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Control of Calcium Regulating Hormones in the Vertebrates: Parathyroid Hormone, Calcitonin, Prolactin, and Stanniocalcin

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I. Introduction

Control of ionic calcium levels of the extracellular fluids is mandatory for all vertebrates, since only minor changes of calcium, in particular the ionized calcium fraction, have pronounced effects on the permeability of cell membranes to ions and, consequently, on important physiological processes such as muscle contraction, nerve signal transduction, and control of cellular metabolism. Hypocalcemia leads to increased excitability of cellular membranes, which may result in tetany and seizures. Hypercalcemia reduces neuromuscular transmission and induces myocardial dysfunction and lethargy. Both conditions can be lethal, at least for mammals and birds. Lower vertebrates, in particular fish, seem more resistant to chronic changes in extracellular calcium, although they eventually show the same symptoms (Pang, 1971; Peacock, 1980). It is likely that the dramatic effects of extracellular Ca^{2+} perturbations are the reason that Ca^{2+} concentrations are more tightly controlled than the concentrations of other extracellular ions. The total of extracellular calcium represents less than 0.1% of total body calcium. About 0.5% is located intracellularly and 99.4% in the skeleton. The large amounts of calcium in the skeleton not only give strength to the body, but also represent an easily accessible supply of calcium that can be mobilized when the calcium concentration of the extracellular fluid is low, and a large reservoir in which calcium can be deposited when the extracellular calcium level tends to rise. The external sources of calcium are either exclusively dietary in origin, as in the terrestrial tetrapods, or are represented by diet and the ambient water, as in the aquatic vertebrates that possess gills. The gut, or the gut and the gills, respectively, are the organs involved in calcium uptake in terrestrial and aquatic vertebrates.

In terrestrial animals calcium is taken up with the food—an episodic process—and transported from the gut to the blood. In general calcium

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uptake is balanced by renal calcium excretion, with exception of periods of body growth or, in females, periods of reproduction and parental care. During growth, large amounts of calcium are taken up, transported via the blood, and deposited in the skeleton. In female animals large amounts of calcium are accumulated in the eggs or, in mammals, transferred to the offspring via placenta or milk glands.

In the aquatic vertebrates the uptake of calcium from the environment is essentially a continuous process and takes place via the gut as well as the gills, for which the water serves as a practically limitless supply. Similar to the terrestrial vertebrates, during periods of growth and, in females, reproduction, substantial amounts of calcium are taken up, transported to the blood, and deposited in the skeleton or the ovaries, respectively.

Thus, under all conditions animals experience influx and efflux of calcium into the body circulation, and since this extracellular calcium concentration is maintained at a fairly constant level or "setpoint" (Brown *et al.*, 1987), effective homeostatic control mechanisms must be present. During periods of growth and during the female reproductive periods the influxes and effluxes are highly increased, and consequently the demand on these control mechanisms is much higher than usual.

As will be discussed later in this review, the setpoint for the concentration of ionic calcium of the extracellular fluid is obviously not fixed for a lifetime in an individual, and we feel that the homeostatic control of calcium may be more flexible than often has been assumed, in particular in the lower vertebrates. Nevertheless, it is beyond doubt that the ionic calcium concentration generally is controlled within narrow limits and that this control is effected by complex hormonal mechanisms.

Many hormones have effects on the handling of calcium at the cellular and organismal levels, which is obviously connected with the circumstance that calcium has so many diverse functions. Traditionally, parathyroid hormone (PTH) and calcitonin (CT) are considered the main calcium-regulating hormones for hypercalcemic and hypocalcemic control, respectively, by regulating calcium influx and efflux to the blood at the level of the gut, the bone and the kidneys. Vitamin D, in particular its metabolite $1,25(OH)_2D_3$, is the third important calcium-controlling factor, but its action concerns total body calcium balance rather than homeostasis of the extracellular fluid. The steroid will therefore not be considered in this review. PTH rather than CT is considered the dominating hormone in calcium homeostasis, mainly because the plasma calcium level has the tendency to fall in the absence of PTH, whereas CT deficiency is often without serious consequences.

It may be surprising, given the importance of PTH for calcium homeostasis in mammals and birds, that true parathyroid glands are present only

in the terrestrial vertebrates and in those amphibians that spend most of their lifetime outside the water (see Section II,A). These glands have not been found in typical aquatic vertebrates: fishes and amphibians with a predominantly aquatic lifestyle, such as many salamandrids (Fig. 1). The absence of PTH in the aquatic vertebrates contrasts with the presence of calcitonin in all vertebrates except the jawless fishes (see Sections II,B and III,B). A hypercalcemic hormone homologous with PTH has not been identified in animals without parathyroid glands, although prolactin, a hormone that occurs in all major vertebrate groups, exerts effects in several amphibians and fish that show some similarities with the action of PTH. The characteristics of prolactin as a calcium-regulating hormone will be discussed in Section III,A.

In this review we want to stress that there is what we consider a fundamental difference between the homeostatic control of plasma calcium levels between terrestrial and aquatic vertebrates. We conclude from the available literature that in the aquatic vertebrates the plasma calcium levels do not fall in the absence of regulatory hormones, but may remain constant or tend to rise. The evidence will be discussed in Section IV. This can explain why a predominating hypercalcemic hormone such as PTH is not found in the purely aquatic animals, and, consequently, why the parathyroid glands may have evolved during the water-to-land transition. This new concept also provides the explanation for the presence of a potent hypocalcemic or antihypercalcemic hormone in bony fishes that is unique for these animals: stanniocalcin, the hormone of the Stannius corpuscles (Section III,C; Fig. 1).

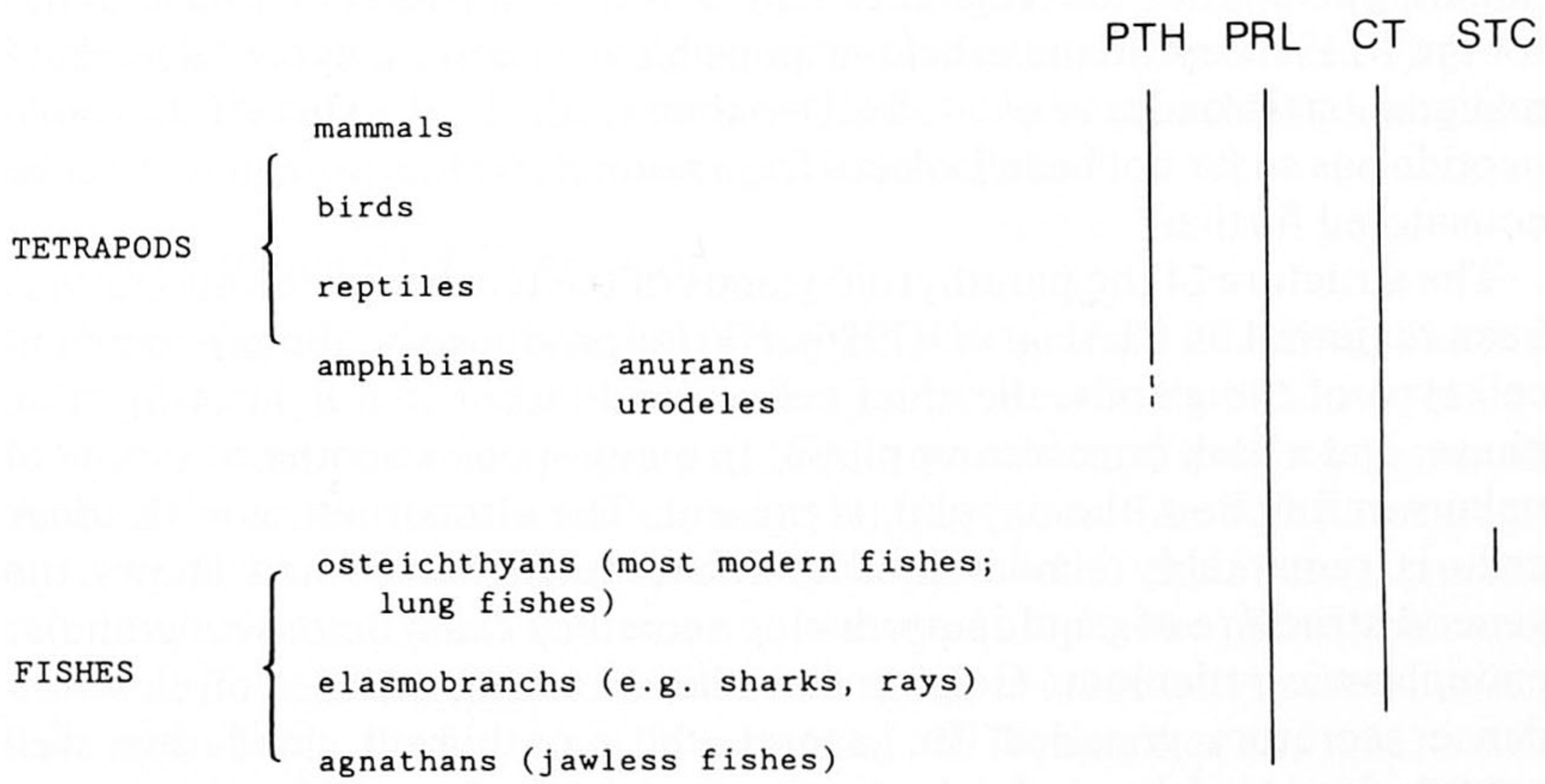

FIG. 1. The occurrence among the vertebrate classes of the four calcium-regulating hormones discussed in this paper.

These differences are sufficient reason for us to treat the terrestrial and aquatic vertebrates separately in this review, which is aimed at defining the function of PTH, calcitonin, prolactin, and stanniocalcin in the control of extracellular Ca^{2+}, by analyzing the factors controlling biosynthesis and release of these hormones.

II. Terrestrial Vertebrates

A. Parathyroid Hormone

1. Introduction

PTH dominates the control of the Ca^{2+} concentration of the extracellular fluid by its calcium-mobilizing action on bone and, indirectly, via its control of the synthesis of $1,25(OH)_2D_3$, intestinal calcium absorption. PTH further regulates renal phosphate excretion and has anabolic effects on bone tissue. The physiology of the hormone has been reviewed recently by Aurbach (1988) and Hurwitz (1989). PTH is a single-chain nonglycosylated protein. In the few mammalian species in which the primary structure has been elucidated, native PTH contains 84 amino acid residues and has a molecular weight of 9600. Analysis of the PTH sequences of human, rat, cow, pig, and dog has shown a great deal of structural homology (Cohn *et al.*, 1986; Kemper, 1986). The PTH molecule of chicken, the only nonmammalian species of which the hormone has been sequenced so far, consists of 88 amino acids, with striking homology with the mammalian hormones in the 1-32 region (Khosla *et al.*, 1988). Recently a factor sharing homology with the 1-13 region of PTH has been isolated that has affinity for the PTH receptor and is held responsible for humoral hypercalcemia of malignancy (Broadus *et al.*, 1988; Donahue *et al.*, 1990). This PTH-related peptide has so far not been isolated from normal PTH tissue and will not be considered further.

The structure of the parathyroid glands of the terrestrial vertebrates has been reviewed by Clark *et al.* (1986). PTH is produced by the predominant cell type of the glands, the chief cells, which occur in a light or inactive phase, and a dark or secretory phase. In many species another cell type of unknown function, the oxyphil, is present. The ultrastructure of the dark cells is remarkably similar in amphibians to mammals and shows the general structure of peptide-producing secretory cells: extensive granular endoplasmic reticulum, Golgi areas, and a varying number of electron-dense secretory granules. In general, the parathyroid glands are well vascularized and have a rich nervous supply. In mammals the nervous innervation is mainly adrenergic; in birds both adrenergic and cholinergic.

In both groups most nerves are associated with the vascular system, indicating that they regulate the blood flow through the gland (Clark *et al.*, 1986). For man and some birds, direct nervous contacts with parathyroid cells have been reported. In many avian species the parathyroid gland is associated with a carotid body. The function of this association is unknown (Clark *et al.*, 1986).

2. Biosynthesis and Release of PTH

The synthesis of PTH follows the general pattern of protein hormones. Since any of these steps represents a potential target for extracellular regulatory factors, this process will be discussed briefly. It has been reviewed in detail by Cohn and MacGregor (1981), Habener *et al.* (1984) and, more recently, by Kemper (1986) and Aurbach (1988). After formation of prepro-mRNA by transcription and posttranscriptional processing of mRNA, the message is translated in the prepro form of the hormone by ribosomes bound to the endoplasmic reticulum. The signal- or presequence typically consists of 25 amino acid residues, and is removed rapidly from the prohormone upon its entering the cisternal space of the endoplasmic reticulum. The prohormone is subsequently transported via vesicles to the Golgi compartment, where additional processing and packaging into secretory granules takes place. The prosequence typically consists of six amino acids for all mammalian and avian species examined. The conversion of proPTH to PTH takes place in the Golgi compartment by proPTH-converting enzyme. Autoradiography of the parathyroid glands *in vivo* or *in vitro* with labeled amino acids has shown that within 2 minutes after administration of the label it is found primarily in the endoplasmic reticulum, after 10 minutes in the Golgi area, and by 20 to 30 minutes in the secretory granules. After packaging of the hormone in the secretory granules, the hormone is either stored in the cytoplasm, released into the circulation by exocytosis, or degraded intracellulary.

3. Intracellular PTH Degradation

The blood circulation contains many PTH fragments in addition to the native molecule, as has been reported first by Berson and Yalow (1968). Most of these are carboxy-terminal (C) fragments, and only a very small amount may represent amino-terminal (N) fragments. The high percentage of C-terminal fragments may partially be related to their low turnover rate in circulation when compared to the intact hormone (Silverman and Yalow, 1973). The production of these fragments is located in the Kupffer cells in the liver, the kidneys (Hruska *et al.*, 1977; Segré *et al.*, 1981), and, surprisingly, the parathyroid glands. The intracellular degradation of PTH was first demonstrated for rat parathyroid glands *in vitro* (Chu *et al.*, 1973).

Flueck *et al.* (1977) identified both C-terminal and N-terminal PTH fragments in addition to intact PTH in the venous effluent blood of parathyroid glands of patients suffering from parathyroid adenomas. The release of intact PTH and PTH fragments from human parathyroid cells *in vitro* was shown by Hanley and Ayer (1986), in a study on tissue of hyperplastic glands. Evidence that PTH fragments were indeed, and in significant amounts, secreted into the circulation by normal functioning parathyroid glands was reported by Mayer *et al.* (1979), who showed that venous effluent plasma samples of the parathyroid glands of young calves contained C-terminal and N-terminal fragments.

Morissey and Cohn (1979b) showed that, although both newly synthesized and stored PTH are affected, the stored hormone is degraded preferentially. Degradation of newly synthesized PTH did not start until 20 minutes after the formation of proPTH, indicating that PTH and not proPTH is degraded and that this process takes place after packaging in the secretory granules. The degradation has been assumed to take place after fusion of secretory granules with lysosomes (Cohn and Elting, 1983). The primary enzymes responsible for PTH degradation are cathepsins B and D, which, at least in bovine parathyroid cells, yield different C-terminal and N-terminal fragments.

The biological activity for most functions of PTH is located in the first (N-terminal) 34 amino acids, which form one of the two domains of the molecule. However, the presence of the carboxy-terminal region 37-84, although in itself without biological activity, ensures that the intact hormone has a higher potency than the 1-34 terminal in several assay systems. Moreover, only 1-84 PTH but not 1-34 PTH is taken up by the liver and stimulates hepatic glucose mobilization, as has been shown in dogs. The C-terminal fragments are considered as biologically inert (Cohn and MacGregor, 1981; Habener *et al.*, 1984). From these structure–activity studies it can therefore be concluded that the intracellular degradation pathway substantially reduces the ratio of active to inactive PTH molecules that are released.

The degradation pathway of the parathyroid cells seems part of a remarkable mechanism of control of hormone release that probably is characteristic of the parathyroid glands and which will be discussed in Section II,A,6.

4. Control of PTH Secretion

a. *Extracellular Calcium.* Extracellular calcium has been considered the primary regulator of PTH secretion since the pioneering studies of Patt and Luckhardt (1942) about 50 years ago. Following sodium oxalate injection in normal and thyroparathyroidectomized dogs, they observed that

serum calcium was rapidly reduced and returned to normal only in the intact animals. When they perfused decalcified blood through a thyroid/parathyroid preparation and injected the perfusate into normal dogs they observed a rise in serum calcium. This effect did not occur when normal blood was used. They concluded that low blood calcium is a direct stimulus for the parathyroid glands to secrete more hormone into circulation. This conclusion has been confirmed frequently by later studies, and the results have shown a close and inverse relationship between extracellular calcium, in particular the ionic calcium fraction, and hormone release by the parathyroid glands, *in vivo* and *in vitro* (Copp and Davidson, 1961; Sherwood *et al.*, 1966; Kemper *et al.*, 1974; Brown, 1983).

