INDICATIONS FOR TWO BIOACTIVE PRINCIPLES IN THE CORPUSCLES OF STANNIUS

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Summary

For a long time it was thought that the corpuscles of Stannius (CS) of holostean and teleostean fishes produce a single hormone reducing Ca\(^{2+}\) influx from the water via the gills. We here present data showing that two separate bioactive principles are present in the CS: stanniocalcin (STC), a 56 kDa glycoprotein, and teleocalcin (TC), a 3 kDa glycopeptide. STC indeed inhibits Ca\(^{2+}\) influx (as reported many times before) but does not affect the Ca\(^{2+}\)- and Mg\(^{2+}\)-dependent phosphatase activity located in the gill plasma membrane. TC does not affect Ca\(^{2+}\) influx but inhibits the Ca\(^{2+}\)- and Mg\(^{2+}\)-dependent phosphatase activity. Thus, the Ca\(^{2+}\)- and Mg\(^{2+}\)-dependent phosphatase activity appears not to be involved in transbranchial Ca\(^{2+}\) transport. We conclude that STC is the pivotal calcium-regulating hormone in fish and that TC has an as yet unidentified role in gill physiology through its phosphatase-reducing activity.

Introduction

The corpuscles of Stannius (CS) are endocrine organs found exclusively in holostean and teleostean fish. Studies with several species (killifish, trout, salmon, eel, goldfish) have shown that a product from the CS has hypocalcaemic effects in fish (Wendelaar Bonga and Pang, 1986). Presently, there is a consensus that the major protein from the CS, ‘stanniocalcin’ (STC), is a 39 kDa to 60 kDa glycoprotein that represents the hypocalcaemic principle. In earlier studies, various products were isolated from the glands and were suggested to be the hypocalcaemic principle (Fenwick, 1982; Ma and Copp, 1978; Pang et al. 1981; Wendelaar Bonga et al. 1985). In a study on salmon CS, Ma and Copp (1978) isolated a glycopeptide of 3 kDa, which they considered to be the active principle of the CS, and named it teleocalcin (TC).

When STC (originally termed ‘hypocalcin’) is isolated from CS extracts (Lafeber et al. 1988b), molecules with a molecular mass smaller than 5 kDa are normally discarded. In the present study, however, we also purified a 3 kDa glycoproteinaceous product from CS extract that appeared TC-like. We compared the bioactivity of this TC and STC.

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Salmon TC (sTC) inhibits a Ca\(^{2+}\) (or Mg\(^{2+}\))-dependent phosphatase (CaP\(_{\text{ase}}\)). This activity was erroneously proposed to be the driving force for Ca\(^{2+}\) uptake (Ma et al. 1974; Ma and Copp 1978). Later it was argued and demonstrated by Flik et al. (1984) that a high-affinity Ca\(^{2+}\)-ATPase is responsible for transcellular Ca\(^{2+}\) uptake. Ma and Copp (1982) claimed that sTC reduces Ca\(^{2+}\) influx in eels, but this claim is not justified since in their influx experiments CS extracts containing both STC and TC were used. We have examined the effects of STC and TC from two different species, trout and carp, on branchial Ca\(^{2+}\) influx in tilapia and carp. We used CS material from these two species to reduce the risk of measuring some species-specific effect. Furthermore, we tested the effects of TC and STC in the assay for CaP\(_{\text{ase}}\) activity developed by Ma et al. (1974). Trout TC (tTC), trout STC (tSTC) and synthetic hormone fragments of Australian eel STC (eSTC), peptides U, V and W (Butkus et al. 1989), were compared. The CaP\(_{\text{ase}}\) activity was determined on purified basolateral membranes that are essentially free of apical and intracellular membranes (Flik et al. 1985b). The role of the two bioactive principles in branchial Ca\(^{2+}\) handling will be discussed.

