

IONIC AND TOTAL CALCIUM LEVELS IN THE BLOOD OF THE EUROPEAN EEL (*ANGUILLA ANGUILLA*): EFFECTS OF STANNIECTOMY AND HYPOCALCIN REPLACEMENT THERAPY

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Summary

Removal of the corpuscles of Stannius (STX) in the freshwater European eel causes a marked increase in the concentrations of blood ionic calcium and protein-bound calcium. The hypercalcaemia peaks 20 days after STX and lasts at least another 20 days. In stanniectomized eels hypocalcin decreased both blood ionic and total calcium concentrations. The reduction of plasma total calcium concentration by hypocalcin is attributed to a reduction in blood ionic calcium concentration. We conclude that hypocalcin regulates blood ionic calcium levels in fish.

Introduction

The corpuscles of Stannius (CS) of holostean and teleostean fish produce an important hypocalcaemic hormone. For a variety of teleost species it has been shown that removal of the CS (stanniectomy, STX), results in hypercalcaemia (Fontaine, 1964; Butler, 1969; Chan, 1970; Pang, 1971a; Fenwick, 1974; Wendelaar Bonga & Greven, 1978; Urasa & Wendelaar Bonga, 1987). This hypercalcaemia was found to be transitory (Fontaine, 1967; Chan, 1970; Fenwick, 1978). Injection of CS extracts (Fontaine, 1964; Pang, 1971a; Kenyon *et al.* 1980) or homotransplantation of the corpuscles (Fenwick & Forster, 1972; Pang, 1973) restored normocalcaemia. Contradictory results have been reported on the regulation by the CS of the levels of other plasma electrolytes such as Na^+ , K^+ , Mg^{2+} , PO_4^{3-} , and Cl^- . Presumably, the CS are not directly involved in the regulation of these ions (Chan, 1972; Pang *et al.* 1975; Fenwick, 1985).

Although a negative feedback between CS and plasma ionic calcium has been suggested (Bailey & Fenwick, 1975; Aida *et al.* 1980; Urasa & Wendelaar Bonga, 1987) evidence has been circumstantial. In their experiments with frog hearts McLean & Hastings (1935) provided evidence that ionic calcium is the major

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physiologically active form of calcium in the body. This is now generally accepted (Heining *et al.* 1983). Calcium in blood or plasma can be subdivided into a protein-bound and an ultrafiltrable (or dialytic) fraction, which consists of calcium complexed to anions and of ionic calcium. For the freshwater European eel it was reported that about 70 % of the plasma calcium is ultrafiltrable, 54 % being ionized (Chan & Chester Jones, 1968). The plasma ionic calcium concentration in the freshwater eel appears rather constant (concentrations reported range from 1.3 to 1.6 mmol l⁻¹; Chan & Chester Jones, 1968; Chan, 1972; Fenwick, 1974) and comparable with values found in mammals.

In this report we re-evaluated calcium levels in the blood of the European eel using a Radiometer ionized calcium analyser. Eels were cannulated to allow serial stress-free sampling. Blood ionic and total calcium levels were manipulated by stanniectomizing eels or by hypocalcin replacement.

Materials and methods

Animals

Sexually immature freshwater eels (*Anguilla anguilla*), weighing about 300 g, were purchased from a commercial fish dealer in The Netherlands. Upon arrival in the laboratory the fish were kept unfed in 300-l well-aerated tanks supplied with running tapwater (total calcium 0.7 ± 0.1 mmol l⁻¹). The temperature of the water was approximately 12°C. Before surgery the eels were acclimated to these conditions for at least 14 but no longer than 42 days.

Preparation of eel CS extract and eel hypocalcin

Freshly dissected eel CS were homogenized in ice-cold saline (0.6 % sodium chloride) and the homogenate was centrifuged (5 min at 9000 g). The supernatant (CS extract) was immediately injected as described below. Eel hypocalcin was isolated using concanavalin-A affinity chromatography (Lafeber *et al.* 1988a). The CS material without affinity to concanavalin-A is referred to as residue and was devoid of hypocalcin as judged by sodium dodecylsulphate polyacrylamide gel electrophoresis (Laemmli, 1970).

