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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 56kDa glycoprotein, and teleocalcin (TC), a 3kDa 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 (Wagner *et al.* 1986) to 60kDa (Flik *et al.* 1989) 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 3kDa, 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.* 1988*b*), molecules with a molecular mass smaller than 5kDa are normally discarded. In the present study, however, we also purified a 3kDa glycoproteinaceous product from CS extract that appeared TC-like. We compared the bioactivity of this TC and STC.

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Key words: fish, *Oreochromis mossambicus*, *Cyprinus carpio*, *Oncorhynchus mykiss*, gills, Ca^{2+} transport, phosphatase, stanniocalcin, teleocalcin.

Salmon TC (sTC) inhibits a Ca^{2+} (or Mg^{2+})-dependent phosphatase (CaP_iase). 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_iase 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_iase activity was determined on purified basolateral membranes that are essentially free of apical and intracellular membranes (Flik *et al.* 1985*b*). 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 60–100g and those used for gill membrane isolation around 200g. 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 $HCO_3{}^-$, pH7.2) at 27°C. Common carp, *Cyprinus carpio*, weighing 70–120g 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 ($\leqslant$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.* 1988*b*). 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 $50mmol\,l^{-1}$ 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.* 1988*b*) 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; 10mm×450mm, 5–8mg 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 280nm, was collected (Fig. 1) and freeze dried. The apparent size of the product was around 3kDa 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.

Ca^{2+} influx

The branchial Ca^{2+} influx was determined as described by Verbost *et al.* (1989). Fish were placed in opaque Perspex boxes (volume 1.5l) and $^{45}CaCl_2$ ($1.0 MBq l^{-1}$) was added after the water flow had been stopped. Influx of Ca^{2+} was calculated from the

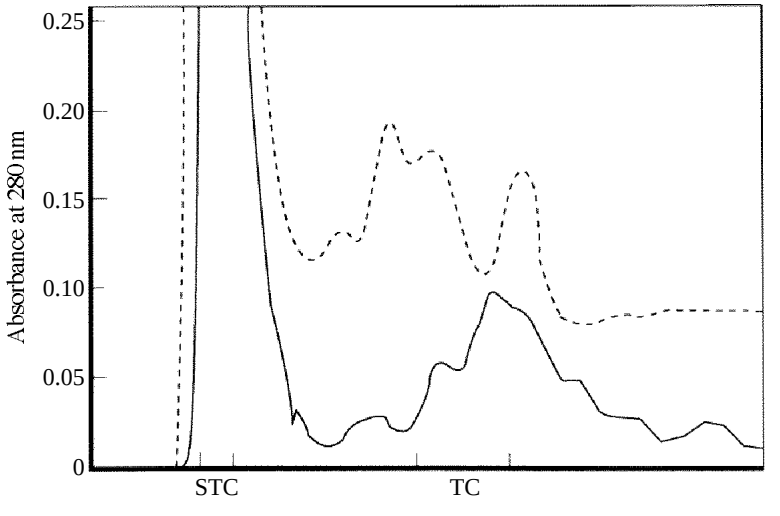

Fig. 1. Elution pattern of aqueous corpuscle of Stannius (CS) extracts (—) and kidney extracts (dashed line) using gel permeation chromatography (G-25, fine). The STC (void volume) and TC fractions of trout CS extract and the corresponding fractions of trout kidney extract are indicated as vertical lines.

radioactivity accumulated in the fish after 3h 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.* (1985*a*) and expressed in $\mu mol\,h^{-1}\,100\,g^{-1}$ fish.

Hormones were administered by intraperitoneal injection 1h 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.* (1985*b*). After quick anaesthesia in Na_2CO_3-buffered MS-222 ($1\,g\,l^{-1}$, pH7.4) 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 pH7.4, 0.1 EDTA and $25TIU\,l^{-1}$ 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***, 10min). The supernatant (H_0) was centrifuged (250000 ***g***, 30min), 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***, 10min and 10000 ***g***, 10min. Finally, the membranes were pelleted (50000 ***g***, 20min) and resuspended in $0.3mol\,l^{-1}$ 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.* (1985*b*) 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, $70mmol\,l^{-1}$ NaCl, $20mmol\,l^{-1}$ Hepes/Tris (pH8.0), $1mgml^{-1}$ ouabain (to exclude Na^+/K^+-ATPase activity) and $3\,mmol\,l^{-1}$ ATP in a total volume of 350 µl. Assays were run at $37^{\circ}C$ for 30min.

