RAINBOW TROUT HYPOCALCIN STIMULATES BONE RESORPTION IN EMBRYONIC MOUSE CALVARIA IN VITRO IN A PTH-LIKE FASHION

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Summary

Hypocalcin, the major hormone with hypocalcaemic action in fish, was isolated from trout corpuscles of Stannius (SCs). The bioactivity of hypocalcin was assessed in a parathyroid hormone (PTH) bioassay involving bone resorption in embryonic mouse calvaria. Calcium and phosphate release and lactate production were stimulated in a dose-dependent manner by hypocalcin. On a molar basis about equal amounts of hypocalcin and PTH were required to obtain similar effects in this assay. Hypocalcin did not stimulate cyclic AMP production either in mouse calvaria or in cultured osteoblasts. In this respect hypocalcin resembles shortened or N-terminus-modified PTH molecules that induce bone resorption without increasing cyclic AMP levels. Since hypocalcin and PTH have comparable bioactivity in this mammalian bioassay (as well as in fish bioassays), we tentatively suggest that both hormones are structurally similar and that both hormones may act via the same receptors. The two hormones show no resemblance to one another in primary structure, so we suggest that they have similarities in tertiary structure.

Introduction

Calcium homeostasis in terrestrial vertebrates largely depends on the actions of parathyroid hormone (PTH) and vitamin D metabolites, which raise the blood calcium concentration, and of the hormone calcitonin, which induces a fall in the blood calcium concentration (Arnoud, 1983). Fish, like terrestrial vertebrates, regulate blood calcium levels efficiently. The hormones involved in fish calcium metabolism, however, differ from those of the terrestrial vertebrates. Calcium regulation in fish is dominated by the hypercalcaemic action of prolactin or cortisol (Flik et al. 1986; Flik & Perry, 1988) and the hypocalcaemic action of the hormone from the Stannius corpuscles, hypocalcin (Wendelaar Bonga & Pang, 1986). In fish, calcitonin seems of minor importance as a calcium-regulating factor (Fein-

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blatt, 1982). Removal of the Stannius corpuscles (SCs) results in an increase in the plasma calcium concentration, that may be reversed by SC implants or by injections of SC extracts or hypocalcin (Kenyon et al. 1980; Pang et al. 1974; Lafeber et al. 1988b; Hanssen et al. 1989). Hypocalcin isolated from trout SCs (Lafeber et al. 1988a) has an N-terminal amino acid sequence which shows substantial similarity to the hypocalcaemic principle of eel (Butkus et al. 1987) and salmon (Wagner et al. 1986).

Antigenic resemblance between hypocalcin and PTH is indicated by cross-reactivity of antisera raised against bovine PTH (bPTH) with a substance in eel blood plasma (Milet et al. 1982). This immunoreactive PTH-like substance disappears from the blood after removal of the SCs (stannectomy, STX). Moreover, the same antiserum to bPTH cross-reacts with a substance in the SCs of the eel and this cross-reactivity disappears when the glands are stimulated to release their presumed hypocalcaemic principle by experimentally induced hypercalcemia (Lopez et al. 1984).

Injections of both PTH and SC extracts decrease blood calcium levels in fish (Wendelaar Bonga et al. 1986) and increase blood calcium levels in the rat (Milet et al. 1980). Recently, we reported biochemical and histological evidence for a similarity in bioactivity of SC products and of PTH. Products released during in vitro incubation of the SC induced bone resorption in embryonic mouse calvaria in vitro, in a way comparable to PTH (Lafeber et al. 1986).

PTH-stimulated bone resorption depends on the activity of osteoclasts and evidence is increasing that their activity is controlled via osteoblasts. PTH is thought to act directly on the osteoblast; the subsequent signalling from osteoblast to osteoclast may result in bone resorption (Perry et al. 1987; Rodan & Martin, 1981). PTH activity may, therefore, be measured by the activity of osteoblasts. Cyclic AMP has long been considered the most important second messenger for the action of PTH on osteoblasts (Chase & Aurbach, 1970; Herrmann-Erlee & Konijn, 1970). However, as originally suggested by Rasmussen & Tenenhouse (1968), evidence is increasing that Ca\(^{2+}\) also fulfils a second messenger function (Herrmann-Erlee et al. 1983, 1977; Rasmussen & Barret, 1984; Löwik et al. 1985). Activation of either second messenger pathway separately may result in bone resorption (Herrmann-Erlee et al. 1983).

