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HYPOCALCAEMIC ACTIVITY OF TROUT HYPOCALCIN AND BOVINE PARATHYROID HORMONE IN STANNIECTOMIZED EELS

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Summary

Hypocalcin isolated from trout corpuscles of Stannius (CS) and bovine parathyroid hormone (bPTH) induce hypocalcaemia when injected intra-arterially in hypercalcaemic, stanniectomized (STX) eels. On a molar basis, both hormones have almost the same potency. Upon injection of hypocalcin we observed a decrease in blood ionic calcium level which fully accounts for the decrease in total calcium. Injections of bPTH also resulted in a decrease of total and ionic calcium concentrations in the blood. The decrease in ionic calcium level upon injection of bPTH, however, did not fully account for the decrease in total calcium concentration of the blood. Nevertheless, hypocalcin and bPTH showed striking similarity in bioactivity in stanniectomized eels. Some similarity in three-dimensional structure of both hormones may underlie this phenomenon.

Introduction

Corpuscles of Stannius (CS) are small endocrine glands characteristic of bony fish. Eels have one pair of CS ventrocaudal to the kidney. The CS have been suggested to be pivotal in hypocalcaemic control in fish (Fontaine, 1964; Pang *et al.* 1980; Wendelaar Bonga & Pang, 1986), more important than the calcitonin-secreting ultimobranchial glands.

Removal of the glands (stanniectomy, STX) results in an increase in the blood calcium concentration, whereas injections of CS homogenates or reimplantations of CS correct STX-induced hypercalcaemia (Fontaine, 1964; Fenwick & Forster, 1972; Kenyon *et al.* 1980).

Several secretory products have been reported to be secreted by the CS (Idler & Freeman, 1966; Ogawa & Sokabe, 1982; Tisserand-Jochem *et al.* 1987; see Wendelaar Bonga & Pang, 1986), but the main bioactive secretory product appears to be a glycoprotein (Wagner *et al.* 1986; Lafeber *et al.* 1988*a*). This

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glycoprotein has been isolated from coho salmon CS (Wagner *et al.* 1986) and was named teleocalcin; we have recently isolated the glycoprotein from trout (Lafeber *et al.* 1988*a*). We have called the principle hypocalcin, the name originally proposed by Pang *et al.* (1974) for the active factor of the CS. The products from salmon and trout show only small differences in relative molecular mass and substantial similarity in the *N*-terminal amino acid sequence (Wagner *et al.* 1986; Lafeber *et al.* 1988*a*). The *N*-terminal sequence predicted from DNA base analysis of the principle of the Australian eel is also similar to that of trout and salmon (Butkus *et al.* 1987). Wagner *et al.* (1986) have shown that the isolated salmon principle inhibits $^{45}Ca^{2+}$ uptake in juvenile rainbow trout, whereas we showed that trout hypocalcin inhibits branchial Ca^{2+} inflow in adult trout (Lafeber *et al.* 1988*b*).

This CS principle resembles the parathyroid hormone (PTH) of terrestrial vertebrates (Milet *et al.* 1979*a*, 1980*a*,*b*, 1985; Lopez *et al.* 1984) and it has been suggested that the CS are homologues of the parathyroid glands. CS tissue extracts and PTH show similar activities in mammals and fish (Milet *et al.* 1979*a*, 1980*a*, 1985; Lafeber *et al.* 1986; Lopez *et al.* 1984; Wendelaar Bonga *et al.* 1986). The latest report on the similarity between CS extracts and PTH shows a hypocalcaemic activity of bovine PTH (bPTH) in low-calcium-acclimated killifish and tilapia (Wendelaar Bonga *et al.* 1986). These authors have questioned, however, the homology of CS and parathyroid glands on the basis of reported differences in embryological origin (Wendelaar Bonga & Pang, 1986). Furthermore, Butkus *et al.* (1987) have concluded that there is no similarity in the predicted amino acid sequences of eel hypocalcin and of PTH.