Materials and methods

Fish

Tilapia, Oreochromis mossambicus, were obtained from a laboratory stock. Tilapia used for Ca\(^{2+}\) flux studies weighed 60–100 g and those used for gill membrane isolation around 200 g. Fish were held in Nijmegen city tapwater (containing in mmol l\(^{-1}\); 0.8 Ca\(^{2+}\); 0.20 Mg\(^{2+}\), 0.61 Na\(^{+}\), 0.05 K\(^{+}\), 0.66 Cl\(^{-}\), 0.32 SO\(_{4}\)^{2−}, 3.15 HCO\(_{3}\)^{−}, pH 7.2) at 27°C. Common carp, Cyprinus carpio, weighing 70–120 g were obtained from laboratory stock (Agricultural University, Wageningen, The Netherlands) and held in Nijmegen city tapwater at 23°C. Rainbow trout, Oncorhynchus mykiss, were obtained from a commercial dealer in Beek near Nijmegen.

Analytical techniques

The protein contents of cell membrane preparations and cell suspensions were determined with a commercial reagent kit (Biorad) according to the method of Bradford (1976). Concentrations of hormones were quantitated according to the method of Lowry et al. (1951); this method is more suited for the determination of small peptides (≤3 kDa).

Radiotracer activities were determined with a Wallac 1410 liquid scintillation counter (Pharmacia/LKB).

Hormones

Trout STC (tSTC) was purified in two steps. The first step was concanavalin A affinity chromatography as described previously (Lafeber et al. 1988b). In the second step, a size separation was performed with the SMART system (Pharmacia/LKB) on a Superdex 75 HR 10/30 column (high performance gel filtration column) using 50 mmol l\(^{-1}\) ammonium acetate as eluent. This step was found to be more effective in removing low molecular weight contaminants than the previously used method (Lafeber et al. 1988b) of
desalting by ultrafiltration. A Western blot of purified tSTC has been published before (Flik et al. 1990).

Trout and carp teleocalcin (tTC and cTC) were purified from a CS homogenate using a Sephadex G-25 column (fine, Pharmacia; 10 mm × 450 mm, 5–8 mg protein per run, 0.5 ml min⁻¹ elution rate) and ammonium acetate as eluent according to the procedure described by Ma and Copp (1978) for the purification of salmon TC. The major peak, measured spectrophotometrically at 280 nm, was collected (Fig. 1) and freeze dried. The apparent size of the product was around 3 kDa and it contained a carbohydrate moiety, as determined with a glycan detection kit (Boehringer). This teleocalcin has a negative charge (in polyacrylamide gel it runs faster than the front marker) and contains carbohydrates with terminal mannoses as determined with a glycan differentiation kit (Boehringer).

Synthetic fragments of Australian eel, *Anguilla australis*, STC (eSTC) were prepared at the Howard Florey Institute peptide laboratory (Butkus et al. 1989) based on the cDNA amino acid sequence of the mature hormone (Butkus et al. 1987). The following three peptides were used: peptide U (N-terminal 1–20 sequence), peptide V (mid 103–136 sequence) and peptide W (C-terminal 202–231 sequence). Numbering of the amino acids starts at the N terminus of the mature hormone.

**Ca²⁺ influx**

The branchial Ca²⁺ influx was determined as described by Verbost et al. (1989). Fish were placed in opaque Perspex boxes (volume 1.51) and ⁴⁵CaCl₂ (1.0 MBq l⁻¹) was added after the water flow had been stopped. Influx of Ca²⁺ was calculated from the

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**Fig. 1.** Elution pattern of aqueous corpuscle of Stannius (CS) extracts (—) and kidney extracts (dashed line) using gel permeation chromatography (G-25, fine). The STC (void volume) and TC fractions of trout CS extract and the corresponding fractions of trout kidney extract are indicated as vertical lines.
radioactivity accumulated in the fish after 3 h of exposure to $^{45}$Ca (the radioactivity in the entire fish was determined) and the mean $^{45}$Ca specific activity of the water. Ca$^{2+}$ influx ($F_{in}$) data were normalized to fish mass according to Flik et al. (1985a) and expressed in $\mu$mol h$^{-1}$ 100 g$^{-1}$ fish.

Hormones were administered by intraperitoneal injection 1 h before the flux determination. Saline served as vehicle and was used in the controls.