We report here on the effects of trout hypocalcin in a PTH bioassay involving bone resorption in embryonic mouse calvaria. We show that hypocalcin is the bone-resorbing component in an SC tissue homogenate. Hypocalcin is shown to stimulate bone resorption independently of cyclic AMP production.

**Materials and methods**

**Isolation procedure**

Isolation of hypocalcin from rainbow trout (Salmo gairdneri) was performed by concanavalin-A Sepharose-4B affinity chromatography as described in detail elsewhere (Lafeber et al. 1988a). In short, approximately 100 mg of lyophilized SC
tissue (400 mg wet mass obtained from 40 kg of trout) was homogenized and applied to a column. Products without affinity for concanavalin-A are referred to as residue proteins. Material with affinity for concanavalin-A is referred to as hypocalcin. At least 98% of protein in this fraction was pure hypocalcin. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970), in combination with a silver staining procedure (Morrissey, 1981), was routinely performed as a purity check.

**Hormone administration**

Bovine PTH (bPTH) was purchased from Sigma (TCA powder) and dissolved in 0.005 mol l\(^{-1}\) acetic acid containing 1% Pentex albumin. Solutions of 1 i.u. µl\(^{-1}\) were stored in liquid nitrogen. Immediately before use, bPTH was diluted to the desired concentration with culture medium. No differences were observed between the bone-resorbing effect of this bPTH and synthetic bPTH(1–34). Doses are expressed in i.u. per ml of calvarium culture medium.

Approximately 100 mg dry mass of SC yielded 3.0 ± 0.3 mg of purified hypocalcin and 4.5 ± 0.1 mg of residue proteins. The amount of hypocalcin that was added to the calvaria cultures is expressed in mg of BSA equivalents per ml of calvarium culture medium. In accordance with the ratio of hypocalcin and residue proteins obtained after isolation (2/3), 50% more residue protein than hypocalcin was always added in the assays.

**Calvarium culture technique**

Calvaria were removed from 18-day-old mouse embryos and each calvarium was bisected. The left half of one calvarium and the right half of a second one or *vice versa* were fixed in a roller tube containing 1 ml of culture medium (Herrmann-Erlee et al. 1972). The culture medium consisted of 90% Hanks' balanced salt solution (Hanks' BSS) and 10% heat-inactivated human serum. After 24 h of incubation at 37°C, calvaria were removed from the incubation medium. The medium was subsequently analysed for calcium, phosphate and lactate.

**Cyclic AMP production**

For measurements of the cyclic-AMP-stimulating activity of PTH and of hypocalcin, mouse calvaria were incubated in Hanks' BSS containing 0.5% Pentex albumin. After a 15-min incubation at 37°C, calvarial cyclic AMP was extracted by ultrasonication in propanol, and measured using a phosphodiesterase-binding assay according to Lust et al. (1976). Results are presented in pmol of cyclic AMP per two calvarium halves produced per 15 min.

Cyclic-AMP-stimulating activity was also measured in cultured chicken OB-cells. To potentiate cyclic AMP production, the adenylate cyclase activator forskolin (10\(^{-7}\) mol l\(^{-1}\)) was added. The phosphodiesterase inhibitor methylisobutyloxanthine (MIX; 0.22 mg ml\(^{-1}\); Sigma) was added to prevent breakdown of the cyclic AMP produced. Cells were isolated and cultured as described elsewhere (Herrmann-Erlee et al. 1983). The DNA content of the cell cultures was
analytical methods

The total calcium content of the medium was determined with a commercial calcium kit (Sigma). Inorganic phosphate was measured according to the method of Delsal & Manhourin (1958). Combined calcium/phosphorus standards (Sigma) were used as a reference. The lactate concentration of the medium was measured as described by Lowry et al. (1964) using an autoanalyser method (Hekkelman et al. 1974). Lithium lactate (Sigma) was used as reference. Results are expressed in p mol of lactate per two calvarium halves, cultured for 24 h. The protein content of the isolated fractions was determined with a commercial protein kit (Biorad) using bovine serum albumin (BSA) as reference.

