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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.* 1988*b*; Hanssen *et al.* 1989). Hypocalcin isolated from trout SCs (Lafeber *et al.* 1988*a*) 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 (stanniectomy, 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 hypercalcaemia (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.* 1988*a*). 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 methylisobutylxanthine (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 determined according to Karsten & Wollenberger (1977). Results are expressed in pmol of cyclic AMP per μg of DNA per 15 min.

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 μmol 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 $P<0{\cdot}05$. 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 ± 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 ($SA_{ext}=1/4{\cdot}5=0{\cdot}22$ 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

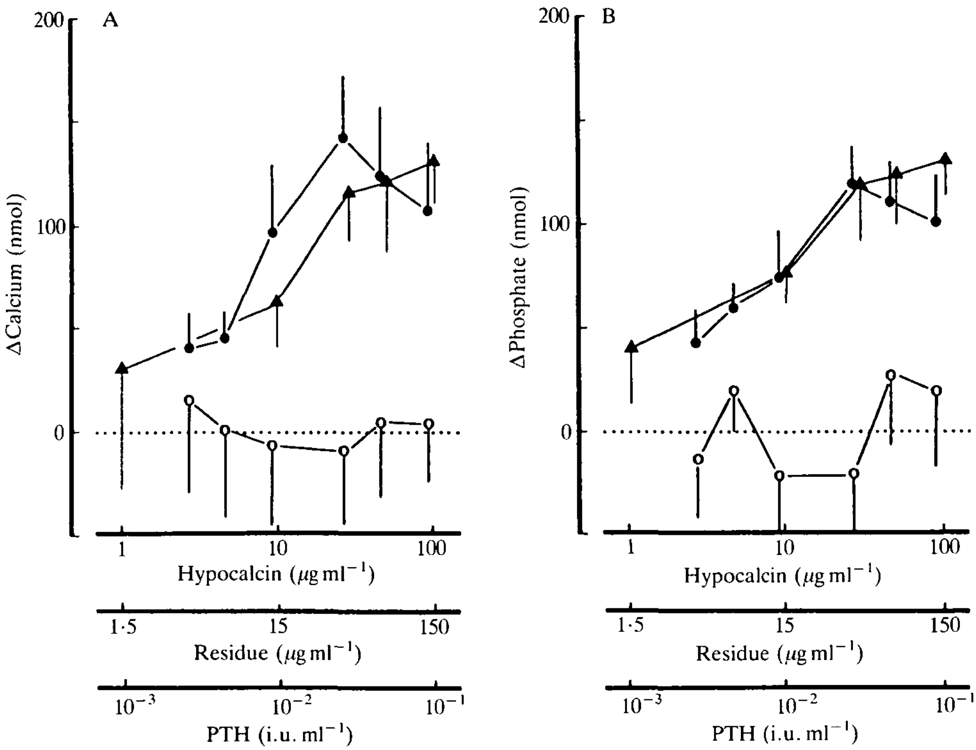

Fig. 1. (A) Effects of hypocalcin (●), residue proteins (○) and bovine PTH (▲) on calcium release from two calvarium halves, cultured for 24 h. Mean values +S.E.M. are given (hypocalcin and residue proteins, $N = 8$; PTH, $N = 4$). (B) Effects of hypocalcin, residue proteins and bovine PTH on phosphate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

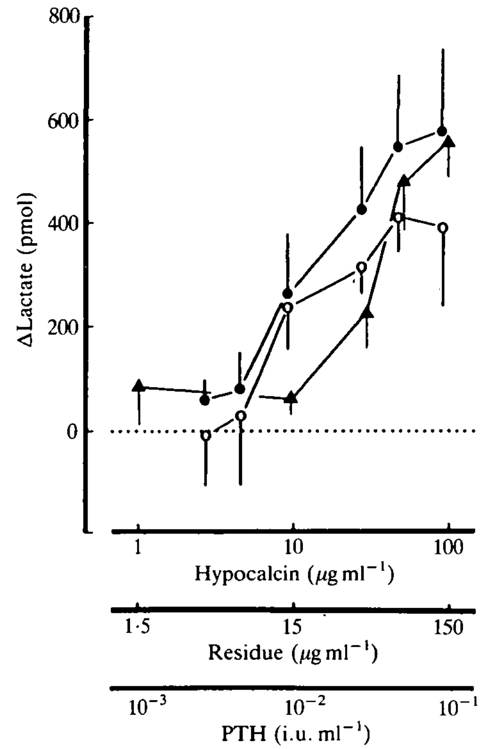

Fig. 2. Effects of hypocalcin (●), residue proteins (○) and bovine PTH (▲) on lactate production of two calvarium halves, cultured for 24 h. Mean values +s.e.m. are given (hypocalcin and residue proteins, $N = 8$; PTH, $N = 4$).