Whereas in earlier reports this relationship has been described as inversely linear over a wide range of Ca^{2+} concentrations, more detailed analysis has shown a sigmoid relationship, with PTH release stimulated markedly by small decreases in the physiological range, around the setpoint for Ca^{2+} (the extracellular Ca^{2+} concentration resulting in 50% inhibition of the maximum rate of PTH release; ± 1.5 m*M*), and with a basal release not suppressible by high Ca^{2+} levels (Habener and Potts, 1976; Brown, 1982, 1983). Around the setpoint, a change in extracellular Ca^{2+} of about 1 m*M* causes a 5-fold change in PTH release, as has been shown both *in vivo* and *in vitro* (Fig. 2). Not only are PTH cells very sensitive to minute changes in extracellular Ca^{2+} around its setpoint, their response is also extremely rapid. Increased PTH release follows a drop in extracellular Ca^{2+} within seconds *in vivo* and *in vitro* (Brown *et al.*, 1987). In newborn animals plasma calcium may be maintained at a higher level than at an older age. Keaton *et al.* (1978) and LeBoff *et al.* (1983) have shown, *in vivo* and *in vitro*, respectively, that the hypercalcemia of newborn calves is most likely caused by a higher setpoint for calcium (Fig. 2). Within a few weeks the setpoint is reduced to the level typical for adult animals. Increased setpoints have also been reported for patients with primary hyperparathyroidism (Brown *et al.*, 1987).

The precise mechanism underlying the control of PTH secretion by Ca^{2+} has long been unclear. The observation that the above mentioned effects of extracellular calcium on PTH release are also shown by dispersed parathyroid cells *in vitro* implies that Ca^{2+} exerts its effects directly on the cell membrane and not indirectly via hormonal or nervous mechanisms. Bruce and Anderson (1979) have shown that the resting potential of mouse parathyroid cells has a unique sensitivity to changes in the extracellular Ca^{2+} concentration when compared to other endocrine tissues such as the thyroid gland or the pancreatic beta cells. The authors connected the striking changes in the membrane potential of the parathyroid cells over a small range of ionic calcium concentrations (1.5–2.5 m*M*)

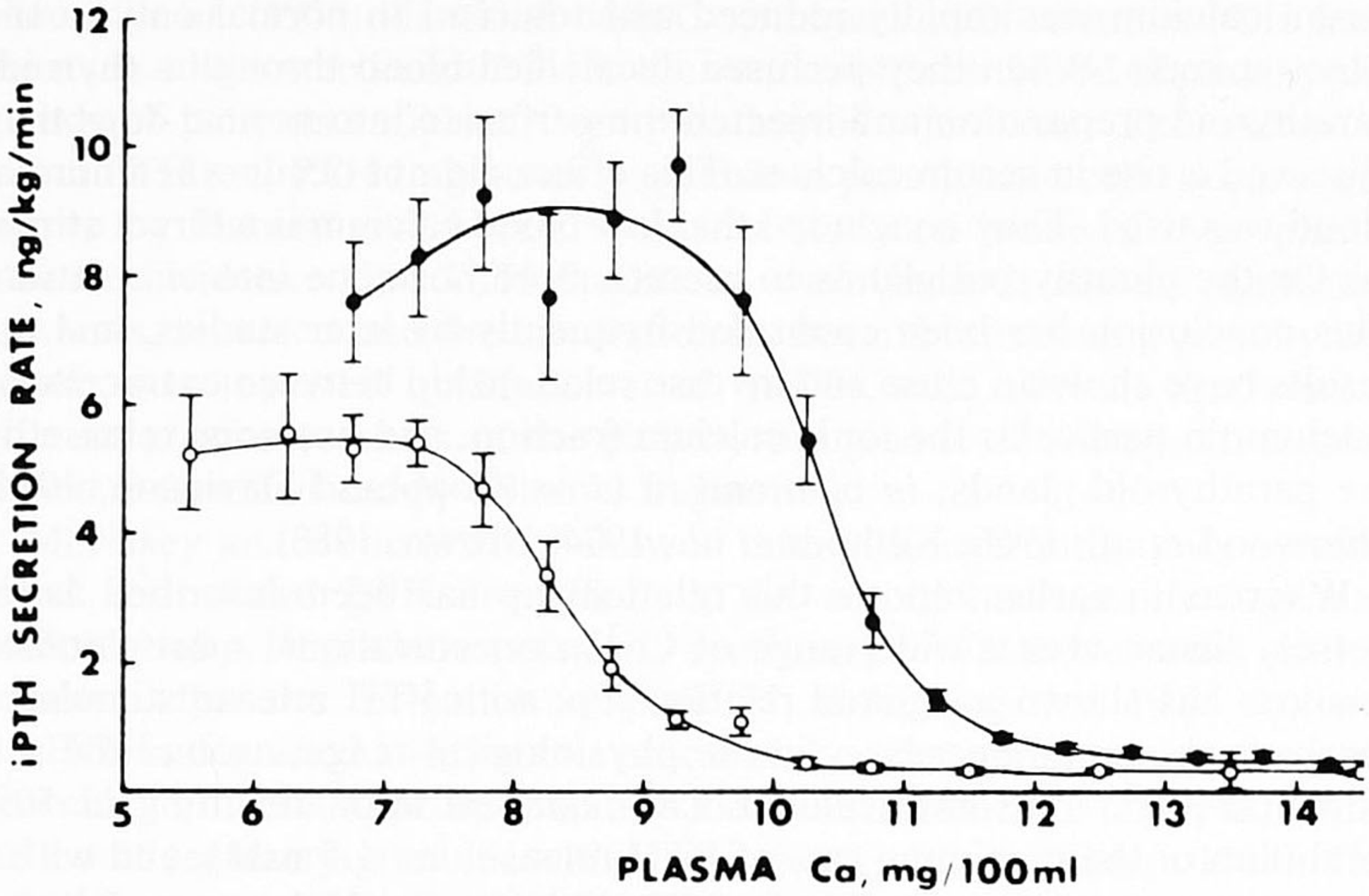

FIG. 2. Comparison of the relationship between immunoreactive PTH release and plasma total calcium concentration in neonatal and older calves, based on measurements of blood from veins draining the parathyroid glands. The values for PTH release in the neonates were grouped according to their associated plasma calcium concentration. Each group spanned a calcium concentration range of 0.5 mg/100 m (0.125 m*M*). Mean release rate ± SE of each group. The relationship between PTH release and plasma calcium was sigmoid in nature in both groups, with the curve for the neonates shifted to the right of the curve of older calves. PTH release was greater at all plasma calcium concentrations in the neonates (Keaton *et al.*, 1978). [●, Neonatal calves (age, 1–3 days); ○, older calves (age, 2–14 weeks).]

with the high calcium sensitivity of the PTH release of these cells in the same concentration range. Calcium ions depolarize the cells by altering the plasma membrane conductance to K^+, a capacity that is shared with other divalent ions and with La^{3+}. In contrast to other gland cells, stimulated secretion is associated with membrane hyperpolarization instead of depolarization. This difference emphasizes the uniqueness of the PTH release-controlling mechanism.

The unusual and prompt response of the PTH cells to extracellular Ca^{2+} led to the assumption of a Ca^{2+} sensor or receptor on the outer cell membranes (Lopez-Barneo and Armstrong, 1983; Wallace and Scarpa, 1983). This was supported by Juhlin *et al.* (1987), who raised monoclonal antibodies to cell surface components of these cells that could block the response of the cells to extracellular calcium. They further demonstrated that the parathyroid cells of patients with hyperparathyroidism and hypercalcemia displayed reduced binding of these antibodies, indicating that the

reduced sensitivity of these cells to extracellular Ca^{2+} is associated with reduced expression of the antigen. Recently, patch clamp studies by Jia *et al.* (1988) on bovine PTH cells revealed the presence of a specific type of calcium-activated potassium channel in the outer membrane that might represent the calcium-sensing mechanism. The authors found potassium-selective channels dependent on internal Ca^{2+} to open. Different from similar channels in other cells, these channels close when the internal Ca^{2+} concentration rises above 160 μM. This concentration corresponds to extracellular Ca^{2+} concentrations between 0.5 and 1.0 mM. This unusual behavior of the calcium-dependent potassium channels of the parathyroid cells may explain the depolarization of their outer membranes with increased extracellular Ca^{2+} levels. It is still unclear, however, whether the membrane hyperpolarization of the cells mediates the stimulatory effect of low extracellular Ca^{2+} on PTH secretion, since increased PTH release by external K^+ ions is associated with membrane depolarization. Jia *et al.* (1988) and Pocotte and Ehrenstein (1989) suggested that the specific potassium channels are also present in the secretory granules, and that closing of these channels in response to increased intracellular Ca^{2+} could account for stimulated secretion rather than the hyperpolarization of the outer cell membrane. On the other hand, Fitzpatrick *et al.* (1986a,c) have suggested that the interaction of extracellular Ca^{2+} with voltage-gated Ca^{2+} channels mediates the effects of extracellular Ca^{2+} on PTH release (see Section II,A,5,b). Thus, there is no unanimity about the nature of the Ca^{2+}-sensing mechanism of the outer membrane of the PTH cells.

The above data deal with the control by extracellular Ca^{2+} of PTH release. How does extracellular Ca^{2+} affect synthesis, processing, and intracellular degradation of the hormone? Whereas the studies mentioned point to prominent and prompt effects of Ca^{2+} on PTH release, extracellular Ca^{2+} appears to have little more than marginal effects on PTH synthesis, at least in the short term. Heinrich *et al.* (1983) have determined the concentration of mRNA in bovine parathyroid slices *in vitro*. After incubation of the tissue for 5 to 7 hours in the presence of different extracellular Ca^{2+} concentrations no differences were noticed in total mRNA, but poly(A) containing PTH-mRNA levels were changed. Unexpectedly, in the presence of nonphysiologically high external Ca^{2+} (5 mM), which inhibit PTH release, poly(A) containing PTH-mRNA amounted to 30% of total mRNA, substantially more than the 10% found in tissue slices incubated in low-Ca^{2+} media. At variance with these results, Russell *et al.* (1983) showed that in dispersed bovine parathyroid cells *in vitro* the concentration of PTH-mRNA was reduced gradually in 96 hours by almost 70% at high extracellular Ca^{2+} (2.5 mM) when compared to low Ca^{2+} (0.5 mM). A change from high Ca^{2+} to low Ca^{2+} after 36 hours reversed

the decrease. These observations were confirmed by Brookman *et al.* (1986): in similar experiments on cultured parathyroid cells PTH-mRNA levels were unchanged over 72 hours at normal extracellular Ca^{2+} (1 m*M*), and during the first 4 hours at 3 m*M* Ca^{2+}. At the latter concentration a 50% suppression was observed after 24 and 48 hours. Low external Ca^{2+} (0.5 m*M*) was reported to have no effect (Russell *et al.*, 1983) or a slightly stimulating effect by 48 hours (Brookman *et al.*, 1986). These *in vitro* results were not fully consistent with *in vivo* observations in rats. Reduction of serum calcium by sodium phosphate injection resulted in a marked increase of PTH-mRNA at 6 hours. Calcium infusion, causing frank hypercalcemia for 6 hours, did not change PTH-mRNA levels (Naveh-Many *et al.*, 1989). With pulse chase experiments of porcine parathyroid tissue *in vitro* with labeled amino acids it was found that the rates of synthesis and conversion of proPTH were the same irrespective of the Ca^{2+} concentration of the incubation medium (Morissey and Cohn, 1979b).

The absence of consistent effects of extracellular Ca^{2+} on PTH synthesis or processing in short-term experiments is in contrast with more long-term ultrastructural observations *in vivo*, that invariably point to an inverse relation between extracellular Ca^{2+} and the secretory capacity of the parathyroid glands. As reviewed by Clark *et al.* (1986), hypercalcemia induced by exogenous Ca^{2+} caused degenerative changes in parathyroid cells of different mammalian species, which strongly suggested decreased secretory activity. Hypertrophy and hyperplasia of the glands have been reported in response to experimentally induced hypocalcemia, for instance in cows, cats, dogs, chickens, iguanas, and newts. These studies indicate that prolonged hypercalcemia or hypocalcemia profoundly influence the capacity for hormone synthesis of the glands. They further demonstrate that the ultrastructural responses of the parathyroids to prolonged hypocalcemia and hypercalcemia are similar in mammals to amphibians. Nevertheless, the effects of extracellular Ca^{2+} on PTH biosynthesis become noticeable in days rather than hours, and this contrasts with the immediate effects on PTH release. How then are the parathyroid glands, which usually contain only very small hormone stores, capable of maintaining PTH release during the first hours of a prolonged hypocalcemic stimulus? Chu *et al.* (1973) and Habener *et al.* (1975) were the first to reveal what seems to be a unique regulatory mechanism by showing that extracellular Ca^{2+} can effectively modulate the degradation pathway of bovine PTH cells and *in vitro*, with more degradation and thus less bioactive PTH secreted at increasing extracellular Ca^{2+} levels. This mechanism enables the cells to persistently increase the output of intact hormone without changing the rate of gene transcription or translation. It will be discussed in Section II,A,6.

b. *Extracellular Magnesium.* High concentrations inhibit PTH secretion *in vitro* and *in vivo*. It was shown that Mg^{2+} is two-and-a-half to three times less potent than Ca^{2+} in inhibiting PTH release *in vitro* (Habener and Potts, 1976). The effects of Ca^{2+} and Mg^{2+} were reported as independent and additive. In a systematic study on the relative potencies of Ca^{2+} and Mg^{2+} to inhibit PTH secretion from dispersed bovine parathyroid cells, Brown *et al.* (1984a) have shown that the relationship between both cations is more complex. At a physiological free calcium concentration (1.0 m*M* Ca^{2+}), the Mg^{2+} concentration giving half maximal inhibition of PTH release was 1.8 m*M*, which was only slightly higher than the corresponding Ca^{2+} concentration (1.25 m*M*). At a Ca^{2+} concentration of 0.5 m*M*, similar to that used by Habener and Potts (1976), the Mg^{2+} concentration giving half maximal inhibition (3 m*M*) was almost two-and-a-half times that of Ca^{2+} (1.25 m*M*) with a Mg^{2+} concentration of 0.5 m*M*, thereby confirming Habener and Potts' data. However, at low Ca^{2+} concentrations, Mg^{2+} was markedly less potent as an inhibitor of PTH release than at physiological Ca^{2+} levels. Conversely, low Mg^{2+} concentrations had little effect on the responsiveness of PTH release to Ca^{2+} (Brown *et al.*, 1984a). Thus, at low Ca^{2+} concentrations Mg^{2+} was less potent than predicted by the 2.5 : 1 ratio suggested by Habener and Potts (1976), and the effect of Mg^{2+} was dependent on the presence of extracellular Ca^{2+}. Shoback *et al.* (1983) have shown that the enhanced PTH secretion induced by lowering of the Mg^{2+} concentration *in vitro* is associated with a decrease in cytosolic Ca^{2+} as measured with quin2.

In vitro studies of Mahaffee *et al.* (1982) have shown that in Mg-free media PTH secretion is inhibited. Anast *et al.* (1972, 1976) reported hypocalcemia and reduced immunoreactive plasma PTH levels in patients with abnormally low plasma Mg^{2+} levels, caused by impaired intestinal Mg^{2+} uptake. Although at first these observations may seem paradoxical in view of the above mentioned inhibitory effects of high external Mg^{2+} levels, these effects probably are not specific for PTH release but rather a reflection of the general dependency on magnesium of many cellular functions. Magnesium is indispensable as a stabilizer of membrane structures, as a chelator of intracellular ATP, and as a cofactor of many enzyme reactions. It is also essential for hormonal activation of the receptor adenylate cyclase complex of many cell types: there are specific magnesium-binding sites. Using homogenates of rat parathyroid glands, Mahaffee *et al.* (1982) have indeed shown that magnesium ions are essential for adenylate cyclase activation. This dependency probably reflects the action of intracellular Mg^{2+} rather than extracellular Mg^{2+} (see Section II,A,5,a).

In vivo data of the effect of administration of magnesium on PTH secretion are scarce, but seem to support the inhibitory effects of high extracel-

lular Mg^{2+} concentrations as observed *in vitro*. Cholst *et al.* (1984) showed that administration of very high doses of magnesium to pregnant women induced a sharp decrease of plasma immunoreactive PTH (iPTH). In a study on hemodialysed patients, Pletka *et al.* (1971) demonstrated a 20% decrease of plasma iPTH after increasing the magnesium concentration of the dialysis fluid from 0.75 to 1.25 m*M* for 2 months.