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 $P \leqslant 0.05$.

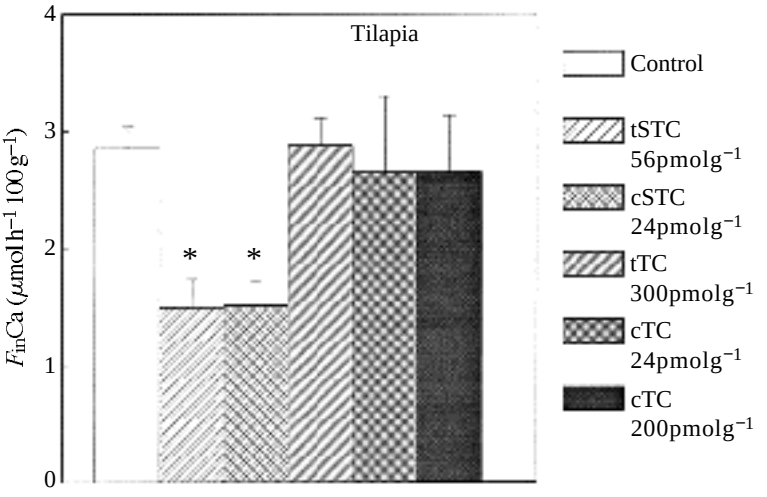

Fig. 2. Effects of STC and TC from trout (t) and carp (c) on whole-body Ca^{2+} influx ($F_{in}Ca$) in tilapia. Values are means (+S.E.M.) for six fish. * indicates a significant ($P<0.05$) difference from the control value.

Results

Effects of STC and TC on Ca^{2+} influx

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

Effects of tSTC and tTC on phosphatase

Table 1 shows the effects of CS extracts, tSTC and tTC on the Ca^{2+}-dependent phosphatase (CaP_iase) activity in tilapia gill plasma membranes. Extracts of CS and tTC inhibit CaP_iase activity, whereas tSTC at concentrations up to 10 μmol l^{-1} does not. Incubation with tTC decreased CaP_iase 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 Mg^{2+}-ATP or Ca^{2+}-ADP was used as substrate. The corresponding controls show that when Ca^{2+} was replaced by Mg^{2+}, ATP hydrolysis decreased by 25%. With Ca^{2+}-ADP as substrate, CaP_iase activity reached 62% of the Ca^{2+}-ATP value. The synthetic eSTC fragments U, V and W did not affect CaP_iase 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 Ca^{2+} influx without affecting the Ca^{2+}-dependent phosphatase (CaP_iase). Second,

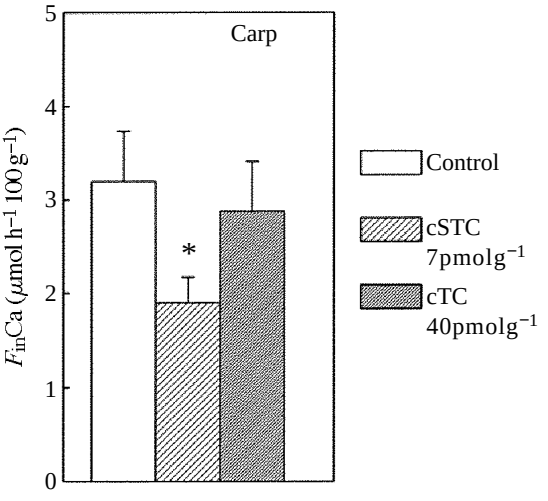

Fig. 3. Effects of cSTC and cTC on whole-body Ca^{2+} influx ($F_{in}Ca$) in carp. Values are means (+S.E.M.) for six fish. * indicates a significant (P<0.05) difference from the control value.