In this paper we compare the effect of bPTH with that of hypocalcin on the elevated plasma calcium levels in STX eels. STX fish were used because substantial effects of CS extracts on plasma calcium levels in intact fish kept in normal fresh water have not been reported (Wendelaar Bonga *et al.* 1986). From a physiological point of view, ionic calcium is more important than the total calcium concentration of the blood (McLean & Hastings, 1934; Andreasen, 1985). We have therefore monitored the ionic as well as the total blood calcium concentration after hormone injections.

Materials and methods

Animals

Trout (*Salmo gairdneri*) with a body mass between 200 and 300 g were obtained from a commercial trout farm (Beek, The Netherlands) and used for collection of CS.

European eels (*Anguilla anguilla*), 230–290 g in body mass, were obtained from several commercial fishmongers. They were collected only in the summer and were sexually immature. The eels were acclimated to tap water, for between 3 and 6 weeks, in 500-l opaque well-aerated fibreglass tanks. Water was filtered by recirculation (1000 l h^{-1}) over charcoal and refreshed with a constant inflow of tap

water ($100\,l\,h^{-1}$). Total calcium concentration of the water varied between 0·6 and 0·7 mmol l^{-1}. Water temperature was between 10 and 12 °C.

Analytical techniques

Total plasma calcium was determined using a commercial calcium reagent kit (Sigma). Ionized calcium and pH of whole blood were measured within 30 min after sampling in an automated ionic calcium analyser (ICA-1, Radiometer; Fogh-Anderson, 1981). Protein was measured using a commercial protein reagent kit (Biorad). Osmolality was measured in a Roebling micro-osmometer.

Isolation of hypocalcin

Hypocalcin was isolated as described recently (Lafeber *et al.* 1988*a*) using concanavalin-A affinity chromatography. Material bound to concanavalin-A consisted of a glycoprotein with an apparent relative molecular mass of 41 000 (41K) (hypocalcin). Hypocalcin fractions proved to be at least 95 % pure on a protein basis. The material without affinity for concanavalin-A is referred to as residue proteins and was devoid of hypocalcin as judged by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) and bioassay (Lafeber *et al.* 1988*b*).

Stanniectomy and cannulation

Eels were anaesthetized in sodium bicarbonate buffered (pH 7·8) ethylaminobenzoate (MS 222, $2\,g\,l^{-1}$). During an operation that lasted up to 20 min, fish were kept wet and CS were removed from the kidney *via* a small incision through the body wall (Leloup-Hatey, 1964). Muscle and skin were sutured. The fish were allowed to recover in 200-l well-aerated tanks with a constant flow of fresh tap water ($100\,l\,h^{-1}$).

Ten to twelve days after this operation, fish were again anaesthetized and the pneumogastric artery was cannulated according to procedures described by Chester Jones *et al.* (1966). They were kept in individual opaque polyvinyl tubes with a diameter of 5 cm, provided with a flow of well-aerated tap water ($50\,l\,h^{-1}$). Cannulae were conducted out of these tubes through a longitudinal slit and were fixed above water level by a counter-balance set-up. This set-up allowed free movement of the fish and stress-free blood sampling. Fish were allowed to recover for at least 2 days. The cannulae were used for injection and for blood sampling. Between these procedures the cannulae were filled with a sodium chloride solution (0·6 %) containing polyvinyl pyrrolidone ($0{\cdot}12\,g\,ml^{-1}$) and sodium heparin (500 units ml^{-1}), and closed with a metal pin.

Experimental procedures

Four groups of fish were injected at day 15 after STX with trout hypocalcin, trout residue proteins, bPTH (Sigma, TCA powder) or vehicle (0·9 % saline solution). These injections were immediately followed by injection of a 500-μl blood sample which had previously been drawn from the fish.

50 μg of hypocalcin and 80 μg of residue proteins were injected per 100 g of fish; these doses equal the amount of material obtained from six fish of comparable weight to the experimental fish. Bovine PTH was injected at a dose of 20 i.u. $100\,g^{-1}$ fish.