Doses injected per 100 g fish were the extract of 6 mg of fresh eel CS (six fish equivalents), 50 µg of eel hypocalcin (eight fish equivalents) or 80 µg of residue protein (four fish equivalents); the carrier volume was 200 µl of saline per 100 g of fish. Saline injections served as controls.

Surgery

Eels were anaesthetized in ethylaminobenzoate (MS 222, 2.5 g l⁻¹, pH 7.8) and stanniectomized or sham-operated as described by Leloup-Hatey (1964). Muscle and skin were sutured carefully and the fish were allowed to recover in 100-l tanks supplied with aerated running tapwater. The CS were used for replacement studies.

The pneumogastric artery was cannulated as described by Chester Jones *et al.*

(1966). Cannulated eels were housed individually in opaque cylindrical containers supplied with running tapwater. Between sampling procedures the cannula was filled with saline (0.6%, pH 7.8) containing polyvinyl pyrrolidone (0.12 g ml^{-1}) and sodium heparin ($500 \text{ units ml}^{-1}$), and closed with a metal pin. The eels were allowed 2 days to recover from surgery. The setup allowed free movement of the eels, stress-free blood sampling, and intra-arterial injection.

Experimental protocols

A maximum period of 17 days was found appropriate for serial blood sampling. Therefore, to cover a period of 40 days, three groups of 16 eels were used to study the long-term effects of stanniectomy. In each group eight eels were stannioctomized and eight sham-operated. The first group was cannulated 2 days before, the second and third group 11 and 26 days after surgery, respectively. A $200 \mu\text{l}$ blood sample was taken daily. In one experiment blood was sampled by cardiac puncture from two separate groups of eels 40 days after STX or sham-operation to assess any interference by the cannulation and blood sampling procedures with blood calcium levels. Blood calcium values in STX or sham-operated fish were not affected by these procedures. In STX fish, values were elevated and comparable with values in cannulated STX fish.

CS extract and hypocalcin were injected into STX eels, as a more pronounced blood calcium lowering effect was anticipated in STX than in intact eels (Kenyon *et al.* 1980). Eel CS extract and eel hypocalcin were tested in cannulated eels. On day 15 after STX, between 08.00 and 10.00 h, a single hormone injection was given. Before injection a $500 \mu\text{l}$ blood sample was taken; $200 \mu\text{l}$ was used for analyses, and the remaining $300 \mu\text{l}$ was reinjected immediately following the hormone injection. This ensured that all hormone entered the bloodstream. Blood samples were collected for 3 days.

Plasma analyses

Blood samples were collected in 1.5 ml Eppendorf cups containing $5 \mu\text{l}$ of calcium heparin (Radiometer). Blood ionic calcium and pH were determined with an ionic calcium analyser (ICA-1, Radiometer). The remaining blood was separated into plasma and cells by centrifugation (1 min at 9000 g). Plasma total calcium, magnesium and protein concentrations were estimated with commercial kits (calcium reagent kit, Sigma; magnesium reagent kit, Sigma; protein reagent kit, with bovine serum albumin as a reference, Biorad). Plasma osmolality was measured with a micro-osmometer (Roebeling) using distilled water and a $300 \text{ mosmol kg}^{-1}$ solution (Sigma) as standards. Measurements of ionic calcium in whole blood reflect plasma values (Andreasen, 1985). Indeed we did not observe a significant difference between Ca^{2+} concentrations in blood or plasma.

Calculations and statistics

The data are presented as means \pm S.E.M. The Mann-Whitney *U*-test (one-tailed) was used for statistical evaluation. Significance was accepted at $P < 0.05$.

Table 1. *Values for haematocrit (Hct), blood pH, plasma osmolality, plasma protein concentration and plasma magnesium concentration in 14-day stanniectomized and sham-operated eels*

| | Hct (%) | Blood pH | Osmolality (mosmol kg ⁻¹) | [Protein] (mg ml ⁻¹) | [Mg] (mmol l ⁻¹) |
|------|---------------|---------------------|--|-------------------------------------|---------------------------------|
| STX | 23 ± 2 (7) | 7.81 ± 0.02 (40) | 277 ± 8 (7) | 39 ± 1 (17) | 1.42 ± 0.12 (5) |
| Sham | 22 ± 2 (5) | 7.84 ± 0.01 (33) | 288 ± 4 (6) | 43 ± 2 (8) | 1.41 ± 0.09 (7) |

Values are presented as means ± s.e.m.