Statistical analysis

Statistical evaluation was performed by Student’s t-test (one-tailed). Significance was accepted at $P < 0.05$. Mean values ± s.e.m. are given.

Results

Bone demineralization

The effects of hypocalcin, the residue proteins and bPTH on calcium and phosphate release from mouse calvaria are shown in Fig. 1. Fig. 1A shows that hypocalcin and PTH stimulate calcium release in a dose-dependent manner. The response to both hormones was quantitatively comparable for 1–100 μg ml⁻¹ hypocalcin and $10^{-3}$–$10^{-1}$ i.u. ml⁻¹ PTH. The residue proteins were without effect. Similar results were found for phosphate release (Fig. 1B). Taking 0.1 mg of hypocalcin and 0.1 i.u. of PTH to be equipotent, no significant differences between the degree of stimulation by hypocalcin or by PTH for both parameters could be observed. Over the dose range used for hypocalcin and PTH, the response curves overlap completely (Fig. 1A,B).

A tissue homogenate of 100 mg of trout SC contains bioactivity equivalent to 1 i.u. of PTH (Lafeber et al. 1986). The amount of protein in a tissue extract of 100 mg of trout SC is $4.5 \pm 1.6$ mg ($N = 16$). Therefore, the specific activity of a crude tissue extract (SA_{ext}) can be expressed as 1 i.u. of PTH activity per 4.5 mg of tissue extract protein (SA_{ext} = 1/4.5 = 0.22 i.u. PTH mg⁻¹ protein). The specific activity of purified hypocalcin (SA_{hyp}) is 1.00 i.u. PTH mg⁻¹ protein. According to this bone resorption assay then, the purification factor for hypocalcin (SA_{ext}/SA_{hyp}) is 1.00/0.22 or 4.5.

Lactate production

The effects of hypocalcin, residue proteins and bPTH on lactate production in mouse calvaria are shown in Fig. 2. Doses of hypocalcin and PTH that gave...
Actions of hypocalcin

Fig. 1. (A) Effects of hypocalcin (●), residue proteins (○) and bovine PTH (▲) on calcium release from two calvarium halves, cultured for 24 h. Mean values ±s.E.M. are given (hypocalcin and residue proteins, N = 8; PTH, N = 4). (B) Effects of hypocalcin, residue proteins and bovine PTH on phosphate release from two calvarium halves, cultured for 24 h. Mean values ±s.E.M. are given (hypocalcin and residue proteins, N = 8; PTH, N = 4).

comparable responses when analysed for calcium and phosphate release, also gave a comparable stimulation of lactate production. Taking the SA of hypocalcin calculated above on the basis of calcium and phosphate release (1.00 i.u. PTH activity mg⁻¹ hypocalcin), we found similar dose–response curves for hypocalcin and PTH with respect to lactate production. Residue proteins, which did not stimulate release of calcium and phosphate from bone, stimulated lactate production significantly and in a dose-dependent manner.

Cyclic AMP production

Table 1 shows that SC tissue extract, residue proteins and hypocalcin have no effect on cyclic AMP production in mouse calvaria, whereas PTH does stimulate cyclic AMP production. Also, hypocalcin was unable to stimulate cyclic AMP production in OB-like cells (both in the presence and in the absence of forskolin). PTH (0.05 i.u.) gave a significant stimulation of cyclic AMP production in these cells and its effect was potentiated by forskolin (9 ± 1 and 114 ± 21 pmol cyclic
Fig. 2. Effects of hypocalcin (●), residue proteins (○) and bovine PTH (▲) on lactate production of two calvarium halves, cultured for 24 h. Mean values ± s.e.m. are given (hypocalcin and residue proteins, N = 8; PTH, N = 4).