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

	(pmol/two calvarium halves)
Control	1·4 ± 0·6 (7)
PTH (0·25 i.u. ml^{-1})	50 ± 11 (7)*
(0·5 i.u. ml^{-1})	180 ± 8 (7)*
SC tissue extract (5 mg ml^{-1})	2·1 ± 1·2 (3)
SC tissue extract (5 mg ml^{-1})+ 10^{-7} mol l^{-1} forskolin	1·4 ±3·2 (3)
Hypocalcin (500 μg ml^{-1})	−1·1 ± 1·3 (9)
Residue proteins (600 μg ml^{-1})	2·1 ± 1·8 (6)

Calvaria were incubated for 10 min.
Mean values ± s.e.m. are given with the number of observations in parentheses.
Asterisks indicate statistical significance ($P < 0{\cdot}05$).

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.* 1988*b*). 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 ($1{\cdot}85\times10^{-8}$ 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.* 1988*b*). 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 (Löwik *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

(Lafeber *et al.* 1986). 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^{2+} concentration, as has been shown for shortened PTH fragments (Löwik *et al.* 1985). Unfortunately, tests on the effects of hypocalcin on Ca^{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^{2+} as a second messenger (Herrmann-Erlee *et al.* 1988) – gives indirect evidence that hypocalcin induces Ca^{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.* 1988*b*).

The similarity in bioactivity seems, at first sight, incompatible with the lack of similarity in primary structure between hypocalcin and PTH. Although immuno-cross-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.* 1988*a*; 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.* 1988*a*). 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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References

ARNOUD, S. D. (1983). Hormonal regulation of calcium homeostasis. In *Assay of Calcium Regulating Hormones* (ed. D. D. Bikle), p. 1. New York: Springer-Verlag.

BUTKUS, A., ROCHE, P. J., FERNLEY, R. T., HARALAMBIDIS, J., PENSCHOW, J. D., RYAN, G. B., TRAIHAIR, J. F. & COGHLAN, J. P. (1987). Purification and cloning of a corpuscles of Stannius protein from *Anguilla australis*. *Molec. cell. Endocr.* **54**, 123–128.

CHASE, L. R. & AURBACH, G. D. (1970). The effect of parathyroid hormone on the concentration of adenosine 3′-5′monophosphate in skeletal tissue *in vitro*. *J. biol. Chem.* **245**, 1520–1526.

DELSAL, S. L. & MANHOURIN, H. (1958). Etude comparative des dosages colorimétriques du phosphore. IV. Dosage de l'orthophosphate en présence d'esters phosphoriques. *Bull. Soc. Biol., Paris* **40**, 1623–1626.

FEINBLATT, J. D. (1982). The comparative physiology of calcium regulation in submammalian vertebrates. In *Advances in Comparative Physiology and Biochemistry*, vol. 8. (ed. O. Löwenstein), pp. 74–97. New York: Academic Press.

FLIK, G., FENWICK, J. C., KOLAR, Z. & WENDELAAR BONGA, S. E. (1986). Effects of ovine prolactin on calcium uptake and distribution in *Oreochromis mossambicus*. *Am. J. Physiol.* **250**, R161–R166.

FLIK, G. & PERRY, S. F. (1989). Cortisol stimulates whole body calcium uptake and the branchial calcium pump in freshwater rainbow trout. *J. Endocr.* **120**, 75–82.

HANSSEN, R. G. J. M., LAFEBER, F. P. J. G., FLIK, G. & WENDELAAR BONGA, S. E. (1989). Ionic and total calcium levels in the blood of the European eel (*Anguilla anguilla*): effects of stanniectomy and hypocalcin replacement therapy. *J. exp. Biol.* **141**, 177–186.

HEKKELMAN, J. W., VAN DER VOORT-DUINDAM, C. J. M. & DIBON, A. (1974). Metabolism of bone cells in a superfusion system. II. Hormone effects on the production of lactate by rat calvaria. *Kon. Ned. Acad. Wetensch.* **C77**, 182–186.