Numbers in parentheses indicate *N*.

For the analysis of the effects of STX, values from STX eels were compared with corresponding values from sham-operated eels. In replacement studies, values from hormone-injected eels were compared with corresponding values from saline-injected eels.

Results

In 14-day stanniectomized eels haematocrit, blood pH, plasma osmolality, plasma magnesium concentration and plasma protein concentration were unchanged (Table 1). STX caused an increase of ionic and total calcium levels. (Fig. 1A,B, respectively). The rise in total calcium level surpassed the rise in ionic calcium. Hypercalcaemia reached its maximum around 20 days after STX. Total and ionic calcium level in 40-day STX eels were still significantly elevated and were comparable to values in 20-day STX eels.

Fig. 2A–D summarizes replacement studies in 15-day STX eels. Preinjection values were 3.03 ± 0.15 for blood ionic and 5.77 ± 0.20 mmol l⁻¹ for plasma total calcium concentration (*N* = 37; Fig. 1A,B). Injection of eel CS extract (Fig. 2A) and eel hypocalcin (Fig. 2B) caused a decrease in blood ionic and plasma total calcium level whereas injection of residue (Fig. 2C) or saline (Fig. 2D) had no significant effect. Blood total calcium level in eels injected with CS extract had decreased significantly by 2 h after injection, plasma ionic calcium by 3 h after injection. Blood ionic and plasma total calcium levels in hypocalcin-injected eels had decreased significantly 3 and 5 h after injection, respectively. Maximum effects on both ionic and total calcium levels were found 24 and 48 h after injection, respectively. Reductions in blood calcium levels lasted up to 72 h after hormone injection.

Discussion

Our experiments indicate that hypocalcin controls blood Ca²⁺ levels. Non-endocrine factors, in particular CO₂, pH and temperature, may also affect calcium

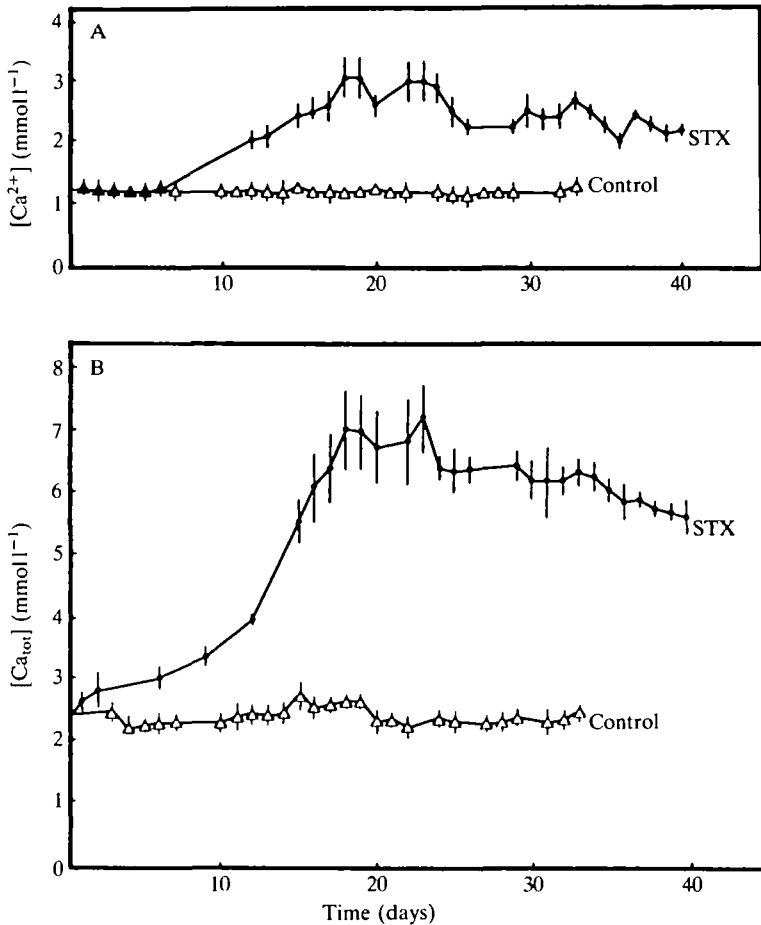


Fig. 1. Effect of stanniectomy (STX) or sham operation (control) on the blood ionic calcium concentration (A) and blood total calcium concentration (B) in cannulated eels. Values are presented as means \pm S.E.M. ($N = 8$).