Blood samples were taken for 3 days. First, a blood sample of 100 μl was drawn to clean the cannula. Subsequently, a 250-μl blood sample was drawn into a 1-ml syringe containing 5 μl of Ca^{2+}-heparin (Radiometer). After determination of ionic calcium and pH, the remaining 140 μl of blood was centrifuged (1 min, 9000 *g*). Plasma was used for determination of the total calcium concentration, osmolality and haematocrit. Two blood samples were taken before injection and eight samples over a 3-day period after injection (t = −2, 0, 1, 2, 4, 5, 6, 24, 48 and 72 h). The pH, osmolality and haematocrit were measured to check the condition of the eels during the experiments.

Statistical evaluation

Mean values ± S.E.M. are given. In Mann–Whitney *U*-tests (one-tailed), significance was accepted at $P < 0{\cdot}05$. For statistical evaluation of differences between experimental- and control-injected groups and differences between the decrease in total and ionic calcium, values obtained at the same time after stanniectomy and after injection were compared.

Results

Stanniectomy of the eels resulted, after 15 days, in a plasma total calcium concentration of $5{\cdot}71 \pm 0{\cdot}21$ mmol l^{-1} and a blood ionic calcium concentration of $3{\cdot}21 \pm 0{\cdot}16$ mmol l^{-1} ($N = 50$). Before injection, there was no significant difference in total or ionic calcium concentration among the four treatment groups (vehicle control, and groups treated with hypocalcin, residue proteins or bPTH).

After saline injection (0·9 % NaCl; controls), no significant change in blood ionic and plasma total calcium concentrations was observed over the 3-day sampling period (Fig. 1). Maximum decreases of $0{\cdot}08 \pm 0{\cdot}04$ and $0{\cdot}22 \pm 0{\cdot}05$ mmol l^{-1} were observed for ionic and total calcium, respectively.

Injection with trout hypocalcin resulted in a decrease of total and ionic calcium level (Fig. 2A). The reduction in blood calcium became apparent after 2 h and reached its maximum after 24 h. Although after 24 h the calcium concentrations had started to climb again, they were still significantly lower than control values 3 days after injection. The decreases in plasma total calcium and blood ionic calcium were similar. The differences between the reductions in ionic and total calcium were not statistically significant. Injection with residue proteins did not cause a statistically significant change in either total calcium or ionic calcium concentrations (Fig. 2B).

Injection with bPTH resulted in a decrease of the plasma total calcium as well as the ionic blood calcium concentration (Fig. 3). The decrease in total calcium