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

		Ca^{2+}-ATP V_{spec} (I_{spec})	Mg^{2+}-ATP V_{spec} (I_{spec})	Ca^{2+}-ADP V_{spec} (I_{spec})
Control		279.5±21.3	213.0±10.8	172.3±19.2
tCS extract	450 μg ml^{-1}	234.1±6.4* (0.7)	176.0±11.8*	142.7±4.6*
	1150 μg ml^{-1}	213.0±19.2* (0.4)	(0.8)	(0.8)
tSTC	275 μg ml^{-1}	284.4±26.7 (0.0)		
	575 μg ml^{-1}	278.8±3.3 (0.0)		
tTC	30 μg ml^{-1}	238.6±11.1* (9.8)		
	75 μg ml^{-1}	150.3±6.7* (12.3)		
Fragment U	1 μmol l^{-1}	277.2±11.5 (0.0)		
	25 μmol l^{-1}	281.6±35.6 (0.0)		
	50 μmol l^{-1}	285.7±11.4 (0.0)		
Fragment V	1 μmol l^{-1}	280.4±10.8 (0.0)		
	25 μmol l^{-1}	284.2±22.0 (0.0)		
	50 μmol l^{-1}	277.0±19.7 (0.0)		
Fragment W	1 μmol l^{-1}	177.6±14.3 (0.0)		
	25 μmol l^{-1}	268.6±16.9 (0.0)		
	50 μmol l^{-1}	277.9±20.2 (0.0)		

Values are means of five experiments (±S.D.).

V_{spec} in μmol P_i h^{-1} mg^{-1} protein.

I_{spec}, between parentheses, is the specific inhibitory activity in unitsmg^{-1}, where 1 unit of (CS) material causes 50% inhibition of CaP$_i$ase activity (Ma and Copp, 1978).

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

teleocalcin (TC), a glycopeptide of around 3kDa from trout CS extracts, inhibits CaP_iase but does not affect branchial Ca^{2+} influx. The biochemistry and bioactivity of this CS fraction indicate similarities with the 3kDa 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 CaP_iase 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 CaP_iase. The activation characteristics match those described previously for CaP_iase 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 CaP_iases in tissues obtained from fish as well as from other vertebrates (Copp and Ma, 1981): TC ($3.8\,\mu g\,ml^{-1}$) inhibited CaP_iase 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 CaP_iase 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 3kDa product corresponding to the CS-TC and none of the kidney compounds reduced the phosphatase activity: trout kidney extract (0.25–1.00mgml^{-1}) or its gel permeation fractions corresponding to STC (0.025–0.575mgml^{-1}) or TC (0.015–0.100mgml^{-1}) from CS extracts had no effect on the CaP_iase (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 CaP_iase activity is changed neither by tSTC nor by the eSTC fragments U, V or W. Branchial Ca^{2+} influx is reduced by tSTC and fragment U in tilapia and trout (Lafeber *et al.* 1988*a*; 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 CaP_iase activity in the gill. It has previously been shown that CaP_iases cannot be responsible for the translocation of Ca^{2+} across the basolateral membrane because the affinity for Ca^{2+} is too low to be stimulated by intracellular Ca^{2+} concentrations and because the substrate specificity and pH optima are not characteristic for a transport Ca^{2+}-ATPase (Flik *et al.* 1984). We conclude now that the CaP_iase does not play a role in active branchial Ca^{2+} 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 3kDa 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

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 Ca^{2+} metabolism. Ca^{2+} 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 Ca^{2+} concentration in American eels by 14% after four daily injections with 340pmol g^{-1} bodymass. However, in their experiments CS, not TC, extracts were used to show the inhibition of gill Ca^{2+} uptake, and CS extracts contain both STC and TC. A possible explanation for the hypocalcaemic effect of TC is that it stimulated Ca^{2+} efflux, thus reducing plasma calcium concentration. The hypothesis that should now be tested is whether TC controls a mechanism for the regulation of Ca^{2+} efflux.

Ma and Copp (1978) called the 3kDa glycopeptide from the Stannius corpuscles TC. Therefore, in retrospect, it was confusing that Wagner *et al.* (1986) also called the 39kDa glycoprotein from salmon CS TC; this protein turned out to be homologous with STC (also named 'hypocalcin' till 1990), the genuine Ca^{2+}-influx-reducing hormone. We suggest that the name TC should be reserved for the 3kDa moiety, the potentially interesting glycopeptide from the CS.

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