levels in blood. Storage (up to 2 h) of our samples under aerobic conditions did, however, not affect blood pH and Ca^{2+} levels. We conclude, therefore, that the Ca^{2+} level in eel blood is not directly influenced by CO_2 levels, as it is in terrestrial vertebrates. This is similar to the pH buffer mechanisms in fish blood, where pH is regulated mainly by plasma bicarbonate adjustment instead of variations in CO_2 ventilation (Höbe *et al.* 1984). In human blood a change in pH of 0.1 unit corresponds to a change in ionic calcium of approximately $0.06\ mmol\ l^{-1}$ (Fogh-Andersen, 1981; Brauman *et al.* 1983). In trout, however, the pH effect on ionic calcium is 3–6 times weaker (Andreasen, 1985). We conclude that changes, if any, in blood pH in our procedures have gone undetected. Since our automated ionic calcium analyser is operated at $37^\circ C$, the ionic calcium level measured is not the *in vivo* ionic calcium concentration. In human blood, temperature is reported to

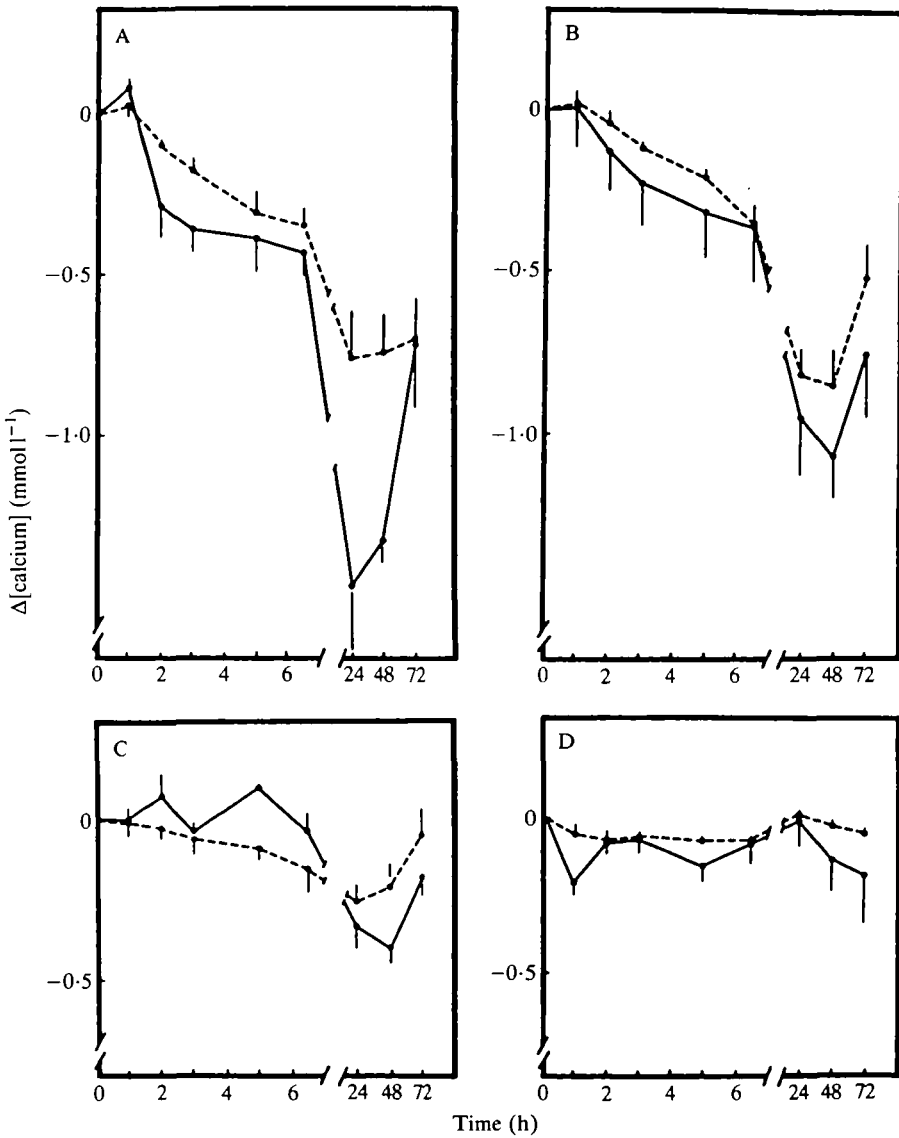


Fig. 2. Effect of hypocalcin replacement therapy on blood ionic (---) and total (—) calcium concentration in cannulated eels 15 days after stanniectomy. Fish were injected with eel CS extract (A), eel hypocalcin (B), eel residue (C) or saline (D). Values are presented as means \pm S.E.M. ($N=11$) and are shown as differences compared with preinjection levels.

affect the protein binding of ionic calcium either directly or indirectly by a temperature-induced change of pH (Heining *et al.* 1983). A rise in temperature will cause more calcium to bind to protein and, as a result, ionic calcium levels will decrease; concomitantly, a rise in temperature will evoke a pH drop which in turn

causes a rise in Ca^{2+} level. In human blood the overall temperature effect on Ca^{2+} levels is an increase of approximately $0.002 \text{ mmol l}^{-1}/^{\circ}\text{C}$ (Heining *et al.* 1983). Assuming a similar relationship for eel blood – the relationship between pH and temperature of trout blood is similar to that of human blood (Perry *et al.* 1985) – a 25°C increase in eel blood temperature would increase blood $[\text{Ca}^{2+}]$ by 0.05 mmol l^{-1} . This deviation is within the error of our measurements.