In the only study on healthy man known to us, Ferment *et al.* (1987) compared the effects of magnesium and calcium injections on plasma PTH levels, using a homologous radioimmunoassay (RIA) directed toward the 53-84 C-terminal PTH fragment. After a single dose of magnesium sulphate which resulted in a 50% increase of plasma Mg^{2+} after 45 minutes, a 30% reduction of PTH levels was measured. Injection of magnesium pyrrolidone carboxylate had no significant effect on plasma PTH although the rise in plasma Mg^{2+} was identical to that following administration of magnesium sulphate. A lower dose of calcium gluconate produced a similar effect on plasma PTH. The authors concluded that Mg^{2+}, on a molar basis, was less potent than Ca^{2+} in inhibiting PTH secretion. The effect of Mg^{2+} was also less sustained than that of Ca^{2+}.

We conclude from the above data that changes in extracellular Mg^{2+} influence PTH secretion. However, there is no clear evidence that Mg^{2+} is of importance as a regulatory factor in the physiological control of PTH secretion. The effects of Mg^{2+} on parathyroid secretion become evident only under conditions of extreme hypo- and hypermagnesemia. As far as hypomagnesemia is concerned, this condition probably leads to magnesium depletion of the parathyroid cells, and the effects on secretion are, therefore, mainly indirect and/or nonspecific. These effects reflect that the parathyroid cells are dependent for proper functioning on adequate extracellular Mg^{2+} levels, a dependency shared with other cell types. This inhibitory effect of low plasma Mg^{2+} levels on PTH secretion will therefore only become noticeable under pathological conditions. The inhibitory effects of high external Mg^{2+} levels on PTH secretion are also unlikely to become noticeable, given the normal ionic levels of Ca^{2+} and Mg^{2+} (1.5 and 1.0 m*M*, respectively) and the low potency of Mg^{2+} at normal plasma Ca^{2+} levels. Thus, variations in extracellular Mg^{2+} at most will have a small modulating effect on the control of PTH secretion by Ca^{2+} under normal physiological conditions.

c. *Hormones and Neurotransmitters. i. Proteins and peptides.* In older literature the pituitary gland has been implicated in the control of the parathyroid glands. In a number of studies in the 1930s, which have been surveyed by Campbell and Turner (1942) and Latman (1980), degeneration of the parathyroid glands was reported for hypophysectomized toads,

pigeons, rabbits, dogs, monkeys, and rats. Anterior pituitary extracts increased serum calcium and induced hypertrophy of the parathyroids of intact dogs, rats, and guinea pigs. Campbell and Turner (1942) concluded from these studies that the stimulatory effects of the pituitary gland on the parathyroids were indirect. More recently, Latman (1980) tested the effects of a pituitary gland extract on parathyroid hormone release by bovine parathyroid glands *in vitro* and found a stimulatory action on C-terminal PTH and, in particular, on N-terminal PTH. Injection of the extract had no effect on the serum iPTH level in calves, which the author ascribed to the relative insensitivity of this parameter to changes in PTH release. Since TSH, LH, GH, ADH, oxytocin, prolactin, ACTH, MSH, and lipotropin did not effect PTH release, he concluded that there was an unknown pituitary factor that can directly stimulate PTH secretion. Injection of the pituitary extract had no effect on the serum iPTH level in calves, which the author ascribed to the relative insensitivity of this parameter to rapid changes in PTH release (Latman, 1980; Fig. 3).

Recently, we have reviewed the literature on the role of growth hormone and prolactin in calcium regulation in mammals and concluded that these hormones have important direct effects on calcium uptake via the gut during periods of high calcium demand, viz. growth, gestation, and lactation, but are not implicated in the control of calcium homeostasis of the extracellular fluid (Wendelaar Bonga and Pang, 1986). Also indirect effects via modulation of the PTH release are unlikely. Data on iPTH levels in hyperprolactinemic patients are conflicting (Raymond *et al.*, 1982; Schlechte *et al.*, 1983; Fiore *et al.*, 1984). Injection of GH has been reported to increase plasma calcium levels, an effect that may be mediated by stimulation of PTH secretion. However, the doses used were excessive and the effect might be relevant only for explaining the hyperparathyroidism and hypercalcemia in acromegalic patients (Lancer *et al.*, 1975).

As will be dealt with in more detail in Section II,B,2,b,i, gastrointestinal hormones are capable of influencing plasma calcium levels, and therefore interactions with the parathyroid glands are indicated. With respect to secretin, stimulatory effects have been reported on bovine PTH release *in vitro* (Windeck *et al.*, 1978; Sethi *et al.*, 1981). Increases in PTH levels following secretin administration were reported for rats and normal human subjects (Sethi *et al.*, 1983). The doses were rather high, however. Infusion of acid in rat duodenum, known to stimulate specifically the release of endogenous secretion, caused a significant rise in PTH release. Somatostatin caused a dose-related decrease of plasma PTH in rats and monkeys. It reduced PTH release from normal bovine and adenomatous human parathyroid tissue *in vitro* (Hargis *et al.*, 1978). Somatostatin antiserum

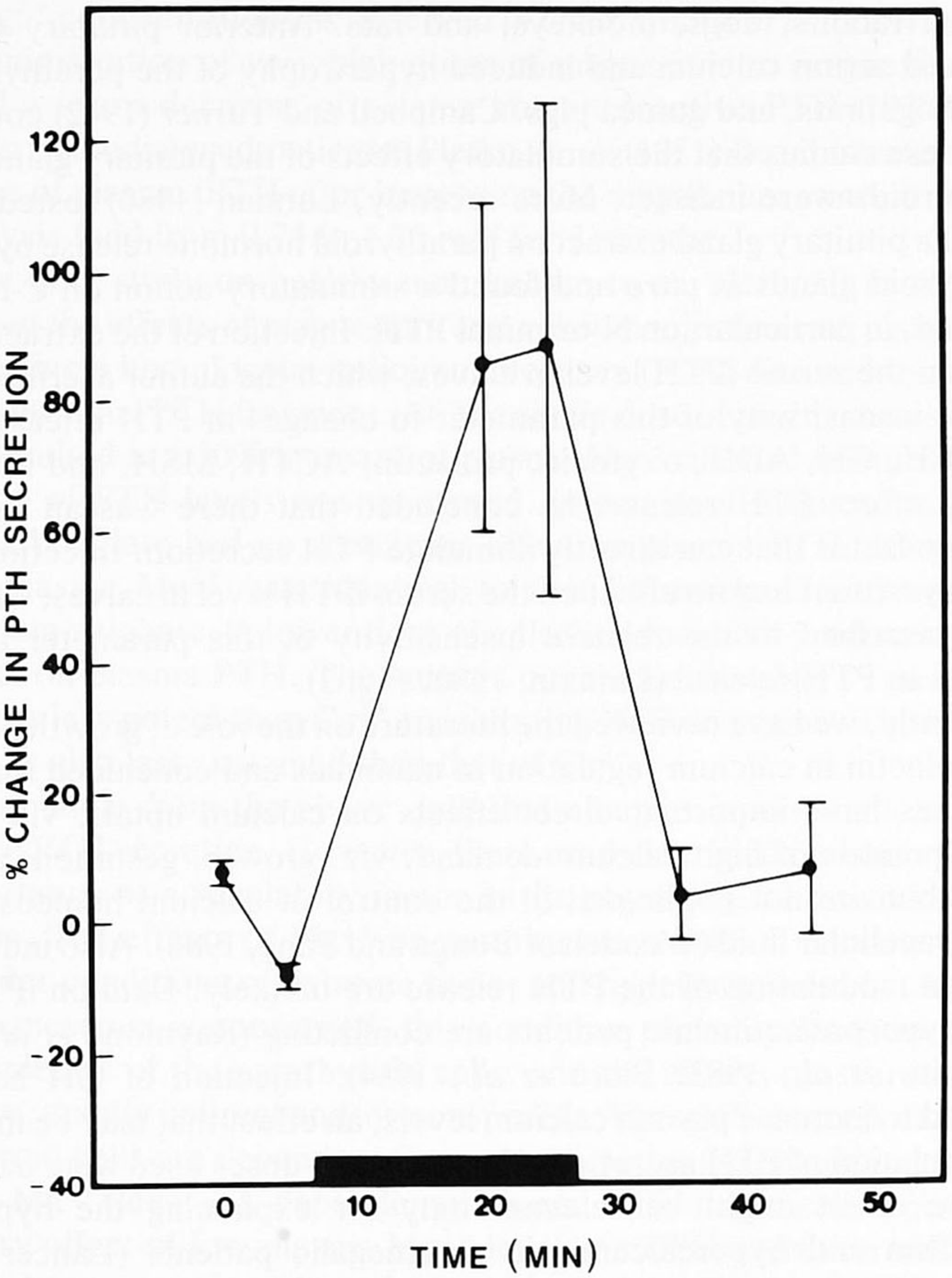

FIG. 3. Stimulation of N-terminal immunoreactive PTH release under slightly hypercalcemic conditions (1.6 m*M* Ca^{2+}) by bovine parathyroid glands perfused *in vitro* with anterior pituitary gland extract (black bar). Perfusion with all known pituitary hormones had no effect; mean percentage change ± SE compared to the level before perfusion (Latman, 1980).

increased plasma PTH in rats, and inhibited PTH release from bovine parathyroid tissue *in vitro*. The hormone was attributed with a function of local regulator of the parathyroid glands (Williams *et al.*, 1979).

ii. Bioamines. Dopamine stimulates PTH secretion of bovine parathyroid cells *in vivo* (Brown *et al.*, 1977) and *in vitro* (Attie *et al.*, 1980), but its functional role is unknown. Injections of epinephrine and norepinephrine in cows (Blum *et al.*, 1978) and men (Kukreja *et al.*, 1975) increase serum PTH immediately, similar to administration of the β-adrenergic agonist

isoproterenol. The stimulatory effects are to some extent additive with those of hypocalcemia and are prevented by the β-blocker propranolol. This indicates that these effects are mediated by β-adrenergic receptors. *In vitro* experiments have shown that the β-adrenergic stimulation is exerted directly on the parathyroid cells, and associated with cAMP production (Williams *et al.*, 1979), via a receptor coupled with adenylate cyclase through a stimulatory guanine nucleotide regulatory protein (Fitzpatrick *et al.*, 1986b,c). α-adrenergic agents inhibit cAMP production and hormone release from bovine parathyroid glands *in vitro* (Brown *et al.*, 1978a). This explains the biphasic effect of epinephrine, a mixed α-adrenergic and β-adrenergic agonist. The β-adrenergic stimulation is observed *in vitro* at low concentrations (up to $10^{-}7$ *M*) whereas at higher concentrations an inhibition becomes apparent. This inhibition is an α-adrenergic response since it is prevented by phentolamine (Brown *et al.*, 1978a). The observation that the inhibitory effect of high doses of epinephrine is blocked by pertussis toxin indicates that the α-adrenergic inhibition of PTH secretion is mediated by the inhibitory guanine nucleotide regulatory protein (Fitzpatrick *et al.*, 1986b). The physiological significance of the α-adrenergic inhibition on PTH secretion is unknown. Conversely, the β-adrenergic influence may be physiologically important since propranolol reduced basal serum levels of PTH in man (Kukreja *et al.*, 1975). Epinephrine was suggested to modulate PTH secretion *in vivo* under stress conditions: the rise of serum PTH during insulin-induced hypoglycemic stress has been attributed to the accompanying rise of (adreno-medullary) plasma epinephrine (Shah *et al.*, 1975). This has not been confirmed by Body *et al.* (1983). The marked rise in plasma epinephrine they recorded following injection in healthy man was paralleled by a reduction of plasma PTH, which was interpreted as an effect of hypercalcemia induced by the experimental treatment. Moreover, epinephrine injections resulting in plasma epinephrine concentration up to the physiological maximum did not influence plasma PTH (or CT). They concluded therefore that β-adrenergic stimulation requires nonphysiologically high adrenomedullary epinephrine levels. Heath *et al.* (1980) examined PTH secretion in adrenalectomized rats and found no indication of important adrenomedullary effects. This seems to limit the function of the β-adrenergic control to catecholamines originating from the sympathetic nerves in the parathyroid glands (Body *et al.*, 1983; Heath *et al.*, 1980). However, chemical sympathectomy by 6-OHDA treatment did not interfere with the homeostatic control of plasma calcium by PTH, and thus the physiological importance of the β-adrenergic control remains unclear.

iii. Steroids. PTH as well as 1,25 dihydroxycholecalciferol ($1,25(OH)_2D_3$) are both important hormones regulating calcium homeosta-

sis of the extracellular fluids. There is, however, a clear difference between the actions of both hormones: PTH dominates the short-term regulation of plasma calcium and can become effective within minutes; the action of $1,25(OH)_2D_3$ becomes noticeable after hours or days, and, as mentioned earlier, relates to the total body calcium balance rather than to the calcium homeostasis of the extracellular fluid. PTH controls $1,25(OH)_2D_3$ formation by regulating 25 hydroxycholecalciferol-1α-hydroxylase, a key enzyme in the synthesis of the steroid (Fraser and Kodicek, 1973). Conversely, $1,25(OH)_2D_3$ influences PTH secretion. It acts directly on the parathyroid cells. High affinity receptors for $1,25(OH)_2D_3$ have been demonstrated in the cytosol of human and chicken parathyroid cells (Oldham *et al.*, 1974; Hughes and Haussler, 1978; Wecksler *et al.*, 1980). Significant binding of tritiated $1,25(OH)_2D_3$ was demonstrated autoradiographically in nuclei of chick and rat parathyroid cells (Henry and Norman, 1975; Wecksler *et al.*, 1977).

Early observations on the effects of $1,25(OH)_2D_3$ on PTH secretion were contradictory. Inhibitory as well as stimulatory effects or no changes have been reported. The effects were predominantly inhibitory, however. Observations on rachitic children suggested a direct inhibitory effect on PTH secretion (Fischer *et al.*, 1973). Chertow *et al.* (1975) reported a reduction of serum PTH by $1,25(OH)_2D_3$ in rats and of PTH secretion *in vitro* in bovine parathyroid slices. Henry *et al.* (1977) showed a reduction of the size of chick parathyroid glands after treatment with vitamin D_3 or $1,25(OH)_2D_3$. The steroid significantly inhibited PTH secretion of normal porcine parathyroid glands and human parathyroid adenomas *in vitro* within 4 hours. This was followed by ultrastructural signs of decreased hormone synthesis during continued incubation (Dietel *et al.*, 1979). Conversely, Canterbury *et al.* (1978) reported a rise in PTH in the thyroid venous blood of dogs given $1,25(OH)_2D_3$ into the thyroid artery. Pharmacological doses were required and no change in peripheral iPTH or plasma Ca^{2+} occurred. The physiological significance of the effect was therefore doubted by the authors. In rachitic or normal dogs no clear effect of $1,25(OH)_2D_3$ on serum iPTH or serum calcium could be demonstrated (Oldham *et al.*, 1979), but the authors found evidence that the steroid increased the responsiveness of the parathyroid glands to the suppressive effects of normal or increased serum calcium levels. An attenuating effect on the secretory response of the parathyroid glands to hypocalcemia was suggested by Hurst and Mayer (1977). Using carboxyterminal N-terminal and C-terminal RIAs for PTH, Golden *et al.* (1980) were unable to find an effect of $1,25(OH)_2D_3$ on PTH release during 4 hours of incubation of bovine parathyroid slices or isolated cells, at variable Ca^{2+} concentrations. An effect could be demonstrated on the patterns of neither secreted

intact hormone nor hormone fragments. Llach *et al.* (1977) found no acute effect of $1,25(OH)_2D_3$ on serum iPTH in humans. Silver *et al.* (1985) showed that $1,25(OH)_2D_3$ inhibits PTH secretion in isolated bovine parathyroid cells. The steroid reversibly and selectively reduced preproPTH-mRNA, as well as the cellular content of the transcript. No effect on total RNA synthesis or total RNA content was found. The effect developed slowly. It became noticeable around 24 hours after the start of the incubation of the parathyroid cells with $1,25(OH)_2D_3$ and was maximal after 48 hours. Similar results were reported for rats. PTH-mRNA levels were reduced about 50% in 6 hours and 75–90% in 24 hours after a dose of 100 pmol without change in serum Ca (Silver *et al.*, 1986; Naveh-Many and Silver, 1988; Naveh-Many *et al.*, 1989; Fig. 4). In other studies of this group the effects of $1,25(OH)_2D_3$ on PTH secretion during short-term (30–120 minutes) and long-term (24-96 hours) incubation of bovine parathyroid cells were compared. Whereas in short-term incubations no effect was found, PTH secretion was inhibited dose-dependently during long-term incubation with the steroid, as was established with both N-terminal and C-terminal PTH assays. At 48 hours a strong correlation was

