level, the glands consisted of lobules of parenchymal tissue traversed throughout by capillaries. A single layer of cells bordered each capillary, and the cell contents were polarized so that the secretory granules containing stored STC were concentrated apically while the nucleus occupied the opposite pole. Most, if not all, CS cells were positively stained for STC protein and mRNA, supporting the view that the CS glands in Atlantic salmon are made up of one endocrine cell type (2, 13), which expresses the STC gene to varying levels of intensity. The only difference that we observed between glands from seawater-adapted Atlantic salmon and those described in freshwater fish was in relation to overall size; the glands were substantially larger in marine fish.

The Ca$^{2+}$ responsiveness of freshwater salmon CS cells has been thoroughly examined in relation to STC synthesis and secretion. At every step in the pathway, increasing the levels of extracellular Ca$^{2+}$ provokes increases in cellular mRNA levels (36), enhances mRNA stability (9), promotes the synthesis of STC from extant mRNA (11), and has potent effects on STC secretion (33, 34, 37). Therefore Ca$^{2+}$ acts as a general stimulus for hormone production and release. The Ca$^{2+}$ sensitivity of seawater CS cells was remarkably similar in magnitude and range of response, in the sense that the responses were concentration dependent within the normal physiological range, plateaued at −2 mM Ca$^{2+}$, and, in terms of overall magnitude, were virtually identical to those observed in cells from freshwater salmon (33, 36). Recent in vivo findings have led us to the same overall conclusion. In a study of freshwater- and seawater-adapted coho salmon, we found that the

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Table 1. Amino acid analysis of ConA bound and void STC

Amino acid analysis was performed on the major peaks eluting from CM Sepharose [run 2 in the case of stanniocalcin 2 (STC2)]. Data are expressed on a mol% basis. Cys and Trp were not determined. ConA, concanavalin A.
STANNIOCALCIN IN SEAWATER SALMON

The Ca²⁺ concentration of the water were hypercalcemia, followed by hypertrophy and hyperplasia of the CS glands. The extent of hyperplasia was such that animals adapted to 5 mM Ca²⁺ (seawater has 10 mM Ca²⁺) underwent a doubling of CS tissue volume over 5 wk (26). As in the case of endocrine neoplasms, the consequences of increased CS tissue mass would be increased hormone output (8). When accompanied by a moderate rise in plasma Ca²⁺ levels, the increase in output would be even more marked. If the hyperplasia scenario outlined above is correct, and the evidence certainly supports it, then this raises an interesting dilemma for the spawning salmon in terms of what to do with these enlarged CS glands on returning to freshwater. The answer may lie in the elegant histological studies that have been carried out on upstream migrating Atlantic salmon, specifically in relation to the changes that occur in gland architecture. Shortly after entering the river, where the Ca²⁺ content is at least 9-fold that of seawater, the CS glands in Atlantic salmon undergo a massive reorganization that becomes more progressive the longer they remain in freshwater (2, 13). The authors of the study characterized this as large-scale cellular necrosis in select regions of the gland and the simultaneous infiltration of an entirely new type of cell, the net effect being destruction of a significant portion of the STC cell population. It therefore appears that a carefully timed program of cell degeneration may be involved in reversing the glandular hyperplasia and in reducing the mass of the glands to the size required for Ca²⁺ homeostasis in a freshwater environment. The new cells infiltrating the glands have not been studied in greater detail since the original study, but they may very well constitute a CS stem cell population that is ultimately recruited to support a new round of glandular hyperplasia when the postspawning salmon returns to sea.

ConA-Sepharose has been employed in the purification of at least three STCs because of its high affinity for the STC carbohydrate moiety. This makes it relatively easy to isolate the hormone to >80% purity from a crude gland extract in a single chromatographic step (17, 32, 35). Atlantic salmon CS glands were no exception in this case, since they also contained a form of STC with a high affinity for ConA-Sepharose. The ConA bound form of Atlantic salmon STC, or STC1, was similar in size to the other salmon STCs that have isolated to date and also had the structure of a disulfide-linked dimer (32, 35). Furthermore, it is likely that both of the monomeric subunits are identical gene products based on the fact that all of the ConA bound forms of salmon STC have proved to be homodimers (17, 32, 35) and that their size difference is due to posttranslational modifications. Of potentially greater interest, however, was the evidence for a second form of STC, STC2, that did not bind to the column, was larger in size and had a different subunit composition. The absence of lectin binding activity in STC2 suggests that it is differentially glycosylated or not glycosylated at all, something that can be resolved in future studies by analyzing the carbohydrate moiety. Similarly, the difference in size

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**Fig. 5.** SDS-PAGE and Western blot analysis of ConA bound and void STC after CM Sepharose chromatography (5 µg/lane in a–c). a: SDS-PAGE of bound and void STC in absence of β-mercaptoethanol (−). Note size difference between bound (42-kDa STC1) and void STC (44-kDa STC2). b: Western blot analysis of bound and void STC in absence of β-mercaptoethanol (−) showing that major bands in both preparations cross-reacted with salmon STC antiserum. c: SDS-PAGE of bound and void STC in presence of β-mercaptoethanol (+); 42-kDa band in bound STC1 was cleaved into 22- and 24-kDa bands, whereas 44-kDa band in void STC2 was cleaved into at least 4 bands of 21–33 kDa.

**Fig. 6.** Bioassay of ConA bound STC (STC1) and ConA void STC (STC2) in rainbow trout. Both preparations significantly inhibited gill Ca²⁺ transport (GCAT). However, ConA bound STC was dearly more potent. Each bar represents mean ± SE of 10 animals. *P < 0.05; **P < 0.01 (ANOVA and Dunnett’s test).
between STC1 and STC2 could reflect differences in polypeptide chain length or complexity of the carbohydrate moiety (27). The two forms of the hormone also shared antigenic determinants in common and hence are both STC related. Other than this, however, we can make no additional inferences as to their compositional differences.

The existence of a second form of STC would not be unprecedented in view of the tetraploid karyotype of salmonids, and yet it could have important implications for STC biology. STC2 may regulate entirely different functions and may be the long-sought-after pressor substance that is reputed to exist in CS glands (5). For these reasons, it is imperative that both forms are purified to homogeneity and fully characterized to establish if they are indeed the products of separate genes. In this regard, it is interesting to note that a second form of STC has recently been reported in mammals (15). A final comment is also warranted on their inhibitory effects on GCAT. Nonetheless, it is important that these results are judged in light of the differences.

We are indebted to Dr. Hugh Bennet, Director of the Endocrine Laboratories, Royal Victoria Hospital, Montreal, for assistance with the sequence analysis. We also thank Lyle F. Wagner for technical assistance and the Brown’s Bay Fish Packing Plant, Campbell River, BC, for the use of their facilities in collecting CS tissue.

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REFERENCES