**Isolation of plasma membranes**

The purification of the branchial plasma membranes was carried out as described by Flik et al. (1985b). After quick anaesthesia in Na$_2$CO$_3$-buffered MS-222 (1 g l$^{-1}$, pH 7.4) the gill arches were excised. Branchial epithelium was scraped off with a glass microscope slide and collected in isotonic buffer (containing in mmol l$^{-1}$: 250 sucrose, 12.5 NaCl, 5 Hepes/Tris pH 7.4, 0.1 EDTA and 25 TIU l$^{-1}$ aprotinin, where TIU is one trypsin inhibitor unit). After homogenization in a douncer device with a loosely fitting pestle, cellular debris and erythrocytes were separated from the membranes by centrifugation (550 g, 10 min). The supernatant ($H_0$) was centrifuged (250 000 g, 30 min), yielding a two-layered pellet of mitochondria and membranes. The fluffy layer of the pellet containing the plasma membranes was collected by mild swirling and subsequently resuspended in isotonic sucrose buffer by 100 strokes in a douncer. The suspension was further purified by differential centrifugation: 1000 g, 10 min and 10 000 g, 10 min. Finally, the membranes were pelleted (50 000 g, 20 min) and resuspended in 0.3 mol l$^{-1}$ sucrose for storage (at $-20^\circ$C for up to 14 days without significant loss of cyclase and phosphatase activity). The Na$^+/K^+$-ATPase activity, a marker enzyme for basolateral membranes, was purified 3.9 times in the final pellet compared to the initial homogenate $H_0$ (determined before freezing the membranes). This is in good agreement with the purification (3.8 times) reported by Flik et al. (1985b) using the same isolation procedure.

**Phosphatase assay**

The phosphatase activity of the isolated membranes was determined as described by Ma et al. (1974) with minor modifications. In a differential assay, with or without 5 mmol l$^{-1}$ Ca$^{2+}$ or Mg$^{2+}$, the Ca$^{2+}$/Mg$^{2+}$-dependent release of inorganic phosphate, $P_i$, from Ca$^{2+}$-ATP or Mg$^{2+}$-ATP was determined. Apart from Ca$^{2+}$ and Mg$^{2+}$, the reaction mixture contained 30 $\mu$g ml$^{-1}$ membrane protein, 70 mmol l$^{-1}$ NaCl, 20 mmol l$^{-1}$ Hepes/Tris (pH 8.0), 1 mg ml$^{-1}$ ouabain (to exclude Na$^+/K^+$-ATPase activity) and 3 mmol l$^{-1}$ ATP in a total volume of 350 $\mu$l. Assays were run at 37$^\circ$C for 30 min.

The phosphatase inhibitory activity of CS compounds has been expressed in CS units per milligram (Ma and Copp, 1978), where 1 unit is the amount of CS material that causes 50% inhibition of Ca$^{2+}$-dependent ATP hydrolysis.

**Statistics**

Results are presented as means ± s.d. (unless otherwise stated). For statistical evaluation the Mann–Whitney $U$-test was used. Significance was set at $P \leq 0.05$. 
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Results

Effects of STC and TC on Ca²⁺ influx

Ca²⁺ influx in tilapia is reduced to 52% of the control level by injection of 56 pmol g⁻¹ tSTC; tTC (up to 300 pmol g⁻¹) had no effect (Fig. 2). Carp STC (24 pmol g⁻¹) reduced Ca²⁺ influx in tilapia to 53% of the control level, whereas cTC (up to 200 pmol g⁻¹) had no effect. In carp, cSTC (7 pmol g⁻¹) reduced Ca²⁺ influx to 59%; injection of 40 pmol g⁻¹ cTC was without effect on Ca²⁺ influx (Fig. 3).

Effects of tSTC and tTC on phosphatase

Table 1 shows the effects of CS extracts, tSTC and tTC on the Ca²⁺-dependent phosphatase (CaPase) activity in tilapia gill plasma membranes. Extracts of CS and tTC inhibit CaPase activity, whereas tSTC at concentrations up to 10 μmol l⁻¹ does not. Incubation with tTC decreased CaPase activity with an average specific inhibitory activity of 11.3. It follows from Table 1 that tTC was purified 20.1 times, based on its specific inhibition. Table 1 also shows that phosphate release was decreased by CS extracts when Mg²⁺-ATP or Ca²⁺-ADP was used as substrate. The corresponding controls show that when Ca²⁺ was replaced by Mg²⁺, ATP hydrolysis decreased by 25%. With Ca²⁺-ADP as substrate, CaPase activity reached 62% of the Ca²⁺-ATP value. The synthetic cSTC fragments U, V and W did not affect CaPase activity at concentrations up to 50 μmol l⁻¹ (corresponding to 99 μg ml⁻¹ U, 186 μg ml⁻¹ V and 161 μg ml⁻¹ W).