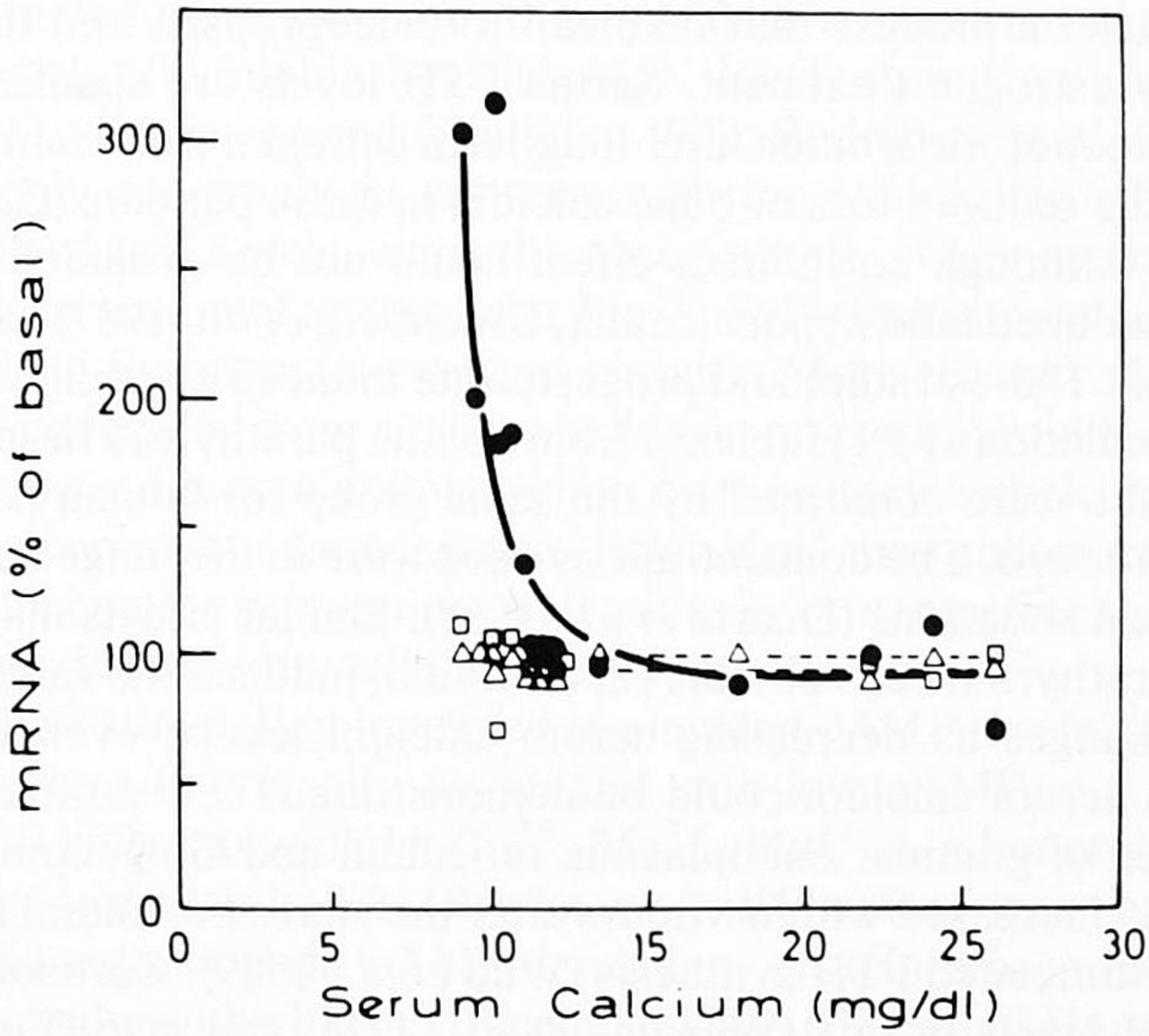

FIG. 4. Effect of changes in serum calcium on mRNA levels for PTH, CT, and action of rat parathyroid glands at 6 hours. Rats were injected intraperitoneally with different amounts of calcium gluconate or monosodium phosphate at 0 and 3 hours. The results at each point are the mean for four rats and expressed as percentage of basal (Naveh-Many *et al.*, 1989). ●, PTH; △, actin; □, calcitonin.

found between the decrease in PTH release and the lowered concentration of preproPTH-mRNA in these cells (Cantley *et al.*, 1985). These studies indicate that $1,25(OH)_2D_3$ inhibits PTH secretion by suppressing gene transcription. The effect is specific. Vitamin D_3 or the metabolites $25(OH)D_3$ and $24,25(OH)_2D_3$ had no effect at physiologically relevant concentrations in primary parathyroid cell cultures (Cantley *et al.*, 1987). The relatively slow response may explain some of the contradictory results reported earlier, although species-dependent differences in the effects of $1,25(OH)_2D_3$ cannot be excluded. At least in the bovine species a negative feedback relationship at the level of hormone synthesis has now been established between PTH and $1,25(OH)_2D_3$. The suppression of secondary hyperparathyroidism by intravenous administration of $1,25(OH)_2D_3$ in uremic patients indicated a similar inhibitory effect in humans (Slatopolsky *et al.*, 1984). Okazaki *et al.* (1988) reported that the steroid decreased the transcription of the human PTH gene introduced in cultured rat pituitary cells, possibly via binding of the $1,25(OH)_2D_3$ to the promotor region of the gene. The physiological significance of the effect of $1,25(OH)_2D_3$ on PTH secretion will be discussed in Section IV.

Gonadal and interrenal hormones may also influence PTH secretion. Estrogens have a profound effect on bone. Bone loss increases markedly at menopause, a process that can lead to osteoporosis, and this can be reduced by estrogen treatment. Serum PTH levels are significantly elevated in osteoporotic women after long-term estrogen treatment, an effect related to the reduced loss of bone calcium in these patients (Gallagher *et al.*, 1980). Although an indirect effect could not be excluded since the treatment induced mild hypocalcemia, Greenberg *et al.* (1987) have shown recently that 17β-estradiol and progesterone induced a specific and dose-related stimulation of PTH release from bovine parathyroid tissue *in vitro*. These results were confirmed by the same group for human parathyroid adenomas *in vitro*. The concentrations used were in the range occurring in physiological situations (Duarte *et al.*, 1988). Similar effects may occur *in vivo*. In parathyroid cells of male rats estradiol induced the same morphometrical changes as decreasing serum calcium levels, even though no changes in serum calcium could be demonstrated: cell surface area and surface area of granular endoplasmic reticulum and Golgi complex were significantly increased within 6 hours after the start of estradiol treatment, indicating stimulated PTH synthesis (Wild *et al.*, 1989). Cortisol increases serum PTH levels in rats (Williams *et al.*, 1974) and man (Fucik *et al.*, 1975). Adrenalectomy in rats decreased thyroidal CT-mRNA concentration. This could be partially corrected by injection of the synthetic glucocorticoid dexamethasone. Plasma CT levels were unchanged (Besnard *et al.*, 1989).

5. *Second Messengers*

a. *Cyclic AMP.* In the early 1970s the first evidence was reported for the implication of adenylate cyclase and its product cAMP in the regulation of PTH release. Studying bovine parathyroid gland slices *in vitro*, Abe and Sherwood (1972) demonstrated that dibutyryl cAMP and the phosphodiesterase inhibitor theophylline caused significant stimulation of PTH secretion. Similar observations were reported by Williams *et al.* (1973). Many following studies, in which (usually) bovine parathyroid slices or dispersed cells were employed, have indicated that intracellular cAMP levels change in parallel with receptor-mediated PTH release *in vitro*, irrespective of the agonists tested, e.g., epinephrine, norepinephrine, isoproterenol, dopamine, prostaglandin E1 and E2, glucagon, calcitonin, secretin, or NaF (see Cohn *et al.*, 1986). The catecholamines act via a-adrenergic receptors. Concomitant stimulation of cAMP and PTH release with a-adrenergic agonists, dopamine, and secretin has also been demonstrated *in vivo* (Blum *et al.*, 1980; Fischer *et al.*, 1982). Furthermore, the stimulatory action on PTH release of reduced extracellular Ca^{2+} and Mg^{2+} levels is associated with increased cAMP concentrations (Brown *et al.*, 1978b).

When studied in homogenates of dog and horse parathyroid glands high calcium levels still inhibit, but high Mg^{2+} levels stimulate, adenylate cyclase activity (Dufresne and Gitelman, 1972; Rodriguez *et al.*, 1978). This effect is only seemingly in contrast with the stimulating effect of low extracellular Mg^{2+} levels, since the observations on homogenates probably reflect the action of intracellular Mg^{2+}. Sufficient intracellular Mg^{2+} is a prerequisite for adenylate cyclase activity. More recently, Mahaffee *et al.*, (1982) concluded from similar studies on rat parathyroid homogenates that Ca^{2+} has a direct inhibitory action on the guanine nucleotide adenylate cyclase complex, whereas intracellular Mg^{2+} may enhance the activity of adenylate cyclase by guanine nucleotides or by competing with Ca^{2+} for binding to a distinct intracellular regulatory site.

While PTH stimulation is related to elevated cAMP levels, suppression of PTH release is typically associated with low cAMP, e.g., following exposure to high extracellular Ca^{2+}, Mg^{2+}, Mn^{2+}, α-adrenergic catecholamines, or prostaglandin $F2_{\alpha}$ (Brown *et al.*, 1978a; Rodriguez *et al.*, 1978). Parathyroid cells contain cAMP-dependent protein kinase activity, and this can be reduced by high extracellular Ca^{2+} (Pines and Hurwitz, 1981). The effects of low extracellular Ca^{2+} on adenylate cyclase are not limited to mere stimulation of the enzyme. Whereas a log–linear relationship between cAMP and PTH release has been established for many agonists for PTH release, including extracellular Ca^{2+} (Fig. 5), extracellular Ca^{2+}

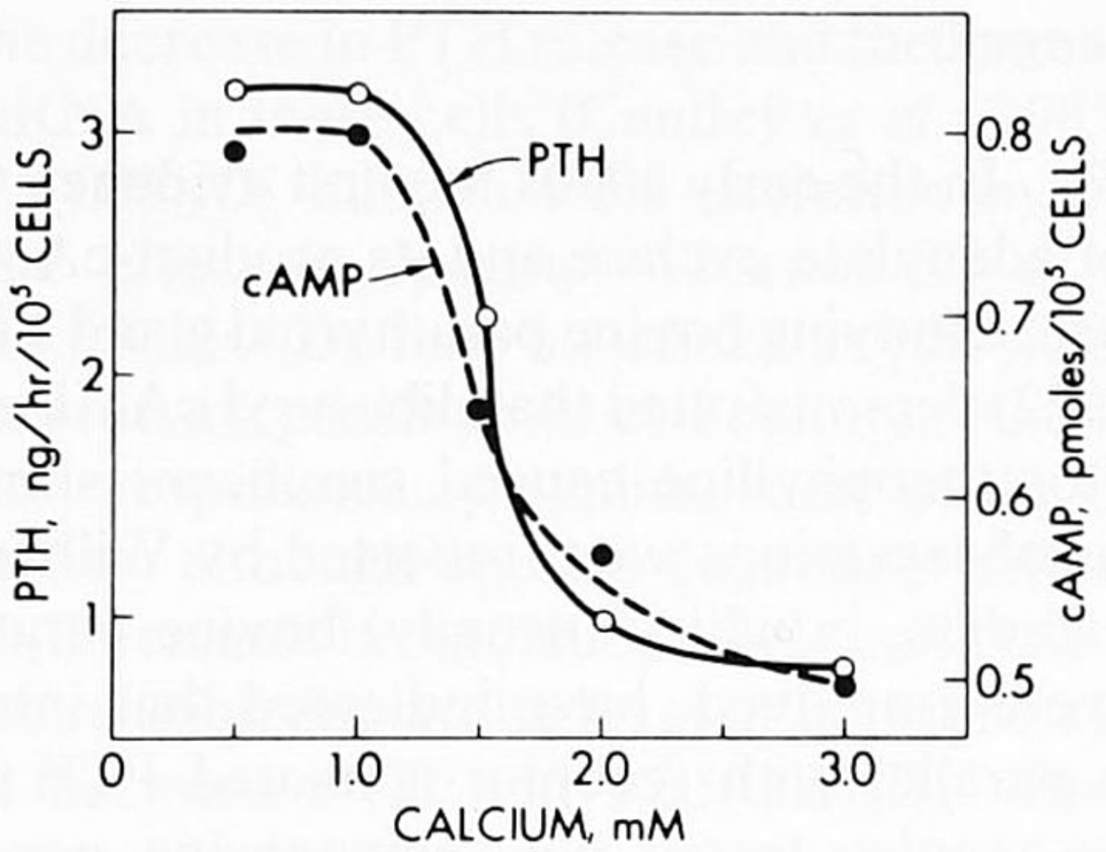

FIG. 5. PTH release and intracellular cAMP as a function of extracellular Ca^{2+} concentration in porcine parathyroid cells in culture (Cohn and Elting, 1983).

appeared to be able to modify this relationship. At high external Ca^{2+} concentrations PTH release induced by a-adrenergic agonists was associated with higher cAMP levels than at low Ca^{2+} concentrations. Thus, variations in external Ca^{2+} apparently modulate the response of the parathyroid cells to a given level of intracellular cAMP.

It was further shown that the inhibition of PTH release by high external Ca^{2+} cannot be explained on a quantitative basis by the observed reduction of cAMP levels (Brown *et al.*, 1978a). At very high external Ca^{2+} concentrations PTH release can be suppressed irrespective of the cAMP levels. Moreover, whereas several secretagogues stimulate cAMP-dependent protein kinase activity in parallel with PTH release, the inhibitory effects of high extracellular Ca^{2+} and Mg^{2+} levels are not mediated by changes in the activity of such enzymes (Brown and Thatcher, 1982). Morissey and Cohn (1979a,b) had concluded earlier, on the basis of pulse-labeling experiments, that PTH release by low extracellular Ca^{2+} was different than that effected by β-adrenergic agonists: whereas low Ca^{2+} stimulated the release of both newly synthesized and stored hormone, with β-adrenergic agonists only stored hormone was released. The release of newly synthesized hormone, in contrast to that of stored hormone, appeared to be independent of the cAMP level in the cells. These data stimulated the search for other intracellular mediators of the effect of extracellular Ca^{2+} on PTH release.

b. *Intracellular Calcium.* Whereas in earlier studies cAMP has been considered the primary second messenger involved in mediating the effects of secretagogues on PTH secretion, more recent data indicate an important role for intracellular Ca^{2+}. In striking contrast to many other

gland cells, in which secretion is stimulated by a rise in intracellular Ca^{2+}, PTH release is associated with a decrease in intracellular Ca^{2+}. Evidence for the implication of intracellular Ca^{2+} was presented by Habener *et al.* (1977), who showed that the calcium ionophores A23187 and X537A inhibited PTH release in the presence, but not in the absence, of extracellular Ca^{2+}. The inhibitory effect of Ca^{2+} ionophores has been frequently confirmed afterward, although not under all conditions (see below).