Table 1. Effects of SC crude tissue homogenates, hypocalcin, residue protein and PTH on cyclic AMP production in mouse calvaria

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cyclic AMP (pmol/two calvarium halves)</th>
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<tr>
<td>Control</td>
<td>1.4 ± 0.6 (7)</td>
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<tr>
<td>PTH (0.25 i.u. ml⁻¹)</td>
<td>50 ± 11 (7)*</td>
</tr>
<tr>
<td>(0.5 i.u. ml⁻¹)</td>
<td>180 ± 8 (7)*</td>
</tr>
<tr>
<td>SC tissue extract (5 mg ml⁻¹)</td>
<td>2.1 ± 1.2 (3)</td>
</tr>
<tr>
<td>SC tissue extract (5 mg ml⁻¹) +</td>
<td></td>
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<tr>
<td>10⁻⁷ mol l⁻¹ forskolin</td>
<td></td>
</tr>
<tr>
<td>Hypocalcin (500 µg ml⁻¹)</td>
<td>−1.1 ± 1.3 (9)</td>
</tr>
<tr>
<td>Residue proteins (600 µg ml⁻¹)</td>
<td>2.1 ± 1.8 (6)</td>
</tr>
</tbody>
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Calvaria were incubated for 10 min. Mean values ± s.e.m. are given with the number of observations in parentheses. Asterisks indicate statistical significance (P < 0.05).
AMP μg⁻¹ DNA, respectively). No significant stimulation of cyclic AMP production by hypocalcin was found.

**Discussion**

From the data presented in this paper we conclude that hypocalcin is the bone-resorbing component of the SC tissue extract and that hypocalcin stimulates bone resorption in a PTH-like fashion. Two parameters characteristic of PTH-induced bone resorption, calcium/phosphate release and lactate production, are stimulated in a dose-dependent manner by hypocalcin.

We have previously reported that rainbow trout Stannius corpuscles (SCs) produce and secrete a product that resembles PTH in its bone-resorbing effect in mouse calvaria (Lafeber et al. 1986). The observations presented here substantiate and extend our previous report and show that a single product in an SC extract, the glycoprotein hypocalcin, is responsible for the PTH-like bone-resorbing activity.

The 4.5-fold purification calculated from the bone resorption assay is in good agreement with the fourfold purification that we calculated from a fish bioassay involving calcaemic responses in eels (Lafeber et al. 1988b). The low purification factor indicates that the SCs store an abundance of hypocalcin. The high degree of granulation of the hypocalcin-containing cells of freshwater fish (Lafeber et al. 1986) is consistent with this notion.

The activity of 1 mg of hypocalcin (1.85×10⁻⁸ mol) is equivalent to the activity of 1 i.u. of PTH (approx. 4×10⁻⁸ mol). On a molar basis, then, about equal amounts of hypocalcin and PTH were needed to obtain a similar bone-resorbing effect in mouse calvaria. Although hypocalcin seems rather potent in this heterologous PTH bioassay, PTH proved to be as potent in an in vivo fish bioassay; we calculated that equimolar amounts of hypocalcin and PTH have similar hypocalcaemic effects in eel (Lafeber et al. 1988b). Apparently, both hypocalcin and PTH can evoke physiological responses in a mammalian and a fish bioassay. We tentatively conclude, therefore, that both calcitropic hormones can activate PTH-dependent as well as hypocalcin-dependent targets.

Hypocalcin is able to increase lactate production in mouse calvaria. Lactate production may serve as a parameter for PTH-induced bone resorption since PTH-induced bone resorption is always coupled to lactate production (Herrmann-Erlee et al. 1972; Herrmann-Erlee & Van der Meer, 1974). PTH fragments reported to induce bone resorption (Herrmann-Erlee et al. 1983) also induce lactate production (Łowik et al. 1984). Residue proteins, which were not able to induce calcium and phosphate release, were also able to stimulate lactate production in a dose-dependent way. This suggests that residue proteins contain a factor which is able to enhance calvarial metabolism without increasing bone resorption. These observations corroborate our previous results where we showed that an SC tissue extract contains two products that can stimulate lactate production: a factor that stimulates bone resorption and one that does not
Although PTH-induced bone resorption is always coupled to lactate production, lactate production is not necessarily coupled to bone resorption (Herrmann-Erlee & Van der Meer, 1974). Therefore, lactate production per se is not a suitable parameter for assessing bone-resorbing activity.