Discussion

Two major conclusions can be drawn from this study. First, stanniocalcin (STC) inhibits Ca²⁺ influx without affecting the Ca²⁺-dependent phosphatase (CaPase). Second,
Fig. 3. Effects of cSTC and cTC on whole-body Ca\textsuperscript{2+} influx ($F_{\text{inCa}}$) in carp. Values are means (+S.E.M.) for six fish. * indicates a significant ($P<0.05$) difference from the control value.

Table 1. The effects of CS extract on the ATP- and ADP-stimulated CaP\textsubscript{ase} and MgP\textsubscript{ase} activity, and the effects of tTC, tSTC and eSTC fragments on ATP-stimulated CaP\textsubscript{ase} activity, in basolateral membranes from tilapia branchial epithelium

<table>
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<tr>
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<th>Ca\textsuperscript{2+}-ATP</th>
<th>Mg\textsuperscript{2+}-ATP</th>
<th>Ca\textsuperscript{2+}-ADP</th>
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<td>$V_{\text{spec}}$ (I_{\text{spec}})</td>
<td>$V_{\text{spec}}$ (I_{\text{spec}})</td>
<td>$V_{\text{spec}}$ (I_{\text{spec}})</td>
</tr>
<tr>
<td>Control</td>
<td>279.5±21.3</td>
<td>213.0±10.8</td>
<td>172.3±19.2</td>
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<tr>
<td>tCS extract 450 $\mu$g ml\textsuperscript{-1}</td>
<td>234.1±6.4* (0.7)</td>
<td>176.0±11.8* (0.8)</td>
<td>142.7±4.6* (0.8)</td>
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<tr>
<td>tCS extract 1150 $\mu$g ml\textsuperscript{-1}</td>
<td>213.0±19.2* (0.4)</td>
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<tr>
<td>tSTC 275 $\mu$g ml\textsuperscript{-1}</td>
<td>284.4±26.7 (0.0)</td>
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<td>tSTC 575 $\mu$g ml\textsuperscript{-1}</td>
<td>278.8±3.3 (0.0)</td>
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<tr>
<td>tTC 30 $\mu$g ml\textsuperscript{-1}</td>
<td>238.6±11.1* (9.8)</td>
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<tr>
<td>tTC 75 $\mu$g ml\textsuperscript{-1}</td>
<td>150.3±6.7* (12.3)</td>
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<tr>
<td>Fragment U 25 $\mu$mol l\textsuperscript{-1}</td>
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<tr>
<td>Fragment V 1 $\mu$mol l\textsuperscript{-1}</td>
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<tr>
<td>Fragment V 25 $\mu$mol l\textsuperscript{-1}</td>
<td>284.2±22.0 (0.0)</td>
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<tr>
<td>Fragment V 50 $\mu$mol l\textsuperscript{-1}</td>
<td>277.0±19.7 (0.0)</td>
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<tr>
<td>Fragment W 1 $\mu$mol l\textsuperscript{-1}</td>
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<td>Fragment W 25 $\mu$mol l\textsuperscript{-1}</td>
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<td>Fragment W 50 $\mu$mol l\textsuperscript{-1}</td>
<td>277.9±20.2 (0.0)</td>
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</table>

Values are means of five experiments ($\pm$S.D.).

$V_{\text{spec}}$ in $\mu$mol P h\textsuperscript{-1} mg\textsuperscript{-1} protein.

$I_{\text{spec}}$, between parentheses, is the specific inhibitory activity in units mg\textsuperscript{-1}, where 1 unit of (CS) material causes 50% inhibition of CaP\textsubscript{ase} activity (Ma and Copp, 1978).