In both bovine and human parathyroid cells the rise in external Ca^{2+} that suppresses PTH release is accompanied by a rise in intracellular Ca^{2+} (Fig. 6). An increase in intracellular Ca^{2+} is also associated with the effects of other divalent cations that inhibit PTH release (albeit at higher concentrations than Ca^{2+}), such as Mg^{2+}, Sr^{2+}, and Mn^{2+} (Shoback *et al.*, 1983, 1984a; Larsson *et al.*, 1984; Nemeth and Scarpa, 1987). It was further shown that the parathyroid cells of patients with hyperparathyroidism not

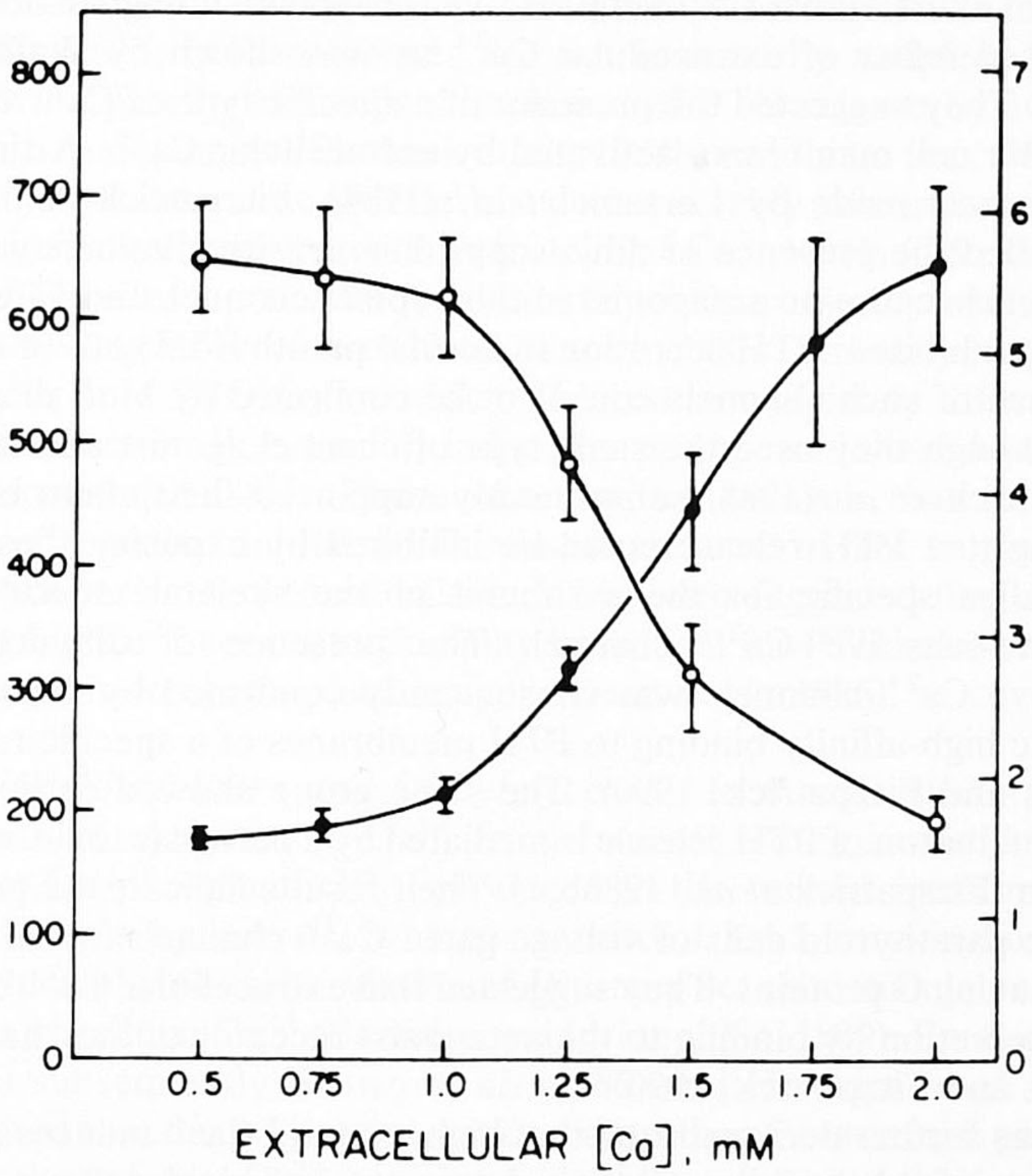

FIG. 6. Relationship between the extracellular Ca^{2+} concentration and PTH release or cytosolic Ca^{2+} in quin2-loaded bovine parathyroid cells *in vitro*. Note the inverse relationship between PTH release and cytosolic Ca^{2+} (Shoback *et al.*, 1983).

only displayed a reduced response to a rise in extracellular Ca^{2+}, but also a chronically reduced level of intracellular Ca^{2+} (Larsson *et al.*, 1984).

The question presents itself whether the rise in intracellular Ca^{2+} is of extracellular or of intracellular origin. In many endocrine cells hormone release is initiated by the influx of Ca^{2+} into the cell through voltage-gated Ca^{2+} channels. A role for such channels in the inhibition of PTH release has been suggested on the basis of results obtained with Ca^{2+} channel blockers such as Verapamil and D600. The results were inconsistent, however. Inhibitory (Hove and Sand, 1981) as well as stimulatory effects (Wallace and Scarpa, 1983) have been reported. These blockers did not influence cytoplasmic Ca^{2+} in the parathyroid cells (Wallace and Scarpa, 1983) and this finding, together with the observation of Wallfelt *et al.* (1985) that membrane depolarization by potassium ions did not influence Ca^{2+} fluxes across the cellular membrane, pointed to the absence of voltage-gated Ca^{2+} channels in parathyroid cells. Nevertheless, the inhibitory effect of extracellular Ca^{2+} on PTH release appeared to be associated with increased influx of extracellular Ca^{2+} as was shown by Wallfelt *et al.* (1985). They suggested the presence of a specific type of Ca^{2+} channel in the outer cell membrane, activated by extracellular Ca^{2+}. A similar suggestion was made by Larsson *et al.* (1984). Fitzpatrick *et al.* (1986a) concluded the presence of dihydropyridine-sensitive voltage-gated Ca^{2+} channels because an antagonist of this type of channel stimulated, and an agonist inhibited, PTH secretion in bovine parathyroid cells *in vitro*. The presence of such channels could not be confirmed by Muff *et al.* (1988), even though they used the same type of channel agonist and antagonist. Fitzpatrick *et al.* (1988) subsequently supported their claim by demonstrating that PTH release could be inhibited by exposing these cells to antibodies specific for the α-subunit of the skeletal muscle dihydropyridine-sensitive Ca^{2+} channel. The presence of dihydropyridine-sensitive Ca^{2+} channels was subsequently confirmed by demonstrating specific high-affinity binding to PTH membranes of a specific radioligand (Jones and Fitzpatrick, 1990). The same group showed earlier that the Ca^{2+} inhibition of PTH release is mediated by a pertussis toxin sensitive G protein (Fitzpatrick *et al.*, 1986b,c). Their results indicate the presence in bovine parathyroid cells of voltage-gated Ca^{2+} channels linked to signal-transducing G proteins. They suggested that extracellular Ca^{2+} can inhibit PTH secretion by binding to the antagonist receptor of the channel itself (Jones and Fitzpatrick, 1990).

It was further demonstrated that high external Ca^{2+} induces a biphasic increase of intracellular Ca^{2+} in bovine parathyroid cells: a sustained increase from extracellular origin was preceded by a transient rise of intracellular Ca^{2+} from internal, nonmitochondrial stores (Nemeth and

Scarpa, 1987; Hawkins *et al.*, 1989). In the same studies it was shown that inositol phosphates were involved in the transduction of the extracellular signals to these stores. Thus, as in other secretory cells, Ca ions of extracellular and intracellular origin contribute to the rise of cytoplasmic Ca^{2+} associated with hormone release in the PTH cells, albeit that changes in intracellular Ca^{2+} had effects different from those in other cells. In most secretory cells release increases sigmoidally with a rise in intracellular Ca^{2+}, with half-maximum stimulation at about 10^{-7} *M* intracellular Ca^{2+}. In bovine PTH cells maximum release was observed at 10^{-7} *M* Ca^{2+}, with secretion at both higher and lower Ca^{2+} concentrations (Pocotte and Ehrenstein, 1989). However, maximum release at much higher intracellular levels (4×10^{-4} *M*) was reported by Oetting *et al.* (1987) for calf PTH cells. The difference was related to the difference in the setpoint for extracellular Ca^{2+} between adult and juvenile bovine PTH cells, but remains difficult to interpret.

Whereas changes in PTH release are frequently associated with changes in cytosolic Ca^{2+}, both responses are not necessarily coupled. In dispersed bovine parathyroid cells stimulation of PTH release by secretagogues such as dopamine (Shoback *et al.*, 1984b) or lithium ions (Nemeth *et al.*, 1986), that increase cAMP levels, do not notably influence cytosolic Ca^{2+}. In similar cells incubated at extracellular Ca^{2+} levels (0.5 m*M*) that stimulate PTH release, the addition of ionomycin increased cytosolic Ca^{2+} without inhibiting PTH release (Nemeth *et al.*, 1986). Similarly, exposure of such cells to $1,25(OH)_2D_3$, which does not immediately affect PTH release (see Section II,A,4,a,iii), induced a prompt and dose-dependent rise in intracellular Ca^{2+}. These observations indicate that receptor-mediated effects that inhibit PTH release are not, or not exclusively, dependent on a rise in cytosolic Ca^{2+}. It has been suggested that a localized change in Ca^{2+} concentration may be critical for PTH release rather than a change in total cytoplasmic Ca^{2+} (Dean *et al.*, 1986).

c. *Inositol Phosphates and Diacylglycerol.* In many secretory cells the increase of cytoplasmic Ca^{2+} from internal stores is mediated by inositol 1,4,5-triphosphate (IP_3), produced in the cellular membrane by phosphoinositide hydrolysis, whereas inositol 1,3,4,5-tetrakisphosphate (IP_4), produced by phosphorylation of IP_3, may act as a stimulator of the influx of extracellular Ca^{2+} (Berridge and Irvine, 1984, 1989). Epstein *et al.* (1985) and subsequently Brown *et al.* (1987) and Hawkins *et al.* (1989) demonstrated that the primary secretion-controlling factor of the parathyroid cells, the external Ca^{2+} concentration, also influenced the formation of several inositol phosphates in these cells, in particular IP_3 and IP_4. In permeabilized bovine parathyroid cells (Epstein *et al.*, 1985), IP_3 pro-

moted the release of intracellular Ca^{2+} stores. A relationship between IP_3 and the initial intracellular Ca^{2+} transient, and of IP_4 with the subsequent more sustained elevation of intracellular Ca^{2+}, was indicated. The accumulation of inositol phosphates likely was dependent on guanine nucleotide regulatory protein (G protein) since it could be promoted by fluoride (Chen *et al.*, 1988; Hawkins *et al.*, 1989), which is known to activate the G proteins that stimulate inositol phosphate formation. The G_i blocker pertussis toxin prevented the inhibitory effects of Ca^{2+} on PTH release (Fitzpatrick *et al.*, 1986c) and cAMP accumulation (Chen *et al.*, 1988). However, pertussis toxin did not suppress the effect of high extracellular Ca^{2+} on IP_3 and IP_4 accumulation, indicating that the formation of these phosphates is mediated by fluoride-stimulated but pertussis toxin-insensitive G protein.

The above data showed that the inhibition of PTH release by extracellular Ca^{2+} could be mediated by IP_3. This is unlikely for IP_4. Substantial formation of IP_4 was only observed at external Ca^{2+} concentrations (5 m*M*) that were well above those producing maximal inhibition of PTH release (Hawkins *et al.*, 1989).

Recently it was suggested that another phospholipid metabolite, phosphatidic acid, may be involved in signal transduction in PTH cells (McGhee and Shoback, 1990), but its role has still to be defined. More data are available on the role of diacylglycerol (DG), a recognized second messenger in many cell types (Berridge, 1987). As in other cells, in the parathyroid cells the formation of IP_3 is stimulated concomitantly with the generation of DG. Diacylglycerol generally stimulates secretion by activating protein kinase C, a group of enzymes connected with secretion and other cell functions, and a stimulator of the plasma membrane calcium pump (Furukawa *et al.*, 1989). Because high extracellular Ca^{2+} stimulates IP_3 as well as DG, one would expect that activation of protein kinase C is associated with inhibition of PTH release. However, in bovine parathyroid cells the relation between DG and hormone release probably is complicated, as appeared in studies on the effects of the activation of protein kinase C by phorbol esters. In most studies PTH release was stimulated (Brown *et al.*, 1984b; Nemeth *et al.*, 1986; Kobayashi *et al.*, 1988), even at high extracellular Ca^{2+} levels. Intracellular Ca^{2+} levels were unaltered (Brown *et al.*, 1984b) or decreased (Nemeth *et al.*, 1986). Kobayashi *et al.* (1988) showed that acute exposure of the cells to low extracellular Ca^{2+} (0.5 m*M*) increased, and high Ca^{2+} (1.75 and 2.5 m*M*) decreased cell membrane protein kinase activity. The authors concluded that the effects of extracellular Ca^{2+} are at least partly mediated by regulation of protein kinase C activity. Membreño *et al.* (1989) came to a different conclusion. They examined the effects of several phorbol esters at high as well as low

extracellular Ca^{2+} concentrations. They could not confirm that these protein kinase C agonists stimulated PTH release in the presence of high extracellular Ca^{2+}. They further showed that the rise in intracellular Ca^{2+} that normally occurred in the presence of high extracellular Ca^{2+} was suppressed in the presence of phorbol esters, which indicated that the stimulation of PTH by this treatment was mediated by reduced intracellular Ca^{2+} levels. Surprisingly, the PTH release normally induced by low extracellular Ca^{2+} was inhibited by these substances. Membreño and co-workers concluded that protein kinase C agonists suppress PTH release at low extracellular Ca^{2+} and enhance PTH release at high extracellular Ca^{2+}. The function of protein kinase C in the regulation of PTH release needs further clarification, although at present the route leading to activation of this enzyme seems the most important one for stimulus-secretion coupling in the PTH cells.

6. *Control of Intracellular PTH Degradation*

As discussed above (Section II,A,3), a substantial part of the newly synthesized and stored PTH is not secreted as intact molecules but as fragments produced by intracellular proteolysis. Chu *et al.* (1973), studying rats fed normal or low calcium diets, concluded that extracellular Ca^{2+} may control the amounts of intracellular PTH through regulation of the rate of intracellular degradation rather than the rate of synthesis. Habener *et al.* (1975) showed that release of intact PTH from bovine parathyroid tissue slices was stimulated 5-fold when extracellular Ca^{2+} was reduced from 2 to 1 m*M*, with little change in the rate of PTH biosynthesis. At 2 m*M* Ca^{2+} intracellular stores of PTH were higher by only 30–40%, much less than predicted on the basis of the observed reduction of PTH release and estimated new formation. They concluded that at high extracellular Ca^{2+} levels up to 50% of the newly synthesized PTH was degraded intracellularly, whereas at low extracellular Ca^{2+} less than 10% was degraded. These results were confirmed for porcine parathyroid tissue *in vitro* by Morissey and Cohn (1979b), who further demonstrated preferential degradation of stored PTH. Interestingly, they demonstrated that only the degradation of newly synthesized PTH was controlled by external Ca^{2+}. Evidence for extracellular Ca^{2+} as a factor controlling the catabolic pathway in the parathyroid cells was also obtained *in vivo*. Starting from the notion that the degradation products are predominantly C-terminal fragments, Mayer *et al.* (1979) determined the ratio of C-terminal and N-terminal fragment immunoreactivity in the venous effluent plasma of neonatal calves. The C : N ratio increased from about 1.3 in hypocalcemic animals to more than 3 during hypercalcemia. They concluded that a relatively high percentage of intact hormone molecules was released dur-

ing hypocalcemia, whereas mostly inactive fragments were secreted during hypercalcemia. These results have been confirmed for human parathyroid tissue *in vitro* by Hanley and Ayer (1986). They showed that at high Ca^{2+} levels proportionally more carboxy-terminal PTH fragments were released than intact PTH, whereas the reverse was found at low Ca^{2+} (Fig. 7).

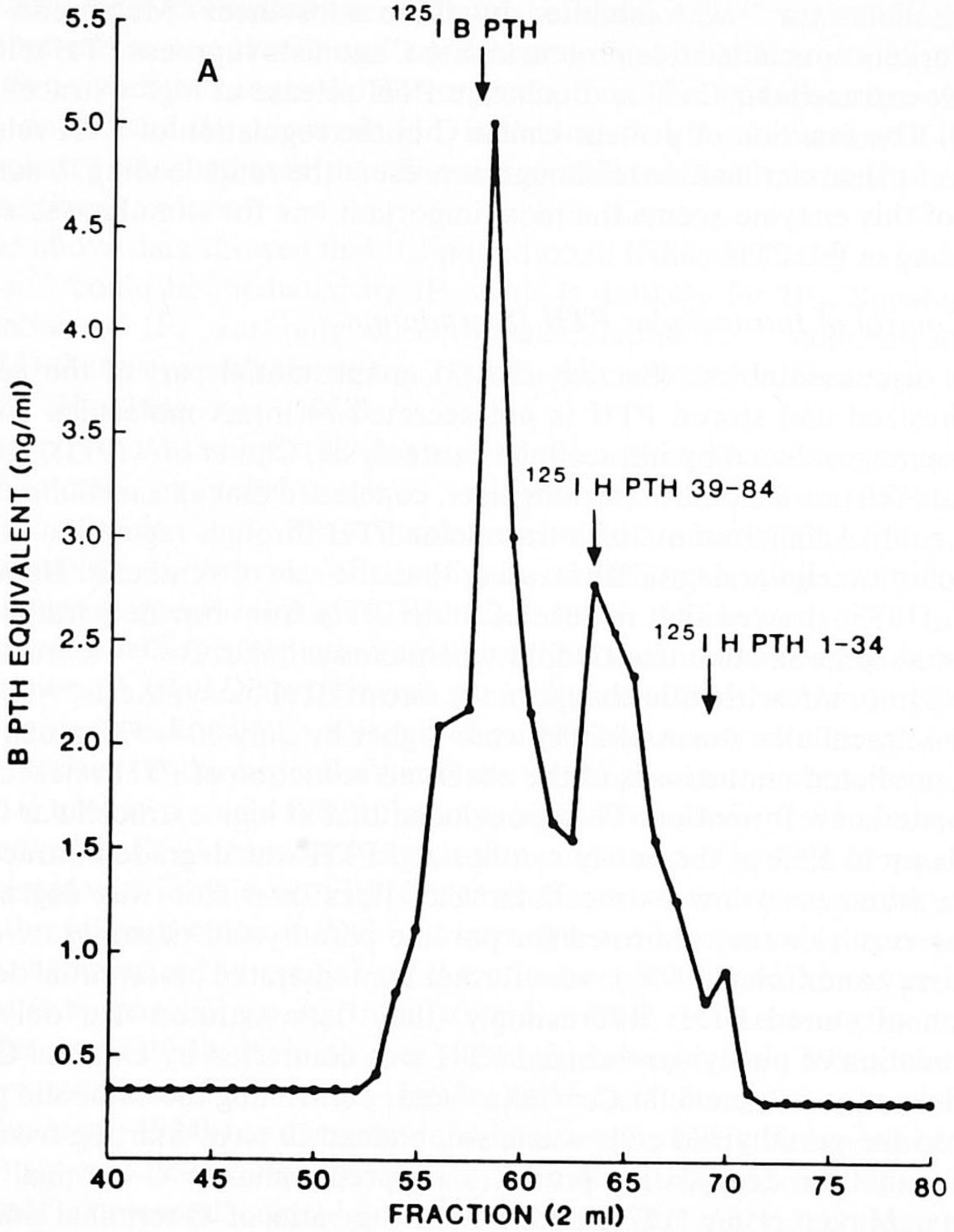

FIG. 7. Chromatographic profile of perifusates of hyperplastic human parathyroid tissue perfused with low calcium (A, 0.5 m*M* Ca^{2+}) or high-calcium (B, 2.0 m*M* Ca^{2+}) fluid. (A) Note the prominent first peak of PTH coeluting with bovine PTH and smaller peaks coeluting with human PTH (39-84) and human PTH (1-34). (B) The first peak is smaller than the second peak, indicating that proportionally more fragments than intact PTH were released from the tissue when exposed to the high Ca^{2+} concentration (Hanley and Ayer, 1986).

