Studies on stanniocalcin: characterization of bioactive and antigenic domains of the hormone

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Summary

Stanniocalcin (STC) decreases branchial Ca2+-uptake in fish. In order to determine its bioactive domain, synthetic fragments (U amino acids (aa) 1–20; V aa 103–136; W aa 202–231) of eel STC were tested for their effect on Ca2+ uptake in tilapia (Oreochromis mossambicus). Ca2+ uptake was inhibited by an N-terminal fragment but not by a midfragment nor a C-terminal fragment of the mature hormone. We provide theoretical and experimental evidence that a midportion of STC, which is included in the synthetic fragment V, is the most antigenic site of the molecule. Polyclonal antibodies against stanniocalcin are directed against this midportion although this region of STC appears not to be essential for signal transduction. These results suggest that the currently available antibodies will recognize inactive STC fragments in the circulation. We conclude that the bioactive portion of STC does not correspond with the major antigenic portion of the hormone. The results imply that studies on plasma STC levels employing a polyclonal antiserum against STC should be interpreted with care.

Introduction

The corpuscles of Stannius (CS) are unique endocrine glands in bony fishes, associated with the kidneys. They produce the glycosylated hypocalcemic hormone stanniocalcin (Flik, 1990). It is generally accepted that stanniocalcin (STC) is an inhibitor of calcium uptake in fish (Wagner et al., 1986; Lafeber et al., 1988a). Lafeber et al. (1988b) provided the first direct evidence that the gills are a primary target for STC. They measured Ca2+ fluxes in intact trout and in a trout isolated head preparation and concluded that the degree of Ca2+ influx inhibition by STC was comparable in both preparations. The branchial Ca2+ efflux was not affected by the hormone (Lafeber et al., 1988a, b). Thus STC specifically inhibits branchial Ca2+ influx.

Using recombinant DNA techniques, Butkus et al. (1987) revealed the nucleotide sequence of STC (eSTC) in the Australian eel (Anguilla australis). Proceeding from the deduced amino acid (aa) sequence of this eSTC, hormone fragments were synthesized; an N-terminal, a C-terminal and a midfragment. In juvenile trout (Oncorhynchus mykiss) the N-terminal fragment of eSTC showed Ca2+ influx inhibiting activity (Butkus et al., 1989; Milliken et al., 1990). In the present study we provide more data in favor of the hypothesis that the N-terminus is the bioactive portion of the molecule which may be related to the high degree of amino acid sequence conservation in teleostean species (Butkus et al., 1989). The reasoning was that the N-terminus should inhibit Ca2+ influx in other fish species as well. We determined the effects of the N-terminus on Ca2+ influx in tilapia (Oreochromis mossambicus) and, for comparison, tested the effects of a midfragment and a C-terminal fragment. Milliken et al. (1990) found stimulatory effects of the C-terminal peptide in juvenile trout, but only when the Ca2+ influx in these fish was very low. The midfragment has never been tested in the Ca2+ uptake assay before.
The bioactive domain of STC does not necessarily correspond with the antigenic domain. However, when circulating hormone levels are to be measured in a radioimmunoassay (RIA) or in an enzyme-linked immunosorbent assay (ELISA) the precise location of the epitope should be known. It is possible for an antibody to be directed against an inactive portion of the hormone that is split off and stays in the blood circulation much longer than a bioactive fragment. This is because an antibody, raised against a protein, generally recognizes just a small portion of the molecule. The early measurements of parathyroid hormone (PTH) levels in terrestrial vertebrates illustrate the problem. Antibodies initially used in PTH RIAs were directed against the mid or carboxyl-terminal portion of the hormone (Nussbaum et al., 1985). These RIAs detect biologically inactive fragments of the hormone present in approximately 10-fold higher concentration than intact hormone. Such assays, at best, are only an index of PTH secretion (Nussbaum et al., 1985).