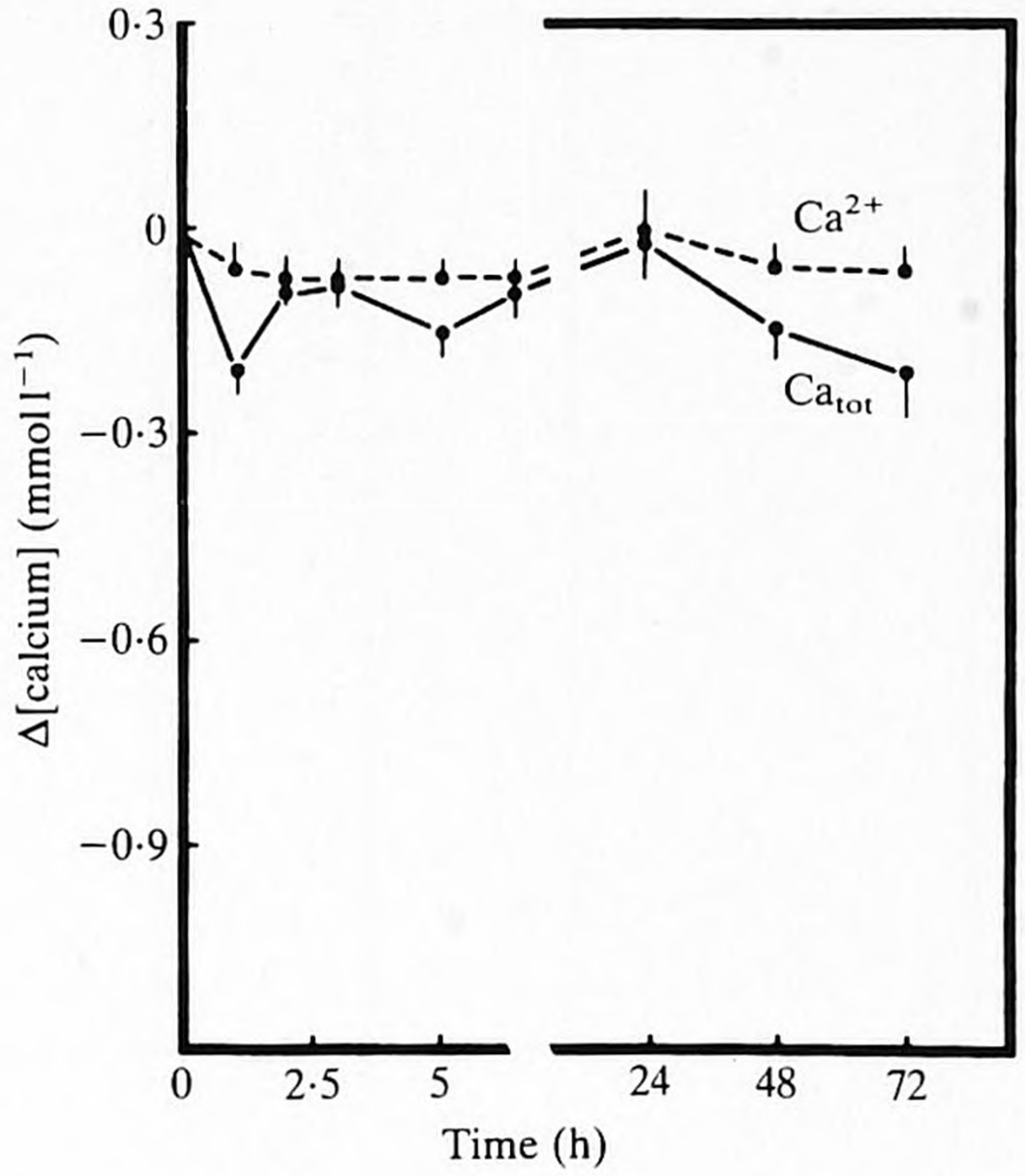

Fig. 1. The effect of NaCl injection (control) on plasma total calcium (Ca_{tot}) and blood ionic calcium (Ca^{2+}) in eels stanniectomized 15 days earlier. Means ± S.E.M. are given. At time zero, for this group of fish $Ca_{tot} = 5{\cdot}58 \pm 0{\cdot}22\,mmol\,l^{-1}$, $Ca^{2+} = 3{\cdot}12 \pm 0{\cdot}22\,mmol\,l^{-1}$ ($N = 15$).

concentration, however, was more pronounced than, and statistically different from, the decrease in ionic calcium.

The pH was measured for all blood samples and did not change during the experiments ($pH = 7{\cdot}9 \pm 0{\cdot}1$). Osmolality and haematocrit were not affected by any treatment given (osmolality: $289 \pm 6\,mosmol\,kg^{-1}$, $N = 25$; haematocrit: $35 \pm 12\,\%$, $N = 25$).

Discussion

Our results show that the glycoprotein isolated from trout CS (which we have tentatively named hypocalcin; Lafeber *et al.* 1988*a*), induces a decrease of plasma total calcium level in hypercalcaemic STX eels. Since the CS residue proteins do not exert a significant calcium-reducing activity, in contrast to the isolated product, we conclude that the isolated glycoprotein may indeed be equated with the hypocalcaemic principle of the CS for which Pang *et al.* (1974) have proposed the name hypocalcin. The decrease in calcium concentration after a single injection with hypocalcin reaches its maximum 1 day after injection and persists for at least 3 days. This sustained decrease of plasma calcium after hypocalcin treatment agrees with the observation that it takes some days at least before plasma calcium levels become elevated after removal of the CS (e.g. Fenwick, 1974; Kenyon *et al.* 1980).