Our observation that STX increases total calcium concentration in the plasma of eels is in agreement with reports in the literature on a variety of species (Fontaine, 1964; Butler, 1969; Pang, 1971a; Chan, 1972; Fenwick, 1974; Wendelaar Bonga & Greven, 1978; Urasa & Wendelaar Bonga, 1987). The hypercalcaemia was not caused by an increased bone demineralization (Lopez, 1970) but resulted from an increased net branchial calcium uptake that was already obvious 7 days after STX (So & Fenwick, 1977; Milet *et al.* 1979). We have shown here that blood ionic and plasma total calcium levels rise concomitantly after STX. Blood ionic and plasma total calcium levels both reach maximum values 20 days after STX and stay elevated for at least another 20 days. This observation contrasts with reports by Fontaine (1967), Chan (1972) and Fenwick (1978), who found that plasma total calcium levels returned to normal 56, 42 or 35 days after STX, respectively. The authors suggested that activation of the ultimobranchial bodies after STX may have resulted in the restoration of normocalcaemia. Reports on the effect of calcitonin on plasma calcium levels in fish are contradictory. Hypocalcaemic effects of mammalian or fish calcitonins in fish (Chan *et al.* 1968; Wendelaar Bonga, 1981) are minor at all doses tested, and such effects have not always been confirmed by others (Pang, 1971b). The present results indicate that the ultimobranchial bodies are unable to restore normocalcaemia after STX under our experimental conditions, and confirm that the hypocalcaemic potency of these glands is low compared with that of the CS.

The increase in blood ionic calcium concentration after STX does not completely account for the increase in plasma total calcium concentration, which implies that the protein-bound calcium level also rises after STX. This observation is consistent with earlier observations by Pang (1971a) and Chan (1972) who found that both dialytic and nondialytic calcium contribute to the increase of total plasma calcium level in stanniectomized killifish and eel, respectively. We did not measure a rise of blood ionic calcium level in the first 6 days after STX as reported by Chan (1972). However, plasma total calcium level did increase during the first 6 days after STX. This observation is in agreement with observations by Fenwick (1974) that plasma dialytic calcium in the American eel is unchanged on the fifth day after STX. A significant increase in blood ionic calcium level was observed 12 days after STX and thereafter. Fenwick (1974) reported that the dialytic calcium fraction was elevated 10 days after STX. Since the hypercalcaemia following STX is caused by an increased net branchial uptake of Ca^{2+} (So & Fenwick, 1977; Milet *et al.* 1979), one would expect a rise of plasma ionic calcium level. Since only protein-bound calcium increases in the first days after STX, we conclude that the increased Ca^{2+} influx in the blood is buffered by binding to plasma proteins. Increased binding of