*Significantly different from the control value ($P<0.05$).
teleocalcin (TC), a glycopeptide of around 3 kDa from trout CS extracts, inhibits CaPjase but does not affect branchial Ca\(^{2+}\) influx. The biochemistry and bioactivity of this CS fraction indicate similarities with the 3 kDa glycopeptide isolated from salmon CS by Ma and Copp (1978). STC is a fast calcium-regulating hormone of the CS, inhibiting branchial Ca\(^{2+}\) influx. Thus, the CaPjase appears not to be involved in the regulation of transcellular Ca\(^{2+}\) influx. An important conclusion from this work is that the CS contain at least two bioactive principles and, therefore, that studies showing the effects of CS extracts on branchial ion handling (Flik, 1990; Mayer-Gostan, 1992) should be re-evaluated.

### Ca\(^{2+}\) influx

Branchial influx of Ca\(^{2+}\) was reduced by tSTC and cSTC, whereas tTC and cTC had no effect. Higher doses of TC than of STC were examined to show that TC did not have an effect on Ca\(^{2+}\) influx, thus taking into account the possibility that TC is a fragment of the active principle and may need a higher dose to produce the same effect. It has been determined, for instance, that an N-terminal fragment of STC inhibits Ca\(^{2+}\) influx in tilapia to a similar extent as the native hormone when it is used at a ten times higher dose than the whole hormone (P. M. Verbost, A. Butkus, P. Willems and S. E. Wendelaar Bonga, in preparation).

To obtain a significant inhibition of influx in tilapia we needed a fairly high dose of tSTC (56 pmol g\(^{-1}\)) compared with the dose that is required to accomplish the same inhibition of Ca\(^{2+}\) influx in trout (10 pmol g\(^{-1}\)). When testing cSTC in carp, 3.5 times lower doses than those needed in tilapia were sufficient to obtain significant inhibition of influx. It is tempting to conclude that there is a species-specificity whereby tilapia and carp STC are more closely related than tilapia and trout STC. However, we cannot exclude the possibility that the different hormone preparations had dissimilar contents of bioactive STC.

### Phosphatase

The phosphatase activity in the plasma membranes from the gills of tilapia is activated by Ca\(^{2+}\) as well as Mg\(^{2+}\). ATP is the preferred substrate for the CaPjase. The activation characteristics match those described previously for CaPjase activity in gill plasma membranes of rainbow trout (Ma et al. 1974) and eel (Flik et al. 1983). The only discrepancy seems to be the almost 30-fold higher specific enzyme activity in tilapia than in trout, which in all probability is explained by the difference in assay temperature (37 °C versus 12 °C). Compared with the phosphatase activity in eel gill plasma membranes measured at 37 °C by Flik et al. (1983) our tilapia value is five times higher.

TC inhibited CaPjases in tissues obtained from fish as well as from other vertebrates (Copp and Ma, 1981): TC (3.8 µg ml\(^{-1}\)) inhibited CaPjase in preparations from dogfish gill, trout gill, chick intestinal mucosa and guinea pig placenta with a comparable degree of inhibition. A smaller but significant reduction of CaPjase activity in a rat kidney preparation was observed with TC. The possible biological function of such a non-specific inhibition awaits further studies on the function of phosphatases in the membranes of tissues involved in Ca\(^{2+}\) transport. The function of phosphatases is unknown for any tissue in which it occurs (Thiede et al. 1988).
It is not known whether TC is released by the CS and no data on plasma levels are available. The possibility that this factor is not released by the glands and is irrelevant for branchial ion regulation should be considered. We did exclude, however, the possibility that the factor originated from kidney tissue, a source of contamination in the collection of CS tissue. Kidney contaminants may be co-purified with CS material and exert effects that would erroneously be ascribed to the CS. However, the kidney extracts did not contain a 3 kDa product corresponding to the CS-TC and none of the kidney compounds reduced the phosphatase activity: trout kidney extract (0.25–1.00 mg ml⁻¹) or its gel permeation fractions corresponding to STC (0.025–0.575 mg ml⁻¹) or TC (0.015–0.100 mg ml⁻¹) from CS extracts had no effect on the CaPᵢase (tested five times, data not shown). This finding also argues against the (remote) possibility that the inhibitory effect is caused by salts that co-elute with TC in the isolation procedure (also see the amino acid composition below).