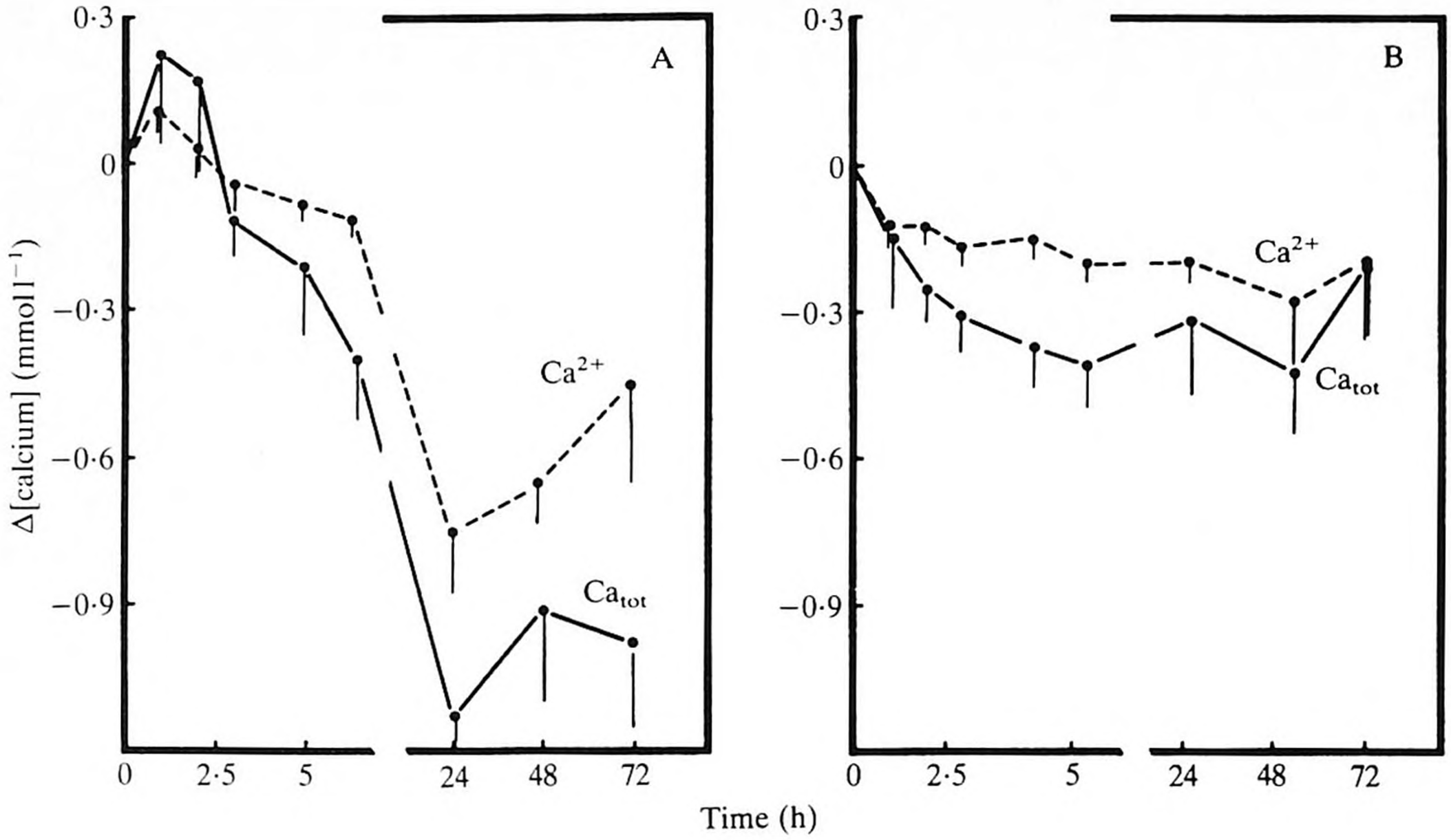

Fig. 2. The effect of trout hypocalcin (A) and trout corpuscles of Stannius residue proteins (B) on plasma total calcium (Ca_{tot}) and blood ionic calcium (Ca^{2+}) in 15-day stanniectomized eels. Means ± S.E.M. are given. For the group receiving hypocalcin injections at time zero, $Ca_{tot} = 4{\cdot}62 \pm 0{\cdot}52$ mmol l^{-1}, $Ca^{2+} = 3{\cdot}06 \pm 0{\cdot}37$ mmol l^{-1} ($N = 5$). For the group receiving residue proteins at time zero, $Ca_{tot} = 6{\cdot}26 \pm 0{\cdot}22$ mmol l^{-1}, $Ca^{2+} = 3{\cdot}07 \pm 0{\cdot}13$ mmol l^{-1} ($N = 7$).

The glycoprotein isolated from trout CS and used for injection experiments has a relative molecular mass of 41K. In a previous study we have reported the same relative molecular mass for trout hypocalcin (Lafeber *et al.* 1988*a*). This value is in good agreement with the apparent relative molecular mass (39K) of the hypocalcaemic principle isolated from salmon CS (Wagner *et al.* 1986). However, the isolated 41K product probably forms the *N*-terminal part of a 54K product (see Lafeber *et al.* 1988*a*). The latter is considered to be the native hypocalcin molecule.

Trout hypocalcin is able to decrease plasma calcium levels in STX eels. The potency of heterologous hypocalcin corroborates the observations of Pang *et al.* (1974, 1981) who have shown a decrease of blood calcium concentrations in killifish after injection with CS tissue homogenates from a variety of fish species.

Intra-arterial injection of hypocalcin results in a decrease of blood ionic and total calcium concentrations. The decrease in ionic calcium fully accounts for the decrease in total calcium. This contrasts with the suggestion made by Bailey & Fenwick (1975) that the CS hormone stimulates the binding of ionic calcium to plasma proteins, without affecting blood total calcium concentration. Our results show that hypocalcin treatment restores the ionic calcium levels of hypercalcaemic STX eels to normal, without causing changes in the other blood calcium fractions. We conclude, therefore, that the hypocalcin regulates the blood ionic calcium