Concomitantly with suppression of PTH release the number of lysosomelike bodies in the cytoplasm increases (Capen, 1971; Roth *et al.*, 1974; Setoguti *et al.*, 1981). These bodies may be involved in hormone degradation, as has been described for other endocrine gland cells after suppression of hormone release (Farquhar, 1976). However, many of the hormone degradation products are released from the parathyroid cells, although in this respect species differences seem to occur. Since the contents of lysosomes in general are not released from the cell–with the lysosomes of the liver as notable exception–a nonlysosomal pathway cannot be excluded.

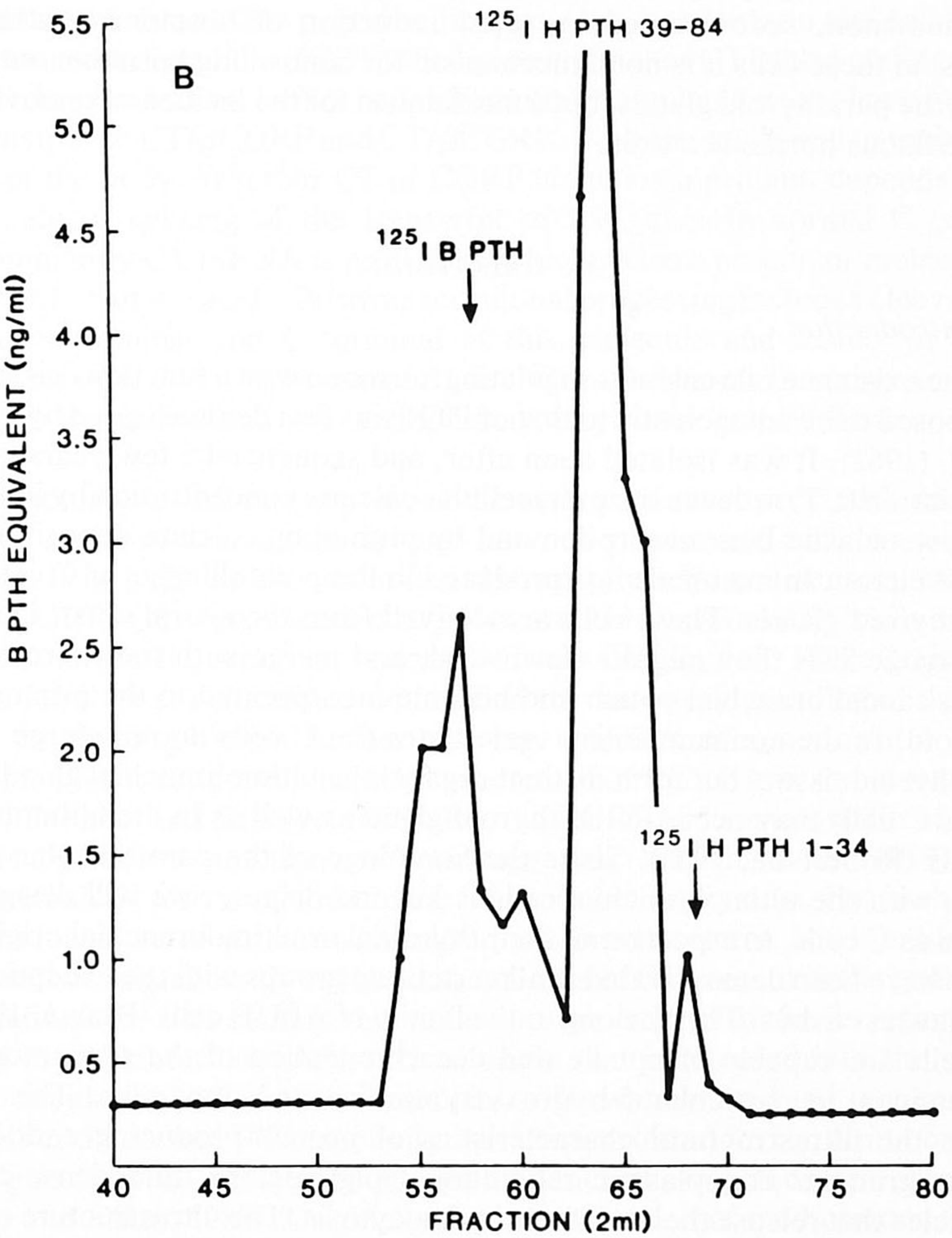

FIG. 7 (*continued*)

Whatever the mechanism involved, the above data strongly suggest that there is a constant overproduction of PTH by the parathyroid glands, and that the physiological demand, reflected by the calcium concentration of the extracellular fluid, determines the ratio of intact to degraded hormone that is released into circulation. Regulation of hormone degradation rather than hormone production as a mechanism of control of hormone secretion seems unique for the parathyroid glands. Although hormone degradation is commonly observed in other endocrine glands, this process concerns a substantial part of hormone production only when the physiological demand is suddenly reduced, e.g., in prolactin cells of lactating rats after removal of the pups (Farquhar, 1976). In these cells it is also a transient phenomenon, accompanied by rapid reduction of hormone synthesis. Thus, in these cells it is not a mechanism for controlling hormone output, as in the parathyroid glands, but a mechanism for the incidental removal of superfluous hormone stores.

B. Calcitonin

1. Introduction

The existence of a calcium-regulating hormone with a function originally supposed to be antagonistic to that of PTH was first demonstrated by Copp *et al.* (1962). It was isolated soon after, and sequenced a few years later. Calcitonin (CT) reduces the extracellular calcium concentration by inhibiting osteoclastic bone resorption and by promoting calcium deposition in the skeleton. In mammals it is produced in the parafollicular or C cells of the thyroid glands. These cells are derived from the neural crest. During embryogenesis they migrate downward, and merge with tissue from the most caudal branchial pouch and become incorporated in the primordial thyroid. In the nonmammalian vertebrates the C cells do not merge with the thyroid tissue, but form distinct organs, the ultimobranchial glands. In birds C cells may occur in the thyroid gland as well as in the ultimobranchials (Robertson, 1986). Since the homology of the parafollicular or C cells with the ultimobranchial cells is beyond dispute, we will designate them as C cells, irrespective of their thyroidal or ultimobranchial origin. C cells have been demonstrated in all vertebrate groups with the exception of the jawless fishes. They belong to the family of APUD cells (Pearse, 1968): C cells are capable of uptake and decarboxylation of the precursors of bioamines, in particular 5-hydroxytryptamine and dopamine. The cells have the ultrastructural characteristics of protein-producing endocrine cells: granular endoplasmic reticulum, Golgi regions, and dense-cored vesicles that release their contents by exocytosis. The ultrastructure of the

C cells is remarkably similar in fish to mammal (Nuñez and Gershon, 1978; Ericson and Sundler, 1984; Robertson, 1986). Thyroid glands and ultimobranchial bodies have in general a rich innervation of autonomic origin. Adrenergic, cholinergic, and peptidergic fibers have been demonstrated (Munson, 1976; Nuñez and Gershon, 1978). As reviewed by Robertson (1986), there is no structural evidence for synaptic innervation of the C cells in mammals, with the exception of dolphins. Synaptic contacts with C cells has also been established for some birds and amphibians, whereas in reptiles and fish no direct neural contacts have been reported, although nerve fibers are common in the connective tissue around the cells.

In addition to CT and varying quantities of bioamines, the C cells frequently produce other peptides, in particular somatostatin and calcitonin gene related peptide (CGRP). The gene coding for CT is also coding for CGRP. In man and rat in fact two differentially regulated genes have been demonstrated: CT/αCGRP and CT/βCGRP. Both are expressed in various parts of the body. Whether CT or CGRP is the main product depends on the mode of splicing of the transcript of the gene. In normal C cells predominantly CT mRNA is produced, which yields a precursor molecule of about 135 amino acids. Posttranscriptional processing includes cleavage of the N terminal and C terminal of this molecule and results in the formation of CT. From mammal to fish CT consists of 32 amino acids, although the amino acid composition may vary considerably (Zaidi *et al.*, 1987).

Calcitonin gene related peptide is the main product expressed in many regions of the central and peripheral nervous system and cardiovascular system. It has effects on cardiovascular, respiratory, and sensory functions. CGRP is also produced in large amounts in transformed C cells and, generally in small quantities, in normal C cells (Zaidi *et al.*, 1987; Breimer *et al.*, 1988). It is colocalized with CT in the secretory granules of the C cells. In these granules further varying amounts of somatostatin are present. This location implies the cosecretion of CGRP and somatostatin with CT (Zabel and Schäfer, 1988). For the CGRP and somatostatin of the C cells a paracrine function has been suggested (Williams *et al.*, 1979).

Calcitonin is also produced in the central nervous system, but in these regions it may act as a neurotransmitter or neuromodulator, and is unlikely to contribute significantly to the total amounts of CT present in the general circulation. We will confine this review to the control of the secretion of CT of thyroidal and ultimobranchial origin that is released into the systemic circulation. CT of ultimobranchial origin was isolated and sequenced in birds and fish, and CT bioactivity was demonstrated for the ultimobranchial glands of all vertebrate groups in which these glands are present (Copp and Kline, 1989). Originally the main function of the hormone was

defined as the control of the extracellular calcium level, in an antagonistic action with PTH. In young mammals, birds, and reptiles, the hypocalcemic effects of CT were generally clear and undisputed (Copp and Kline, 1989). The hypocalcemic response of young rats has frequently been used as a reliable and sensitive bioassay for CT, and salmon and human CT preparations are effective therapeutic agents in treating hypercalcemia in man (Wisneski, 1990). However, the function of CT as a hypocalcemic hormone was reevaluated in the early 1970s, because chronically elevated or reduced circulating levels in mammals or birds were only occasionally associated with substantial disregulation of plasma calcium levels. The cycle in annual plasma calcium concentration in frogs was unchanged by removal of the ultimobranchial glands (Fig. 8) in contrast to removal of the parathyroid glands, which had a depressive effect (Robertson, 1977). Administration of CT, although effective in young animals, frequently did not reduce plasma calcium levels in adult mammals, birds, and reptiles (Copp and Kline, 1989). Observations on plasma calcium levels in amphibians were contradictory (Srivastav and Rani, 1989). In these animals CT stimulated the accumulation of calcium carbonate in the lime sacs (Oguro *et al.*,

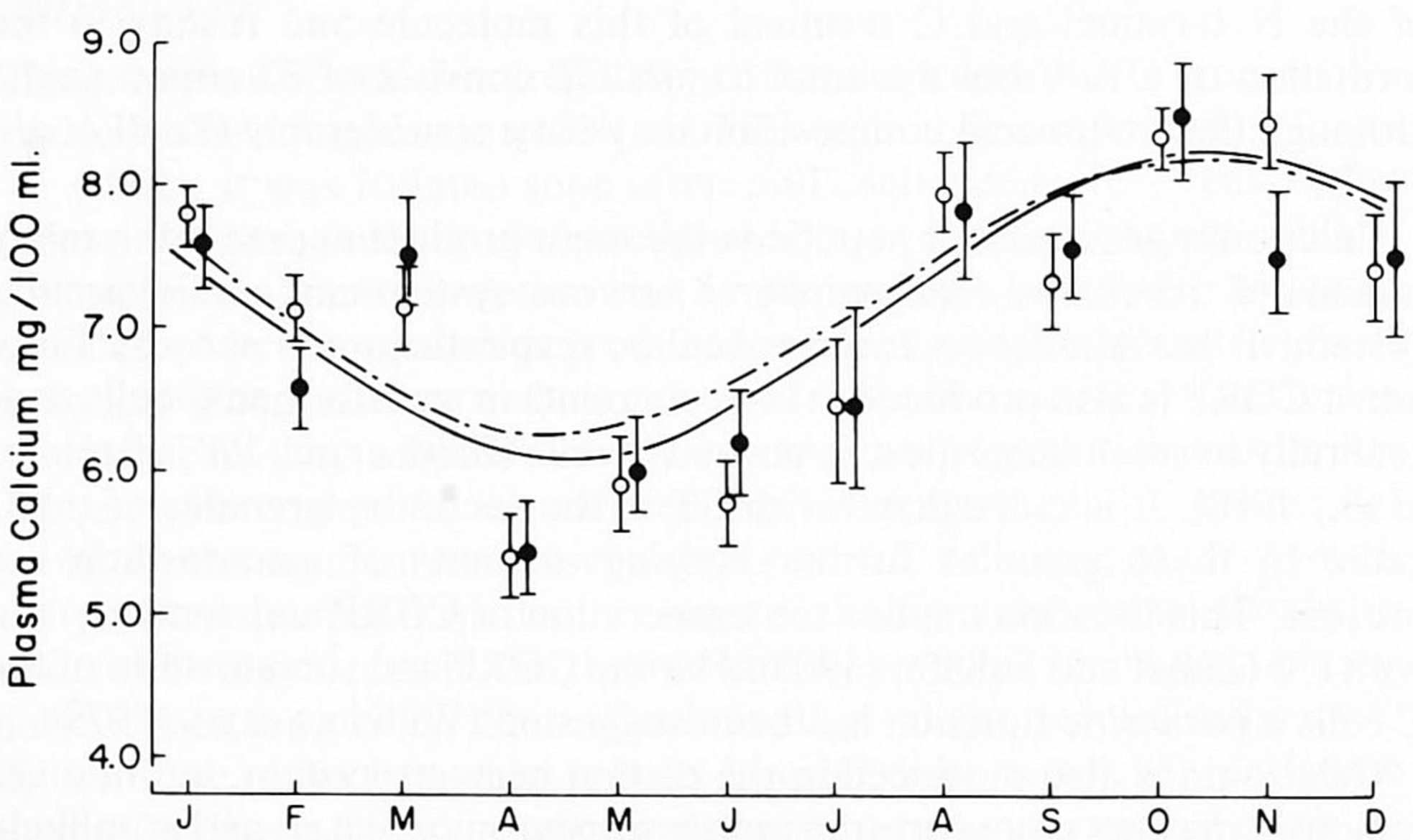

FIG. 8. Annual plasma calcium concentration in sham control and ultimobranchialectomized (UBX) adult male frogs (*Rana pipiens*). The periodic change in calcium levels of intact frogs (solid line) ($N = 247$) throughout the year is expressed: $Ca_p = 7.186 + 1.079 \cos (30r - 315)$; $r = 0.635$; $p = .001$ and not significantly different from controls. Both groups display a significant ($p = .001$) minimum in April–June, while the maximum plasma calcium levels are attained in October–November. Each point is mean ± SD; $N = 15$–30 frogs (Robertson, 1977). ○, Control; ●, UBX.