The CaPᵢase activity is changed neither by tSTC nor by the eSTC fragments U, V or W. Branchial Ca²⁺ influx is reduced by tSTC and fragment U in tilapia and trout (Lafeber et al. 1988a; Milliken et al. 1990; P. M. Verbost, J. Van Rooij, G. Flik, R. A. C. Lock and S. E. Wendelaar Bonga, in preparation: this study). From these results, we conclude that STC does not exert its hypocalcaemic effects through changes in CaPᵢase activity in the gill. It has previously been shown that CaPᵢases cannot be responsible for the translocation of Ca²⁺ across the basolateral membrane because the affinity for Ca²⁺ is too low to be stimulated by intracellular Ca²⁺ concentrations and because the substrate specificity and pH optima are not characteristic for a transport Ca²⁺-ATPase (Flik et al. 1984). We conclude now that the CaPᵢase does not play a role in active branchial Ca²⁺ transport.

Teleocalcin

One could argue that TC is a fragment of the native STC. However, the carbohydrate moiety of TC carries terminal mannoses but no terminal sialic acids as STC does (P. M. Verbost, A. Butkus, P. Willems and S. E. Wendelaar Bonga, unpublished observations, obtained with a Boehringer glycan differentiation kit on dot blots). A polyclonal antibody against tSTC did not recognize TC (tested on dot blot: 1 µg material per dot) but this does not exclude the possibility that TC is a fragment of STC because it may not contain the moiety that is reactive to the antibody.

The biochemical characteristics of tTC and cTC are comparable with those of teleocalcin (Ma and Copp, 1978) from Pacific salmon CS (sTC). Both are protein fractions of CS extracted with ammonium acetate, purified by gel permeation, and show inhibitory actions on the phosphatase. The inhibitory substance resides in the first major peak that elutes from the gel permeation column (after the large amount of high molecular weight proteins in the void volume) and has a specific inhibitory activity of 12 units (Ma and Copp, 1978; this study). Both tTC and sTC contain carbohydrates. The molecular weight determined by analytical gel permeation chromatography was 3 kDa for tTC and cTC (this study), the same as for sTC (Ma and Copp, 1978). An amino acid analysis of the tTC revealed more amino acids than the seven found in sTC (Ala, 4; Asn, 2; Gln, 5; Gly, 5; Ser, 2; Thr, 1; Val, 1; Copp and Ma, 1981), probably because our tTC was not as pure
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as the sTC used for the analysis. However, the ratio of these seven amino acids relative to valine in tTC (Ala, 3.2; Asn, 1.2; Gln, 4.0; Gly, 4.8; Ser, 2.8; Thr, 1.7; Val, 1.0) was similar to that in sTC, indicating that tTC contains the TC sequence. A major difference between tTC and sTC seems to be the effect on Ca²⁺ metabolism. Ca²⁺ influx in tilapia was not influenced by tTC or cTC. Ma and Copp (1978) showed hypocalcaemic effects of purified sTC. They reported that the Sephadex G-50 fraction of salmon CS decreased the total plasma Ca²⁺ concentration in American eels by 14% after four daily injections with 340 pmol g⁻¹ body mass. However, in their experiments CS, not TC, extracts were used to show the inhibition of gill Ca²⁺ uptake, and CS extracts contain both STC and TC. A possible explanation for the hypocalcaemic effect of TC is that it stimulated Ca²⁺ efflux, thus reducing plasma calcium concentration. The hypothesis that should now be tested is whether TC controls a mechanism for the regulation of Ca²⁺ efflux.

Ma and Copp (1978) called the 3 kDa glycopeptide from the Stannius corpuscles TC. Therefore, in retrospect, it was confusing that Wagner et al. (1986) also called the 39 kDa glycoprotein from salmon CS TC; this protein turned out to be homologous with STC (also named ‘hypocalcin’ till 1990), the genuine Ca²⁺-influx-reducing hormone. We suggest that the name TC should be reserved for the 3 kDa moiety, the potentially interesting glycopeptide from the CS.

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References