ionic calcium to plasma proteins may result from an increase in plasma protein concentration or from a change in the Ca^{2+} -binding capacity of plasma proteins. Chan (1972) speculated that the appearance of a calcium-binding protein could explain the rise in nondialytic calcium in 14-day STX Japanese eels. However, we did not find that the rise in blood calcium concentration after STX was accompanied by a rise in plasma protein concentration. The maximum binding capacity for blood ionic calcium in human blood is approximately $0.12 \text{ mmol Ca}^{2+} \text{ g}^{-1} \text{ protein}$, 90 % being bound to albumin (Pedersen, 1972). If we assume a similar maximum binding capacity for eel blood, and take into account a protein concentration in eel plasma of 40 mg ml^{-1} , it follows that in untreated and sham-operated eels ($[\text{Ca}^{2+}] = 1.25 \text{ mmol l}^{-1}$, $[\text{Ca}_{\text{tot}}] = 2.50 \text{ mmol l}^{-1}$) the binding capacity amounts to $0.03 \text{ mmol Ca}^{2+} \text{ g}^{-1} \text{ protein}$. During STX the binding capacity increases until a maximum value of $0.11 \text{ mmol Ca}^{2+} \text{ g}^{-1} \text{ protein}$ is reached 21 days after STX ($[\text{Ca}^{2+}] = 2.50 \text{ mmol l}^{-1}$, $[\text{Ca}_{\text{tot}}] = 7.00 \text{ mmol l}^{-1}$). This is further reflected by the decrease in the $\text{Ca}^{2+}/\text{Ca}_{\text{tot}}$ ratio in blood which is around 0.50 in untreated and sham-operated eels and which decreases to 0.40 in STX eels. We conclude that the increase in Ca^{2+} uptake during the first days of STX is buffered by increased binding to plasma calcium-binding proteins. This might be due to a qualitative change in the plasma proteins.

Injections with homogenates of CS obtained from different teleost species have been reported to decrease total plasma calcium levels in STX fish (Fontaine, 1964; Kenyon *et al.* 1980) and in intact fish adapted to low-calcium water (Pang *et al.* 1981; Wendelaar Bonga *et al.* 1986). So & Fenwick (1979) showed that CS extract injected into STX eels reduced the branchial calcium uptake. Recently Lafeber *et al.* (1988b) concluded that in rainbow trout the reduction of plasma calcium level caused by injection of CS extract, resulted from a hypocalcin-induced reduction of whole-body Ca^{2+} uptake. The experiments presented in this paper show a decrease in blood ionic and plasma total calcium levels after injection of eel CS extract or eel hypocalcin into stanniectomized eels. The hypocalcaemic effect can be observed for more than 48 h. To our knowledge there are no other peptide hormones that can exert their function for such a long period. We observed a decrease of blood ionic calcium level that almost fully accounted for the decrease of blood total calcium level. Bailey & Fenwick (1975) observed a decrease in ionic calcium and not in total calcium level after injection of eel CS homogenates into intact eels. They suggested an influence of the CS extract on calcium binding to plasma proteins. Our experiments do not support their suggestion, since no increase of the calcium-binding capacity of plasma proteins was found. At the time of injection, 15 days after STX, the binding capacity amounted to $0.06 \text{ mmol Ca}^{2+} \text{ g}^{-1} \text{ protein}$ ($[\text{Ca}^{2+}] = 3.03 \text{ mmol l}^{-1}$, $[\text{Ca}_{\text{tot}}] = 5.77 \text{ mmol l}^{-1}$, $[\text{protein}] = 40 \text{ mg ml}^{-1}$). Since the reduction in ionic calcium concentration equals the reduction in total calcium concentration after hypocalcin replacement therapy, no change in the binding capacity will occur. The rather sudden effect of hypocalcin on the elevated blood ionic calcium level in STX eels indicates that hypocalcin exerts its hypocalcaemic action mainly by inhibition of Ca^{2+} uptake. This

inhibition results in a net calcium efflux, as has been shown by Lafeber *et al.* (1988b).

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