HERRMANN-ERLEE, M. P. M., GAILLARD, P. J., HEKKELMAN, J. W. & NIJWEIDE, P. J. (1977). The effect of verapramil on the action of parathyroid hormone on embryonic bone *in vitro*. *Eur. J. Pharm.* **46**, 51–58.

HERRMANN-ERLEE, M. P. M. & KONIJN, T. M. (1970). Effects of parathyroid extracts on cyclic AMP content of embryonic mouse calvaria. *Nature, Lond.* **227**, 177–179.

HERRMANN-ERLEE, M. P. M., NIJWEIDE, P. J., VAN DER MEER, J. M. & OOMS, M. A. C. (1983). Action of bovine PTH and bovine PTH fragments on embryonic bone *in vitro*: dissociation of the cyclic AMP and bone resorbing response. *Calcif. Tiss.* **35**, 70–77.

HERRMANN-ERLEE, M. P. M. & VAN DER MEER, J. M. (1974). The effect of dibutyryl cyclic AMP, aminophylline and propanolol on PTE-induced bone resorption *in vitro*. *Endocrinology* **94**, 424–434.

HERRMANN-ERLEE, M. P. M., VAN DER MEER, J. M., LOWIK, C. W. G. M., VAN LEEUWEN, J. P. T. M. & BOONEKAMP, P. M. (1988). Different roles for calcium and cyclic AMP in the action of PTH; studies in bone explants and isolated bone cells. *Bone* (in press).

HERRMANN-ERLEE, M. P. M., VAN ZUYLEN, J. G. S. & HEKKELMAN, J. W. (1972). Studies on the interrelationships of various parathyroid hormone effects on embryonic bone *in vitro*. In *Proceedings of the Fourth Parathyroid Conference.* (ed. R. V. Talmage & P. L. Munson), pp. 463–466. Amsterdam: Excerpta Medica Foundation.

KARSTEN, K. & WOLLENBERGER, A. (1977). Improvements in the ethidium bromide method for the direct fluorimetric estimation of DNA and RNA in cell and tissue homogenates. *Analyt. Biochem.* **77**, 464–467.

KENYON, C. J., CHESTER JONES, I. & DIXON, R. N. B. (1980). Acute effects of the freshwater eel (*Anguilla anguilla*) on extracts of the corpuscles of Stannius opposing the effects of stanniosomatiectomy. *Gen. comp. Endocr.* **41**, 531–538.

KLEIN, D. C. & RAISZ, L. G. (1970). Prostaglandins: stimulation of bone resorption in tissue culture. *Endocrinology* **80**, 1436–1440.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature, Lond.* **227**, 680–684.

LAFEBER, F. P. J. G., HANSSEN, R. G. J. M., CHOY, Y. M., FLIK, G., HERRMANN-ERLEE, M. P. M., PANG, P. K. T. & WENDELAAR BONGA, S. E. (1988*a*). Identification of hypocalcin (teleocalcin) isolated from trout Stannius corpuscles. *Gen. comp. Endocr.* **69**, 19–30.

LAFEBER, F. P. J. G., HANSSEN, R. G. J. M. & WENDELAAR BONGA, S. E. (1988*b*). Hypocalcaemic activity of trout hypocalcin and bovine parathyroid hormone in stanniecomized eels. *J. exp. Biol.* **140**, 199–208.

LAFEBER, F. P. J. G., SCHAEFER, H. I. M. B., HERRMANN-ERLEE, M. P. M. & WENDELAAR BONGA, S. E. (1986). Parathyroid hormone-like effects of rainbow trout Stannius products on bone resorption of embryonic mouse calvaria *in vitro*. *Endocrinology* **119**, 2249–2255.

LOPEZ, E., TISSERAND-JOCHEM, E., EYQUEM, A., MILET, C., HILLYARD, C., LALLIER, F., VIDAL, B. & MACINTYRE, I. (1984). Immunocytochemical detection in eel corpuscles of Stannius of a mammalian parathyroid-like hormone. *Gen. comp. Endocr.* **52**, 28–36.

LOWRY, O. H., PASSONNEAU, J. K., HASSELBERGER, F. X. & SCHULZ, D. W. (1964). Effects of ischemia, unknown substrates, and cofactors of the glycolytic pathway in brain. *J. biol. Chem.* **239**, 18–21.

LÖWIK, G. W. G. M., VAN LEEUWEN, J. P. T. M., VAN DER MEER, J. M., VAN ZEELAND, J. K., SCHEEVEN, B. A. A. & HERRMANN-ERLEE, M. P. M. (1985). A two receptor model for the action of parathyroid hormone on osteoblasts: a role for intracellular calcium and cyclic AMP. *Cell Calcium* **6**, 311–316.