Although cyclic AMP is reported to be an important second messenger for the bone-resorbing activity of PTH, hypocalcin was, in contrast to PTH and despite its PTH-like bone-resorbing effects, not able to increase cyclic AMP production. However, it has been demonstrated that the bone-resorbing effect of PTH is not fully dependent on cyclic AMP as a second messenger. PTH fragments modified or shortened at the N-terminus induce bone resorption without increasing cyclic AMP levels (Herrmann-Erlee et al. 1983). We therefore suggest that hypocalcin resembles PTH but lacks, as do the PTH fragments, the part of the molecule that is essential for cyclic AMP production.

We have shown before that the active principle of the SCs stimulates osteoclastic activity, as does PTH. Furthermore, the bone-resorbing effects of both substances appeared to be exerted via the same pathway, since no additive effect was observed when maximum stimulating concentrations of the active SC principle and PTH were tested together (Lafeber et al. 1986). On the basis of these results and the similarities in bioactivity of hypocalcin and PTH (N-terminal-shortened fragments) in the PTH bioassay reported in this paper, we hypothesize that both hormones act via the same receptor.

If we assume that both hormones act via the same receptor, then hypocalcin should stimulate bone resorption via changes in cytosolic Ca\textsuperscript{2+} concentration, as has been shown for shortened PTH fragments (Löwik et al. 1985). Unfortunately, tests on the effects of hypocalcin on Ca\textsuperscript{2+} influx in OB-like cells using the Quin-2 fluorescence method (Löwik et al. 1985) were unsuccessful because of a high and interfering autofluorescence of hypocalcin. Our former observation, however, that hypocalcin increases the number of osteoclasts in mouse calvaria (Lafeber et al. 1986) – the increase in osteoclasts is known to be mediated by Ca\textsuperscript{2+} as a second messenger (Herrmann-Erlee et al. 1988) – gives indirect evidence that hypocalcin induces Ca\textsuperscript{2+} influx in bone cells. In this respect hypocalcin may be a useful tool to investigate further the two binding sites of the PTH receptor and the relative contribution of both second messengers to the PTH-induced bone-resorbing activity (Herrmann-Erlee et al. 1988).

Prostaglandins (Klein & Raisz, 1970; MacDonald, 1986) and several PTH-like tumour factors (Mundy et al. 1984) may induce bone resorption without activating the PTH receptor. One could argue, then, that the bone-resorbing activity of hypocalcin could also result from the activation of a pathway different from the one activated by PTH. However, our observations favour a common receptor/second messenger pathway for hypocalcin and PTH. The similarity between PTH and hypocalcin is not restricted to PTH-like bone-resorbing activity in vitro. SC tissue extracts have been reported to exert hypercalcaemic effects in rats (Milet et al. 1980). In contrast, PTH and hypocalcin have been reported to exert hypocalcaemic effects in intact fish (Wendelaar Bonga et al. 1986). We have
recently shown that both PTH and hypocalcin counteract the development of hypercalcaemia in stanniectomized eels (Lafeber et al. 1988b).

The similarity in bioactivity seems, at first sight, incompatible with the lack of similarity in primary structure between hypocalcin and PTH. Although immunocross-reactivity has been reported between some PTH antisera and the products released by the SC (Milet et al. 1982), the amino acid sequence of hypocalcin (Lafeber et al. 1988a; Butkus et al. 1987; Wagner et al. 1986) shows no similarity with that of PTH (or any other known peptide). Furthermore, whereas PTH is a peptide, hypocalcin is a glycoprotein, possibly composed of two subunits (Lafeber et al. 1988a). We postulate, therefore, that these hormones partially resemble each other not in their primary structure but in their three-dimensional configuration. This would be a plausible explanation for the immunological similarities as well as for the similarities in bioactivity of these hormones in homologous and heterologous bioassays.

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