In various studies the specificity of antisera raised against STC has been shown (Butkus et al., 1987; Kaneko et al., 1988; Wagner et al., 1988). It is not known, however, against which portion of the molecule it is directed. In the present study we first analyzed eSTC for possible antigenic sites in the molecule. Secondly, by means of ELISAs and dot blotting immunoassays on eSTC peptides the fragment that is recognized by currently available antisera was determined. Thirdly, we compared the immunoreactivity and the N-terminal amino acid sequence of fully bioactive STC and that of an STC preparation with impaired bioactivity.

Materials and methods

Fish

Freshwater tilapia (Oreochromis mossambicus) ranging in weight from 20 to 40 g were obtained from a laboratory stock. Fish were kept at 27°C in city of Nijmegen tapwater that contains (in mmol·l⁻¹): 0.8 Ca²⁺, 0.20 Mg²⁺, 0.61 Na⁺, 0.05 K⁺, 0.66 Cl⁻, 0.32 SO₄²⁻ and 3.15 HCO₃⁻.

Analytical methods

The calcium content of the water was determined colorimetrically with a diagnostic kit (Sigma). Tracer content of water samples and tissue digestes was determined by liquid scintillation analysis. Aqueous samples (0.5 ml) were mixed with 4.5 ml of Aqualuma scintillation fluid. Protein was measured with the Lowry (1951) method using bovine serum albumin (BSA) as a reference.

Hormone preparation

Trout STC (tSTC) was purified in two steps. The first step was concanavalin-A affinity chromatography as described in detail before (Lafeber et al., 1988a). 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). Preparations thus obtained are essentially free of contaminants as established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Bioactivity is assessed by determining the Ca²⁺ influx reducing potency in trout (results not shown) and tilapia (this study).

To produce STC with impaired activity a batch of freshly isolated corpuscles of Stannius was left at room temperature for 24 h before isolation of STC was started. This STC batch (STC') possessed 50% of the bioactivity of native STC (Fig. 1).

Fragments of the Australian eel STC (eSTC) were synthesized at the Howard Florey Institute Peptide Laboratory (Butkus et al., 1989). The following three peptides were used: peptide U: N-terminal (1-20) sequence of eSTC; peptide V: mid (103-136) sequence of eSTC; peptide W: C-terminal (202-231) sequence of eSTC. Freeze-dried peptides were air-shipped from Parkville, Australia directly following synthesis.

Bioassay (Ca²⁺ uptake)

The bioassays were conducted as described by Verbost et al. (1989). Fish were placed in opaque Perspex boxes (volume: 1.5 liter water) and ⁴⁵CaCl₂ (1.0 MBq·l⁻¹) was added after the water flow had been stopped. Uptake of Ca²⁺ was calculated from the radioactivity accumulated in the fish after 3 h exposure to ⁴⁵Ca and the mean ⁴⁵Ca specific activity of the water. Ca²⁺ uptake (Fᵢₚ) data were normalized to fish weight (Flik et al., 1985) and expressed in μmol·h⁻¹ per 100 g fish weight.

Hormone injections

The peptides were dissolved in demineralized water as 5 mmol·l⁻¹ stock solutions that were diluted in...
saline immediately before injection. STC (0.5 nmol per g fish) and synthetic hormone fragments (5 nmol per g fish) were all freshly dissolved and injected intraperitoneally (10 μl/g) 1 h before tracer exposure. Injection of saline served as control.

Antigenic sites of STC

Using the primary structure of Australian eel STC, the molecule was analyzed for possible antigenic sites. A combination of hydrophilicity, accessibility and mobility parameters has been used in algorithms to predict which sequences of amino acid residues of STC compose antigenic sites. This method (Parker et al., 1986) predicts surface sites (expressed as surface value) that correlate very well with antigenic sites. It should be noted that in this kind of analysis the carbohydrate moiety of STC (Butkus et al., 1987) is not included.

Dot blotting immunoassay

2 μg STC or CS extract and 1 μg synthetic hormone fragment were blotted directly on nitrocellulose membrane using a glass capillary. Immunostaining was performed as described in detail by Flik et al. (1990) for Western blots with RADH-III, a polyclonal antiserum raised against tSTC (Kaneko et al., 1988), as the primary antibody in a 1:5000 dilution. With two other antisera, ECS-I and ECS-III, raised against native eSTC (Butkus et al., 1987), the same type of dotting immunoassay was performed.