LÖWIK, C. W. G. M., VAN ZEELAND, J. K., VAN DER MEER, J. M. & HERRMANN-ERLEE, M. P. M. (1984). Effects of PTH fragments on ornithine decarboxylase activity and lactate production in isolated osteoblasts. In *Endocrine Control of Bone and Calcium Metabolism*, vol. 8B (ed. D. V. Cohn, J. T. Potts & T. Fujita), pp. 248–256. Amsterdam, New York, Oxford: Exerpta Medica.

LUST, W. P., ERNESTINE, D., DEATON, A. V. & PARSONNEAU, J. V. (1976). A modified cyclic AMP binding assay. *Analyt. Biochem.* **72**, 8–12.

MACDONALD, B. R. (1986). Parathyroid hormone, prostaglandins and bone resorption. *Wld. Rev. Natr. Diet.* **47**, 163–166.

MILET, C., HILLYARD, C. J., MARTELLY, E., CHARTIER, M. M., GIRGIS, S., MCINTYRE, I. & LOPEZ, E. (1982). A parathyroid-like hormone from eel corpuscles of Stannius which exhibits hypocalcaemic action. In *Comparative Endocrinology of Calcium Regulation* (ed. C. Oguro & P. K. T. Pang), pp. 181–185. Tokyo, Japan: Scientific Society Press.

MILET, C., HILLYARD, C. J., MARTELLY, E., GIRGIS, S., MACINTYRE, I. & LOPEZ, E. (1980). Similitudes structurales entre l'hormone hypocalcémiante des corpuscules de Stannius (PCS) de l'anguille (*Anguilla anguilla* L.) et l'hormone parathyroidienne mammalienne. *C.R. hebd. Séanc. Acad. Sci. Paris* **291**, 977–980.

MORRISSEY, J. H. (1981). Silverstain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Analyt. Biochem.* **117**, 307–310.

MUNDY, G. R., IBBOTSON, K. J., D'SOUZA, S. M., SIMPSON, E. L., JACOBS, J. W. & MARTIN, T. J. (1984). The hypercalcemia of calcium: clinical implications and pathogenic mechanisms. *New Engl. J. Med.* **310**, 1718–1722.

PANG, P. K. T., PANG, R. K. & SAWYER, H. (1974). Effects of environmental calcium and replacement therapy on the killifish (*Fundulus heteroclitus*) in bioassays for the hypocalcaemic response to Stannius corpuscles from killifish and cod (*Gadus morhua*). *Endocrinology* **94**, 548–555.

PERRY, H. M., SKOGEN, W., CHAPPEL, J. C., WILNER, G. D., KAHN, A. J. & TEITELBAUM, S. L. (1987). Conditioned medium from osteoblast-like cells mediate parathyroid hormone induced bone resorption. *Calcif. Tissue. Int.* **40**, 298–300.

RASMUSSEN, H. & BARRET, P. Q. (1984). Calcium messenger systems: An integrated view. *Physiol. Rev.* **64**, 938–976.

RASMUSSEN, H. & TENENHOUSE, A. (1968). Cyclic adenosine monophosphate, Ca^{2+} and membranes. *Proc. natn. Acad. Sci. U.S.A.* **59**, 1364–1367.

RODAN, G. A. & MARTIN, T. J. (1981). Role of osteoblasts in hormonal control of bone resorption – A hypothesis. *Calcif. Tiss. Int.* **33**, 349–351.

WAGNER, G. F., HAMPONG, M., PARK, C. M. & COPP, D. H. (1986). Purification, characterisation, and bioassay of teleocalcin, a glycoprotein from salmon corpuscles of Stannius. *Gen. comp. Endocr.* **63**, 481–491.

WENDELAAR BONGA, S. E. & PANG, P. K. T. (1986). Stannius corpuscles. In *Vertebrate Endocrinology, Fundamentals and Biomedical Implications,* vol. 1 (ed. P. K. T. Pang & M. P. Schreibman), pp. 439–464. New York: Academic Press.

WENDELAAR BONGA, S. E., PANG, P. K. T. & PANG, R. K. (1986). Hypocalcaemic effects of bovine parathyroid hormone(1–34) and Stannius corpuscle homogenates in teleost fish adapted to low-calcium water. *J. exp. Zool.* **240**, 363–367.