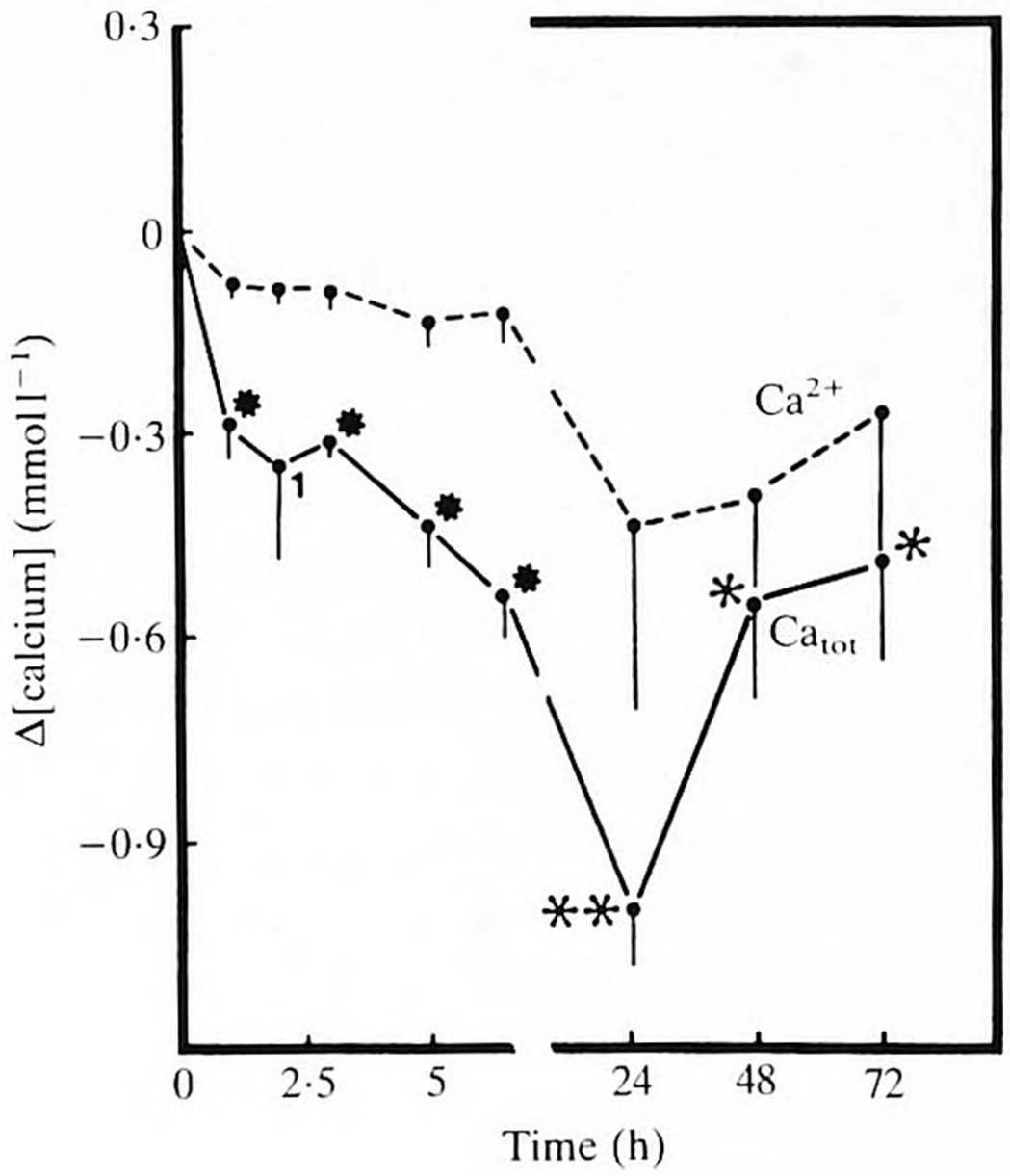

Fig. 3. The effect of bovine parathyroid hormone (Sigma crude extract) on plasma total calcium (Ca_{tot}) and blood ionic calcium (Ca^{2+}) in 15-day stanniectomized eels. Means ± S.E.M. are given. At time zero, $Ca_{tot} = 6{\cdot}47 \pm 0{\cdot}49\,mmol\,l^{-1}$, $Ca^{2+} = 2{\cdot}99 \pm 0{\cdot}41\,mmol\,l^{-1}$ ($N = 4$). The decrease in ionic calcium did not differ statistically from the decrease in controls. Asterisks indicate statistically significant differences between the decrease in ionic and total calcium concentrations; * $P < 0{\cdot}10$; ** $P < 0{\cdot}06$.

fraction. This is probably effected by inhibition of gill Ca^{2+} influx: we have shown that trout hypocalcin injections reduce branchial Ca^{2+} influx without affecting Ca^{2+} efflux, resulting in a net loss of Ca^{2+} *via* the gills (Lafeber *et al.* 1988*b*).

From a physiological point of view, the blood ionic calcium concentration may be more important than the blood total calcium concentration, as has been shown for many higher vertebrates. In 1934, McLean & Hastings elegantly demonstrated that a frog heart acts as a Ca^{2+}-selective electrode. There are, however, few reports on blood ionic calcium homeostasis in fish. Andreasen (1985) reported that the blood ionic calcium level in trout is kept constant under different stress situations, another indication that it is regulated.