1984; Srivastav and Rani, 1989) and prevented the PTH-induced mobilization of these calcium stores (Robertson, 1972). Fish will be discussed separately (see Section III,B). Thyroidectomy in mammals or ultimobranchialectomy in birds or amphibians (Robertson, 1977; Kapoor and Chhabra, 1981) were normally not followed by a rise in plasma calcium. Although such observations could be explained by the assumption that potential hypocalcemic actions of CT are obscured by efficient hypercalcemic control mechanisms, they have raised doubts about the importance of CT as a hypocalcemic hormone. The role of CT in reducing plasma calcium may be limited to redressing the hypercalcemia resulting from intestinal calcium resorption during and after a meal. This explains the well-known postprandial surge in CT secretion (Gray and Munson, 1969). It is also consistent with observations that calcium infusions produced hypercalcemia in ultimobranchialectomized birds or amphibians, but had no or small effect on intact controls (Calamy and Barlet, 1970; Copp *et al.*, 1970; Robertson, 1971a; Sasayama and Oguro, 1976; Sasayama, 1978). CT is further essential for the conservation of ingested calcium by promoting calcium deposition in the bone. It further protects the skeleton against demineralization, by inhibition of osteoclastic activity and osteocytic osteolysis, during periods of potential calcium shortage, such as growth, pregnancy, and lactation and, in the submammalian vertebrates, ovarian growth and yolk formation (Lewis *et al.*, 1971; Stevenson *et al.*, 1979). CT also stimulates phosphate deposition into bone and prevents its loss from bone and bone fluid (Talmage and Van der Wiel, 1979). With respect to plasma calcium, CT may have an antihypercalcemic function rather than being implicated in the minute-to-minute control of plasma calcium homeostasis. The function of CT will be discussed further in Section IV, after we have reviewed the factors regulating CT secretion.

2. *Control of CT Secretion*

In mammals the control of CT secretion has received less attention than that of PTH. This is understandable for two reasons. First, the modes of control turned out to follow the general pattern known from other cell types, whereas the control of PTH secretion is unique for the hormone. Second, the number of CT-producing cells is relatively small (in humans about 0.1% of thyroid mass), and, because of the location of these cells between the thyroid follicles, rather inaccessible for experimental manipulation or *in vitro* studies. To overcome the latter difficulties, several studies concern medullary thyroid carcinoma tissue, which produces large amounts of CT. However, data obtained with transformed cells require verification in normal cells and this has been done only occasionally.

a. *Extracellular Calcium.* Extracellular calcium is considered an important regulator of CT secretion in mammals as well as in the submammalian vertebrates. In general, plasma CT levels parallel daily fluctuation in plasma calcium concentrations, as has been demonstrated for mammals (Milhaud *et al.*, 1972), birds (March and McKeown, 1977), and amphibians (Robertson, 1978). The C cells become activated by experimental hypercalcemia induced by, e.g., injections of calcium salts or vitamin D preparations. Following such treatments, in mammals hypertrophy of the C cells, enlargement of granular endoplasmic reticulum and Golgi regions, degranulation of the cytoplasm, and release of secretory granules by exocytosis were reported (Zabel and Schäfer, 1988; Nuñez and Gershon, 1978; Ericson and Sundler, 1984; Srivastav and Rani, 1988). Such structural changes were accompanied by decreased intracellular CT contents and elevated CT levels (Cooper *et al.*, 1971a; Rix *et al.*, 1984; Zabel and Schäfer, 1988). Drinking of calcium-containing water resulting in rises of plasma calcium within the normal range induced dose-related increases in circulating CT levels in young adult men. This was consistent with the hypothesis mentioned above that CT prevents excessive postprandial hypercalcemia (Austin *et al.*, 1979; Fig. 9). The rise in CT following calcium administration can be a rapid response: a surge of CT occurred in healthy persons within 2 minutes. Plasma calcium returned to normal within 30 minutes after the start of calcium infusion (Rude and Singer, 1977; Parthemore and Deftos, 1978). Ingestion of $CaCl_2$ by newborn rats, which induced a rapid rise of plasma calcium levels, resulted in a biphasic response: within 5 minutes a peak in plasma CT occurred that was followed by a prolonged rise starting after 1 hour (Garel and Besnard, 1980).

The effects of extracellular calcium on CT secretion are, at least partially, direct. This was first indicated by the now classical experiment that led to the discovery of CT (Copp *et al.*, 1962). Perfusion of the thyroid glands of cannulated dogs with calcium-enriched blood produced hypocalcemia in the systemic circulation. In a similar perfusion experiment in pigs, increased CT release was demonstrated by direct measurement in the thyroid effluent blood (Care *et al.*, 1968). This was confirmed for perfused ultimobranchial glands in chicken (Ziegler *et al.*, 1969) and goose (Bates *et al.*, 1969). Rat thyroid slices incubated *in vitro* responded with increased CT release to increased ionic calcium levels in the extracellular fluid (Feinblatt and Raisz, 1971); this was confirmed for different mammalian species including man (Bell, 1975; Cooper *et al.*, 1977b), and for ultimobranchial glands of birds (Feinblatt *et al.*, 1975; Nieto *et al.*, 1975), and fish (see Section III,B,2).

Relatively few studies are available on the effects of extracellular Ca^{2+} on CT synthesis. As reported earlier, experimental manipulation of plasma

calcium for 6 hours in rats revealed that low calcium markedly stimulated PTH-mRNA in the parathyroid cells. However, neither increased nor decreased plasma calcium had any effect on CT-mRNA in the thyroid tissue of these animals (Fig. 4; Naveh-Many *et al.*, 1989). This confirmed the result of an earlier *in vitro* experiment showing that high calcium increased CT release but had no effect on CT-mRNA in rat thyroid slices over 3- and 6-hour periods (Jacobs *et al.*, 1983). However, Segond *et al.* (1984) reported a rapid but transient stimulation of CT-mRNA levels 2–8 minutes after acutely induced hypercalcemia. Administration of depolarizing potassium concentrations in the rat increased plasma CT levels as well as CT-mRNA extracted from the thyroid gland within 30 minutes. (Jousset *et al.*, 1988).

The response of C cells to hypocalcemia is puzzling as far as mammals are concerned. Whereas reduction of secretory activity might have been expected for a hypocalcemic hormone, frequently the reverse has been indicated. In chronically parathyroidectomized rats, which experience hypocalcemia for at least 1 or 2 months, serum CT as measured by bioassay increased, and CT accumulated in the thyroid gland (Gittes *et al.*, 1968). Using a homologous RIA, Peng and Garner (1979) confirmed this observation. After several months they observed C-cell hyperplasia, increased thyroid CT-content and plasma CT levels, and a rise of plasma calcium to normal concentrations. Increased mitotic activity, hypertrophy, and hyperplasia during hypocalcemia were reported several times (Nuñez and Gershon, 1978; Srivastav and Rani, 1988). In addition vacuolar degeneration of C cells was observed (Peng and Garner, 1979), suggestive of physiologically controlled cell death by apoptosis (Wyllie *et al.*, 1980). These responses are difficult to interpret when starting from the hypothesis that CT is involved in the homeostatic control of plasma calcium. They can be explained by the assumption that CT protects the skeleton against demineralization that may occur during prolonged hypocalcemia.

For submammalian vertebrates, data on the response of C cells to hypercalcemia or hypocalcemia are scarce, but in general they point to activation in the former, and inactivation in the latter, condition. Light microscopic indications for C cell activation were reported for birds, lizards, and frogs. Young chickens on a calcium-deficient diet showed cellular atrophy, and granule accumulation was reported during experimental hypocalcemia in iguanas (Copp and Kline, 1989). For fish ultimobranchial cells, similar responses were reported (see Section III,B).

b. *Hormones and Neurotransmitters. i. Proteins and peptides.* Factors originating from the gastrointestinal system frequently have been

reported to influence CT secretion. This is of interest with respect to the antihypercalcemic function ascribed to CT during and following dietary calcium uptake. Attention was drawn to the gastrointestinal hormones by the observation that in pig thyroid venous blood CT levels rose after oral calcium administration even without detectable changes in plasma calcium (Cooper and Deftos, 1970). Subsequently, Cooper *et al.* (1971b) and Care *et al.* (1971a) demonstrated the potent stimulatory effects of gastrin and related peptides on CT secretion, such as the structural analogue pentagastrin and cholecystokinin. The latter hormone has some structural C-terminal homology with gastrin. Infusion of low doses of gastrin into the thyroid artery of pigs stimulated CT secretion, whereas intravenous infusion had no effect, indicating that the hypocalcemic action reported for the peptide was mediated by CT (Cooper *et al.*, 1972). Oral administration of meat or calcium at doses that did not noticeably increase plasma calcium levels increased both plasma gastrin and glucose in pigs (Care *et al.*, 1971b; Cooper *et al.*, 1974). The physiological importance of the gastrin-CT connection therefore seems well established in these animals. Whether it is of significance for other species is unclear. For adult men, McGuire *et al.* (1972) reported that gastrin infusion of physiologically relevant doses induced hypocalcemia. However, a rise in plasma gastrin levels induced by low or high calcium meals was not associated with increased CT levels, whereas plasma CT could not be increased with intravenous pentagastrin infusion, unless at very high doses. At least in adult men the physiological significance of the gastrin-CT relationship is doubtful. In rats it is unlikely that any effects of feeding on CT release are mediated by gastrin. In this species CT release was stimulated by feeding, even when plasma calcium was slightly below normal (Talmage *et al.*, 1975; Toverud and Munson, 1976). In particular CT release was increased in young rats when suckling and in their mothers when eating (Cooper *et al.*, 1977a). Nevertheless, gastrin injections were ineffective in stimulating plasma CT levels in testing and suckling newborn rats or their mothers (Garel and Jullienne, 1977; Cooper *et al.*, 1977b), and attempts to promote CT secretion by thyroid tissue of suckling rats were unsuccessful (Cooper *et al.*, 1977b).

Secretin is another potential secretagogue. In normal human subjects as well as in 8-day-old rats secretin stimulated serum CT levels. The effective doses were about three times those observed in the postprandial state. A dose-related increase in CT release was found during incubation with secretin of rat thyroid tissue (Sethi *et al.*, 1981). Interestingly, in rats, duodenal infusion of acid (known to stimulate secretin but not gastrin secretion) caused a slight but significant rise in serum CT concentration (Sethi *et al.*, 1983).

Another intestinal hormone that could be a mediator of intestinal influence on the C cells is glucagon. Pancreatic glucagon was reported to induce hypocalcemia in several mammalian species (Munson, 1976). For dogs it was concluded that the glucagon-induced hypocalcemia was mediated by CT, because glucagon was ineffective in thyroidectomized animals (Avioli *et al.*, 1969). Glucagon had no effect on plasma CT in 2-hour-old fasted newborn rats, although a slight elevation was noted in 14-day-old fasted rats (Garel and Jullienne, 1977). However, in general no clear effects of glucagon on plasma CT-levels have been demonstrated and its physiological importance as a secretagogue for CT has not been established. Another pancreatic peptide, somatostatin, caused a dose-related decrease of serum CT in rat and monkey (Hargis *et al.*, 1978) and rats receiving somatostatin antiserum had elevated CT levels (Williams *et al.*, 1979). The authors suggested that somatostatin, which can be cosecreted with CT (see Section II,B,1) is a local regulator of CT release.

The autonomic nerves innervating the thyroid of several mammalian species contain neuropeptides such as vasoactive intestinal peptide (VIP), gastrin/cholecystokinin (CCK), and substance P. Only substance P could be demonstrated in nerves innervating the chicken ultimobranchial glands (Ahrén *et al.*, 1980, 1983). With the exception of VIP, these neuropeptides did not stimulate thyronine secretion after intravenous injection in rats. However, VIP, CCK-4, CCK-8, and substance P all stimulated CT secretion in these animals (Ahrén *et al.*, 1983), indicating that nerves ending on or around the C cells could influence CT secretion.

ii. Bioamines. The literature on this subject before 1980 has been reviewed by Heath (1980) and will be summarized briefly. Evidence for catecholaminergic effects on CT secretion was first obtained by perfusion of thyroid glands of young pigs *in situ* with high-calcium blood (Bates *et al.*, 1970). Epinephrine and the β-agonist isoproterenol stimulated CT release, although only in the presence of the α-adrenergic blocker phentolamine. The effect was inhibited by the β-blocker propranolol, confirming the β-adrenergic nature of the stimulation (Bates *et al.*, 1970). The stimulatory effect of epinephrine was confirmed for sheep. However, in these experiments both isoproterenol and phentolamine prevented the stimulation of CT secretion (Philippo *et al.*, 1970). In similar experiments on dogs, epinephrine was ineffective when given alone, but was stimulatory in combination with phentolamine (Avioli *et al.*, 1971). Care *et al.* (1970) demonstrated for pigs that the effect of epinephrine was much more pronounced than that of norepinephrine, an observation that has been confirmed *in vitro* for porcine thyroid gland slices (Bell, 1975). These and other studies established a β-adrenergic stimulation and α-adrenergic inhibition

of CT release in several mammalian species, including man (Munson, 1976; Heath, 1980). The physiological significance of these effects is still unclear, however. Pathologically elevated plasma catecholamine concentrations in man were not generally associated with increased CT levels (Miller *et al.*, 1975), and adrenalectomy in rats did not impair CT secretion or the response of the C cells to hypercalcemia (Heath *et al.*, 1980). Evidence for a role of sympathetic nerves is poor (see below). Since dopamine is produced by the C cells and can be cosecreted with CT, effects on C cells might be anticipated. Dopamine indeed stimulated CT release *in vitro* from porcine thyroid tissue (Bell, 1975). A similar inhibitory effect on CT secretion was reported for the precursor L-dopa when administered to patients with medullary thyroid carcinoma. The substance reduced the stimulatory response to infusion of calcium or pentagastrin. It was also effective on cultured carcinoma tissue (Baylin *et al.*, 1979).

iii. Steroids. For several mammalian species a clear sex difference has been reported in plasma CT levels. In rats, lower basal values were found for males than for females (Peng *et al.*, 1978; Roos *et al.*, 1978) and this difference increased after feeding (Peng and Garner, 1979; Fig. 9). Women had lower basal as well as calcium-stimulated or gastrin-stimulated CT levels than men (Heath and Sizemore, 1977; Hillyard *et al.*, 1978). Nevertheless, in humans as well, exogenous estrogens (but not androgens) stimulated CT secretion (Stevenson *et al.*, 1981, 1983). This effect probably was attributed to the reduction in plasma calcium and reduced bone resorption following estrogen treatment (Riggs *et al.*, 1969; Aitken *et al.*, 1971). More recently receptor-mediated direct effects of estrogens were reported on bone cells, showing that there were also interactions with CT and PTH at the target level (Eriksen *et al.*, 1988; Colston *et al.*, 1989; Takano-Yamamoto and Rodan, 1989). While Morimoto *et al.* (1980) did not observe a change in plasma CT levels in elderly women treated with estrogens for several weeks, possibly because of the time of the day of blood sampling, the increment in plasma CT in response to calcium infusion was significantly higher after estrogen treatment than before, indicating increased sensitivity to CT. There was a close relationship between plasma estrogens and CT in adult women, and the postmenopausal reduction in estrogen levels was associated with lowered CT secretion (Stevenson *et al.*, 1983). Since plasma PTH and $1,25(OH)_2D_3$ were in general unchanged (Stevenson *et al.*, 1981) the low CT levels were the most likely cause of the increased loss of bone minerals in postmenopausal women, which may result in osteoporosis. Very low CT levels were reported for osteoporotic patients (Milhaud *et al.*, 1978). Bone resorption could be prevented or even slightly reversed by administration of estrogens and/or CT, in particular in combination with calcium supplements