Enzyme-linked immunosorbent assay

A competitive ELISA for STC, developed by Mayer-Gostan et al. (1992), was used to quantitate STC levels in plasma samples. This technique is based on competition between free STC in standard or plasma samples and STC immobilized on microtiter plates for the STC antibodies. The trout STC antiserum used (RADH-III; Kaneko et al., 1988), has a high degree of crossreactivity with eel STC. Purified trout STC (Lafeber et al., 1988a) served as a standard.

The wells of microtiter plates were coated with tSTC (1.85 nmol·1⁻¹) in 200 μl of coating buffer (0.05 mol·1⁻¹ sodium bicarbonate buffer, pH 9.6), except for the wells in the first column of the plate which received 200 μl of a BSA solution of equivalent protein content in coating buffer (blanks). Coating lasted for 1 h at 37°C; coated plates were stored at 4°C. Between incubation steps, coated plates were rinsed with washing buffer (0.01 M sodium phosphate buffered saline (PBS), pH 7.4, with 0.05% Tween-20; Bio-Rad). For competition, antigen and RADH-III antiserum were diluted in dilution buffer (i.e. washing buffer containing 2% porcine serum). Equal volumes of antigen and diluted antiserum (1:20,000 to 1:320,000) were incubated in 4 ml Minisorp tubes (Nunc) for 16 h at 20°C. Under these conditions the binding of RADH-III to the STC-coated wells was competitively reduced by STC in a sigmoidal fashion; 50% reduction of signal was obtained with 1.7–2.2 nmol·1⁻¹ STC. Tubes containing diluted antiserum only were incubated under the same conditions. After washing, the wells of coated plates were blocked with 200 μl of dilution buffer (containing 2% pork serum) for 1 h at 37°C. The plates were washed and filled with 200 μl per well of the antigen/antiserum mixture except for the wells in columns 1 (blanks) and 2 (B1) which received 200 μl of the diluted antiserum. Plates were incubated for 2 h at 37°C. The wells were washed and incubated with 200 μl of goat anti-rabbit peroxidase immunoconjugate (Nordic) in dilution buffer (1:5000) for 1 h at 37°C. For quantification of the immunoconjugate bound to the wells, an enzymatic reaction was used with o-phenylenediamine (OPD; Sigma) as a substrate. After washing, 200 μl of substrate (0.05% OPD in citrate/
phosphate buffer; 0.2 mol \cdot 1^{-1} \text{Na}_2\text{HPO}_4, 0.1 \text{mol} \cdot 1^{-1} \text{citric acid, pH 5.0, with 0.025\% hydrogen peroxide) was added. The reaction was allowed to proceed for 20–30 min in the dark and was stopped by adding 50 μl of 2 mol \cdot 1^{-1} \text{H}_2\text{SO}_4. Absorbance was measured at 492 nm in a microplate reader (Titertek).

**Results**

**Effects of hormone fragments on Ca^{2+} influx**

Fig. 1 shows the effects of injection of tSTC or hormone fragments on Ca^{2+} influx. tSTC and peptide U (aa 1–20) decreased the influx, whereas V (aa 103–136) and W (aa 202–231) had no effect. 'STC' isolated from corpuscles left for 24 h at room temperature (tSTC') showed only 50% of the Ca^{2+} influx reducing effect compared to intact tSTC; this tSTC' induced inhibition of Ca^{2+} influx was not significant. Amino acid sequence analysis (by Edman degradation method performed at the Protein Sequencing Facility, Department of Medical Biochemistry, State University of Leiden, Netherlands) shows that 50% of tSTC' lacks the first 4 N-terminal amino acids (Phe-Ser-Ser-Asn). The other 50% carries the complete N-terminus as determined for active tSTC (Phe-Ser-Ser-Asn-Ser-Pro-Ser-Asp-Val-Ala-etc.; Lafeber et al., 1988a). The proportion of bioactivity that is lost correlates well with the amount STC molecules that is truncated.