Injection of bPTH also results in a decrease of total and ionic calcium concentrations of the blood. The dose of 20 i.u. PTH (0·89 nmol) per 100 g of fish results in a response not significantly different from the effect induced by 50 μg of hypocalcin (0·93 nmol) per 100 g of fish. This means that about equimolar amounts of PTH and hypocalcin are required to obtain a similar calcium-lowering effect, which shows that PTH is highly potent in this heterologous bioassay. This statement seems justified by the fact that ratios of more than 1000:1 have been reported for ovine and fish prolactin effects on sodium levels in several fish species (Clarke, 1973; Hasegawa *et al.* 1986).

Parathyroid glands have never been observed in fish, whereas CS have never been observed in terrestrial vertebrates. The effects of PTH in teleost fish have been reported frequently since Budde (1958) tested PTH in the guppy and showed effects on bone mineralization. Milet *et al.* (1985) concluded that CS extracts and hypocalcin affect calcium handling in STX eel gills in a similar way, and that this effect underlies the hypocalcaemic properties of these hormones. Wendelaar Bonga *et al.* (1986) demonstrated a hypocalcaemic effect of PTH in intact killifish and tilapia adapted to low-calcium water. Effects of CS extracts on terrestrial vertebrates have also been reported. Extracts of eel CS caused an increased calcium release from rat bones *in vitro* (Milet *et al.* 1979*a*), which was recently confirmed in our laboratory for trout CS preparations (Lafeber *et al.* 1986). Milet *et al.* (1980*b*) described the immunological resemblance between PTH and a principle from the CS. These reports inspired further studies on the similarity between PTH and hypocalcin. We have isolated hypocalcin from the CS of trout (Lafeber *et al.* 1988*a*) and reported on its PTH-like activity (Lafeber *et al.* 1988*b*). The present results show hypocalcin-like effects of PTH.

All these observations point to a homology between the CS and the parathyroid glands as has been suggested by Milet and by Lopez and coworkers (Milet *et al.* 1980*b*; Lopez *et al.* 1984). However, although both hypocalcin and PTH reduce plasma calcium levels in fish, there is some difference between the effect of hypocalcin treatment and PTH treatment. Whereas the reduction of plasma calcium by hypocalcin is mainly effected by a change in ionic calcium, bPTH induces a decrease in ionic calcium that does not fully account for the decrease in total calcium. Apparently, both ionic and protein-bound calcium are decreased by PTH. This suggests that hypocalcin and PTH may act, possibly partially, *via* different mechanisms. As mentioned before, hypocalcin is reported to inhibit gill Ca^{2+} influx without affecting Ca^{2+} efflux, which results in a net loss of Ca^{2+} *via* the gills (Lafeber *et al.* 1988*b*). PTH was reported to inhibit gill Ca^{2+} influx (Milet *et al.* 1980*b*). In addition, however, PTH may also act on bone mineralization in fish (Budde, 1958).

The presumed homology between PTH and hypocalcin has been questioned recently because the glands could have different embryological origins (Wendelaar Bonga *et al.* 1986). This subject, however, is still in discussion, because cross-reactivity of hypocalcin with PTH antisera, and co-localization and secretion of immunoreactive secretory protein-I (SP-I) have been reported for both parathyroids and CS (Tisserand-Jochem *et al.* 1987). The presence in both glands of SP-I, a marker protein for endocrine cells from the neural crest, indicates that parathyroids and CS may have a similar embryological origin. However, at variance with the supposed homology between both products is their difference in amino acid sequence. The predicted total amino acid sequence of Australian eel hypocalcin, as reported by Butkus *et al.* (1987), shows no similarity with the amino acid sequence of PTH. The amino acid sequence of eel hypocalcin, however, shows a high degree of similarity with the *N*-terminal amino acid sequences of salmon and trout hypocalcin (Wagner *et al.* 1986; Lafeber *et al.* 1988*a*), which were

reported to be bioactive (Wagner *et al.* 1986; Lafeber *et al.* 1986, 1988*b*). Moreover, hypocalcin is glycosylated, whereas PTH is not. Nevertheless, there is a striking similarity in bioactivity between PTH and hypocalcin. Similarity in the three-dimensional structure of both hormones may underlie this fascinating phenomenon.

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