**Antigenic sites of eSTC**

A surface area plot based on the amino acid backbone of eSTC (see Materials and methods) is shown in Fig. 2. The sequences covered by the synthetic fragments U, V and W are indicated with horizontal bars. In native STC the carbohydrate group is linked N-glycosidically at position 29. Residues with composite profile values greater than 50% represent potential antigenic sites. Fig. 2 shows large potential antigenic sites in the regions covered by fragments V and W. Besides these two there are six smaller peaks that surpass the 50% one of which is localized in fragment U.

**Crossreactivity of anti-STC with hormone fragments**

The immuno-dot blots (Fig. 3) show that fragment V, but not U or W, is recognized by the antisera RADH-III, ECS-I and ECS-III, but no recognition of U or W. RADH-III crossreacts with both CS extracts and purified STC of trout and carp.

![Fig. 3. Immuno-dot blot of CS extract, STC and fragments U, V and W. The blots were probed with three independently raised CS antibodies, RADH-III, ECS-I and ECS-III. A1: goat serum (4 μg); A2: trout CS-extract; B1: fragment U; B2: fragment V; B3: fragment W; C1: tSTC; C3: cSTC.](image1)

![Fig. 4. Antigen dilution curves for tSTC and fragment V obtained with a competitive ELISA applying anti-STC (RADH-III, 1:20, 000). The wells of the microtiter plates were coated with tSTC. The results of two separate experiments in duplicate are presented.](image2)
In an ELISA using the trout STC antibody RADH-III, the results obtained with the dot blots were confirmed and extended. We used antibody dilutions from 1:20,000 to 1:320,000 and found no binding of peptide U or W. With peptide V a similar type of binding as with STC seems to occur (Fig. 4) since the linear parts of the dilution curves run parallel (the gradient for STC is $-1.36 \pm 0.10$; the gradient for V is $-1.18 \pm 0.20$). The affinity for V is approximately 100 times lower than for tSTC. To verify the specificity of the staining reactions obtained with fragment V we also tried another rabbit serum (anti-prolactin; in this case the wells were coated with prolactin) but this showed no crossreactivity (results not shown). The binding characteristics of tSTC' (the non-bioactive preparation) tested in the STC ELISA were the same as for bioactive tSTC (as in Fig. 4; results further not shown).

**Discussion**

**Bioactivity**

The N-terminal eSTC fragment, U, inhibits Ca$^{2+}$ influx in tilapia whereas the mid-portion, V, and the C-terminal, W, of the molecule have no effect. We conclude that the N-terminal fragment carries a, and possibly the, bioactive amino acid sequence of STC. It should be noted, nonetheless, that 10 times higher concentrations of the N-terminal fragment (5 nmol/g fish) are needed to obtain a similar Ca$^{2+}$ influx inhibition as with tSTC (0.5 nmol/g). However, at least four conceivable explanations may be given for this difference. Firstly, it could be a species specific difference; eSTC and its fragments could be less effective in tilapia than tSTC. Secondly, it also could be that the fragment is too small to produce the same bioactivity as the native hormone. An analogous situation was reported for parathyroid hormone (PTH, 84 residues) in mammals, where 1–34 PTH gives maximal bone resorbing and hypercalcemic responses whereas 1–28 or 3–34 PTH fragments have a much lower potency (Habener and Potts, 1976; Herrmann-Erlee et al., 1983). Thirdly, the native hormone is a dimer (Lafeber et al., 1988a), in contrast to U. Possibly, this dimeric constitution renders STC its maximal bioactivity. Fourthly, the carbohydrate group which is attached at position 29 in the native hormone (Butkus et al., 1987) may be required for optimal receptor activation and obviously this moiety is absent in U. It is generally believed that the carbohydrates of glycoprotein hormones are essential for their complete biological activity in target cells although the oligosaccharide moiety may not be necessary for receptor binding (Sairam, 1989). In any case it is clear that fragment U reduces Ca$^{2+}$ influx in addition to the effect of the endogenous STC in the fish.

The midportion of the hormone (V) did not affect Ca$^{2+}$ influx. Although V alone appears unable to activate the STC receptor as no decrease in Ca$^{2+}$ influx occurred, it may still be that receptor binding of the midportion is needed to reach full activity or the midportion may play a role in defining the three-dimensional structure of the molecule so that full receptor binding of STC can occur. Also the C-terminal fragment (W) has no effect on Ca$^{2+}$ influx in tilapia. In analogy to V, W appears not to affect the STC receptor. Milliken et al. (1990) showed stimulatory effects of the C-terminus (W) on Ca$^{2+}$ influx in juvenile trout and explained the effect as an antagonistic action on the binding of endogenous hormone. The enhancement in Ca$^{2+}$ uptake by W was only found when the fish were at the nadir of an apparent Ca$^{2+}$ uptake cycle (calcium uptake varied from 0.33 to 4.88 μmol·h$^{-1}$·100 g$^{-1}$). We do not find a cycling in Ca$^{2+}$ uptake in tilapia nor trout. Compared to juvenile trout, tilapia have a constantly high uptake rate. This may explain why we did not see stimulatory effects of the C-terminal fragment on Ca$^{2+}$ uptake in tilapia.

Hormone isolated from corporules of Stannius that had been left at room temperature for 24 h (tSTC') was deficient in reducing Ca$^{2+}$ influx. The molecular size of this tSTC' was checked with polyacrylamide gel electrophoresis and gel permeation chromatography but was not significantly different from bioactive tSTC (results not shown). In an amino acid analysis tSTC' gave two signals, showing that roughly 50% of the tSTC' had lost the four N-terminal amino acids. The other 50% carried the complete N-terminus identical to that of intact tSTC that gives one signal in the amino acid analysis (identical to Lafeber et al., 1988a; results further not shown). This suggests an important role for the N-terminus in determining the bioactivity.

**Antigenicity**

Our results indicate that polyclonal STC-antisera, whether raised against trout or eel STC, recognize a midfragment of eSTC but not the N-terminus that is required for bioactivity, or the C-terminus. According to its amino acid sequence STC has 11 surface areas that are likely to be antigenic. The terminal regions, especially the C-terminal, show large antigenic sites. Still, no antibodies directed against these sites have been produced. The midfragment conceivably contains a major antigenic site of the hormone (Fig. 2). That the site is very antigenic is confirmed by the observation that three independently produced polyclonal antisera raised against STC specifically recognize fragment V (Fig. 3). The main difference between the midregion and the terminal regions lies in the flexibility; compared to the midregion, the N-terminus is rigid and the C-terminus is even more rigid because of frequently occurring prolines in the C-terminus (review by Suzuki, 1988). The flexibility ap-
pears to be very important for the antigenicity of STC portions. This is in agreement with notions from crystallography and nuclear magnetic resonance (NMR) studies (Tainer et al., 1984; Westhof et al., 1984) that the more flexible regions of a protein become epitopes. A high flexibility of the midregion may explain why the different antibodies were directed against this portion of STC.

One could argue that in the determination of surface areas the glyco group of the hormone (positioned at amino acid 29) is disregarded as a potential epitope. Sugar residues are generally highly antigenic. However, this appears not to be the case for STC (Flik et al., 1990); the glyco group does not represent an antigenic site in this hormone.

If V contains the major antigenic site it needs to be discussed why the affinity for the antibody is 100 times lower than that of tSTC. It could be that V forms just a part of the antigenic site. It could also be due to the fact that V is monomeric and lacks three-dimensional folding. Obviously, more fragments need to be tested to be sure about the antigenic site in STC. The results show, however, that the currently available antibodies may recognize inactive STC forms. This conclusion is confirmed by the results obtained with tSTC'.

In conclusion, tSTC' showed similar antibody binding characteristics as intact tSTC, but lost its bioactivity as mentioned before. Thus, non-bioactive STC lacking the first four amino acids would be measured in an ELISA as is native STC. Interestingly, immunization of rabbits with tSTC' resulted in a serum that only recognizes fragments (coupled to a carrier protein for immunization) seems to be the most promising route.

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References