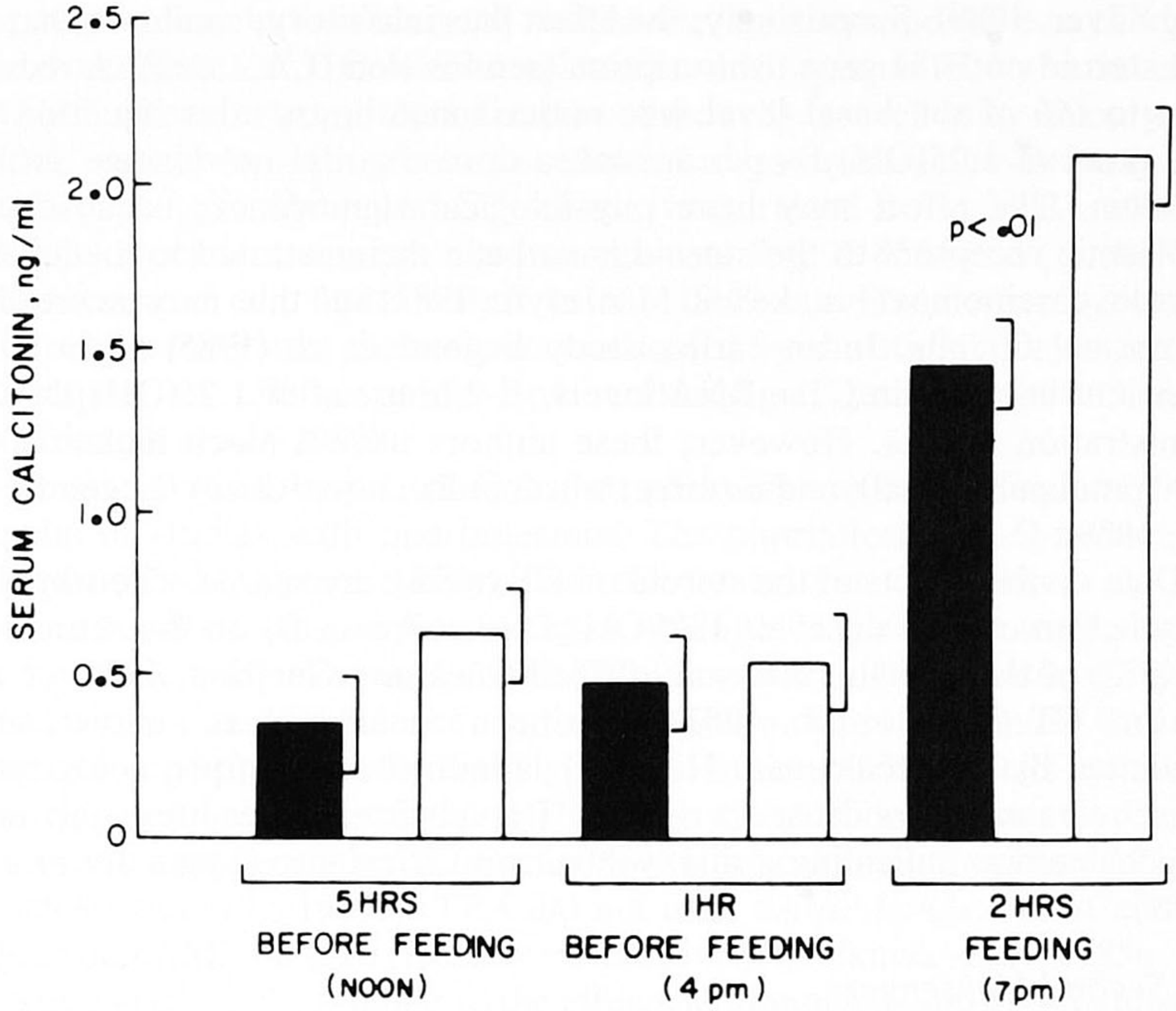

FIG. 9. Sex difference in the rise in CT after feeding in rats on a fixed feeding schedule. Food was given at 5 PM. There were 12 rats in each group. The height of each bar represents the mean values, and the brackets show SE. The *p* value refers to the comparison of means between 6-month-old female (unshaded area) and male (shaded area) rats that were fed for 2 hours (Peng and Garner, 1979).

(Jowsey *et al.*, 1978), although the effect might be transient (Aitken *et al.*, 1971).

Variations in plasma CT were reported during the menstrual cycle (Pitkin *et al.*, 1978), but this observation was not confirmed (Baran *et al.*, 1980). Other ovarian factors might be implicated in addition to estrogens. The role of estrogens in control of CT could be species specific. In adult rats, with higher CT levels in males than in females, ovariectomy induced a transient fall in CT secretion, which could not be corrected by exogenous estrogens (Cressent *et al.*, 1981). After studying CT levels during the estrus cycle in rats (Cressent *et al.*, 1983) a minimum was reported during estrus followed by a peak during diestrus. The relationship with estrogens was not clear, and no correlation was found with plasma calcium levels.

Recently $1,25(OH)_2D_3$ was reported to influence CT-gene transcription rate in rat C cells, at physiologically relevant concentrations (Naveh-Many

and Silver, 1988). Surprisingly, the effect was inhibitory, similar to that of the steroid on PTH gene transcription (see Section II,A,4,c,iii). A reduction to 6% of the basal level was noticed at 6 hours after injection of 100 pmol of $1,25(OH)_2D_3$ per animal, a dose that did not change serum calcium. The effect may have physiological significance, because cytoplasmic receptors to the steroid have been demonstrated in medullary thyroid carcinomas (Freake and MacIntyre, 1982) and thus may be present in normal C cells. In an earlier study Segond *et al.* (1985) reported a transient increase in CT-mRNA levels, 1–2 hours after $1,25(OH)_2D_3$ administration to rats. However, these authors used a much higher dose (500 pmol per animal), and no effect was found at lower doses (Legendre *et al.*, 1989).

Data on the effects of the steroid on CT release are scarce. The stimulatory action of high doses of $1,25(OH)_2D_3$ or vitamin D_2 on the secretory activity of the C cells (Munson, 1976; Nuñez and Gershon, 1978) or on plasma CT (Raue *et al.*, 1983) have been considered as indirect, and mediated by hypercalcemia. However, administration of physiologically more relevant doses caused a rise in CT levels in rats, despite a transient hypocalcemia, indicating a direct effect on CT release (Legendre *et al.*, 1989).

3. Second Messengers

Cyclic AMP, cytosolic Ca^{2+}, and phosphatidylinositides have all been implicated in the external signal transduction leading to increased CT synthesis and release. The stimulating effect of cAMP, in contrast to that of cGMP, was established by the early studies of Bell (1970) and Bell and Queener (1974) on porcine tissue slices, that were soon confirmed and extended for other mammals and for birds (Feinblatt and Raisz, 1971; Nieto *et al.*, 1975). In cultured human medullary thyroid carcinoma (MTC) cells, cAMP decreased cell proliferation, an indication of maturation in these cells, and increased CT gene transcription and CT release (De Bustros *et al.*, 1985). The stimulating effect of cAMP was observed only in the presence of extracellular Ca^{2+}. The suppressive effect of Ca^{2+} entry blockers on the *in vitro* release of CT from rat thyroparathyroid gland complexes was consistent with this observation and indicated the importance of Ca^{2+} influx as a signal for CT release (Ramp *et al.*, 1979). During treatment with the calcium channel agonist Bay K 8644 (Hishikawa *et al.*, 1985a) or after a rise in extracellular Ca^{2+} (Haller-Brem *et al.*, 1987) cytosolic Ca^{2+} and CT release were raised concomitantly in rat MTC cells. Fried and Tashyan (1986) have drawn attention to the unusual calcium sensitivity of cytosolic Ca^{2+} in such cells to changes in extracellular Ca^{2+}. Haller-Brem *et al.* (1987) studied a human MTC cell line that, through an

apparent defect in the outer cell membrane, was unresponsive to changes in extracellular Ca^{2+}. Calcitonin release could be stimulated by extracellular Ca^{2+} after electropermeabilization of the cells, as well as by ionomycin, a Ca^{2+} ionophore acting on intracellular Ca^{2+} stores (Haller-Brem *et al.*, 1987). Cytosolic $Ca^{2}+$ and CT release were raised in rat MTC cells by voltage-dependent calcium channel agonists Bay K 8644 and (+)202-791 (Hishikawa *et al.*, 1985a; Muff *et al.*, 1988). Involvement of voltage-independent calcium channels was indicated by the stimulatory effect of maiotoxin on CT secretion from cultured rat fetal thyroid C cells (Nishiyama *et al.*, 1990).

Involvement of the phosphoinositide pathway was first indicated by the results of studies with phorbolesters. The phorbolester 12-O-tetradecanoylphorbol-13-acetate (TPA) had effects on rat (Hishikawa *et al.*, 1985a,b) and human (De Bustros *et al.*, 1986) MTC cells that were similar to those of cAMP decreased cellular proliferation, and stimulated CT gene transcription and CT release. In the activation of protein kinase C, TPA may act as a substitute for diacylglycerol. In the rat MTC cells synthetic diacylglycerol also stimulated release. The effect of both TPA and diacylglycerol was synergistically enhanced with the calcium ionophore A23187 (Hishikawa *et al.*, 1985b). TPA did not alter cAMP levels: the effects of TPA and cAMP on CT release were additive (Hishikawa *et al.*, 1985b; De Bustros *et al.*, 1986), similar to the effects of ionomycin and TPA in human MTC cells (Haller-Brem *et al.*, 1988). Thus, similar to many other cells, including the parathyroid cells, both the cAMP-dependent protein kinase A route and the Ca^{2+}-dependent phosphatidyl inositol pathway resulting in activation of protein kinase C might be involved in CT secretion. However, in contrast to most other cells, in the C cells the effect of both pathways was stimulatory and closely linked. This was further demonstrated by the observation that forskolin, an adenylate cyclase agonist, increased cAMP levels dramatically in human MTC cells, without changing cytosolic Ca^{2+} or increasing CT levels more than marginally. Ionomycin and TPA did not change cAMP levels but promoted CT release. However, forskolin synergistically enhanced the release-stimulating effects of ionomycin and TPA (Haller-Brem *et al.*, 1988).

III. Aquatic Vertebrates

Plasma calcium levels in amphibians and fish are directly influenced by the calcium concentration of the environment, because of the intimate contact between the blood and the water via the skin and, in particular, the gill surface. Changes in water Ca^{2+} concentration as well as, for instance,

periods of rapid growth may have pronounced effects on the plasma calcium level (Fig. 10). This indicates that extracellular calcium is less precisely controlled in these animals than in the higher vertebrates, even though the aquatic animals are very well equipped to rapidly reduce experimental hypercalcemia (Ma and Copp, 1978; Lafeber and Perry, 1988).

As discussed in Section I, control of calcium homeostasis and calcium metabolism in the purely aquatic vertebrates is different from that of the terrestrial vertebrates: parathyroid glands are missing, the pituitary glands may act as a source of hypercalcemic hormones, in particular prolactin, and some bony fishes possess a unique hypocalcemic hormone in addition to CT– stanniocalcin.

The group of amphibians includes terrestrially oriented as well as purely aquatic species, and this difference is reflected in the control of their

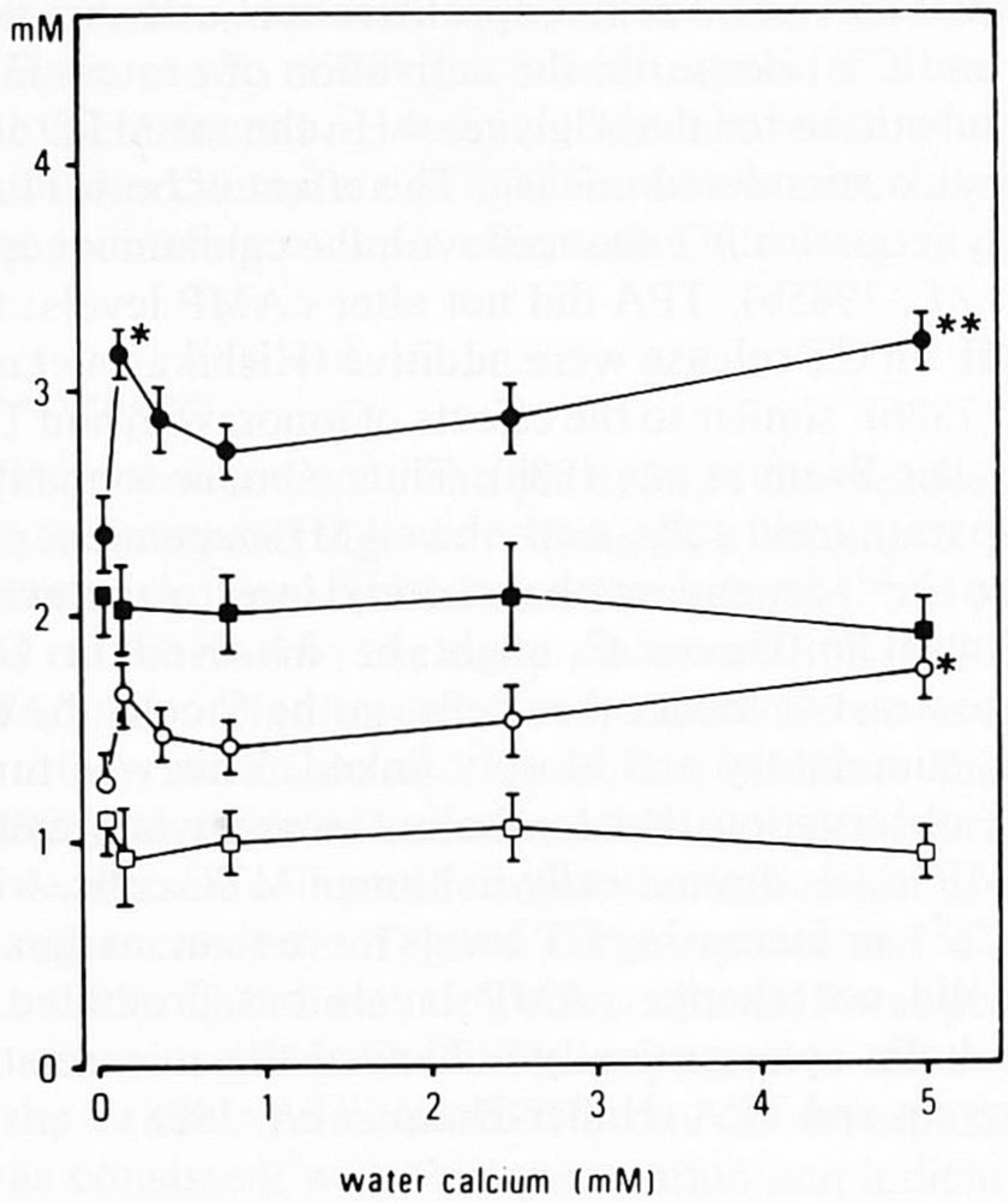

FIG. 10. Effect of the water calcium concentration of freshwater (FW) on plasma total and ultrafiltrable calcium and phosphate levels of male teleost fish (*Oreochromis mossambicus*). Fish from water with 0.8 m*M* calcium (controls) were exposed for 5 weeks to water with higher or lower calcium concentrations; means ± SD of eight fish per group; *, significantly different from controls, $p < .05$; ** $p < .01$. The peak at left corresponds to the calcium concentration (0.2 m*M*) that gives the highest growth rate (Urasa and Wendelaar Bonga, 1987). ●, Total calcium; ○, ultrafiltrable calcium; ■, total phosphate; □, ultrafiltrable phosphate.

calcium metabolism. Parathyroid glands are found in the more advanced and partially or fully terrestrial anurans (frogs and toads), and in some of the urodeles (newts and salamanders). Interestingly, in these animals the glands develop around the time of metamorphosis of the purely aquatic larvae into the adult life form. In many purely aquatic urodeles and in the fishes, parathyroid glands are missing. Of the systematically highly diverse group of fishes only the modern rayfinned fish, the teleosts, have been studied to an extent that allows some general, albeit tentative, conclusions with respect to the control of calcium metabolism.

A. Prolactin

1. Introduction

The absence of parathyroid glands in some lower, purely aquatic amphibians and in fish stimulated the search for a hormone in these animals that exerts the function of PTH as a hypercalcemic endocrine factor. Several studies on the aquatic vertebrates have indicated the pituitary gland as the endocrine organ dominating hypercalcemic control, with prolactin (PRL) as the active principle.

The involvement of PRL in the control of calcium metabolism is certainly not limited to the aquatic animals. In the terrestrial vertebrates PRL has effects on calcium metabolism that may become functional during specific life periods, such as pregnancy and lactation in mammals and yolk formation in the lower vertebrates. During these periods prolactin secretion may be markedly increased. During lactation in rats, suppression by bromocriptine of the high rate of PRL release reduced the elevated intestinal calcium uptake to the levels found in nonlactating animals. The effect may be mediated by $1,25(OH)_2D_3$ (Robinson *et al.*, 1982). During lactation, prolactin may stimulate calcium mobilization from the maternal skeleton via a vitamin D-independent mechanism (Brommage and DeLuca, 1985), and promote the transfer of calcium to the fetus (Barlet, 1985). Although there are several reports stating that PRL has hypercalcemic effects and that the rate of PRL secretion is influenced by the circulated calcium level, the literature is inconsistent. In a recent review we concluded that the available information does not support a role for PRL in the homeostatic control of extracellular calcium, and that its involvement in calcium metabolism is limited to the regulation of the transfer of calcium to the offspring (Wendelaar Bonga and Pang, 1989).

Interestingly, in some amphibians the role of the pituitary gland in calcium control seems more important the more time the animals spend in the water. In frogs and toads the extracellular calcium concentration seems mainly under control of PTH and CT. Although the available infor-

mation does not allow definite conclusions, the function of both hormones in these animals is comparable to that of the higher vertebrates (see previous sections). Nevertheless, about 60 years ago the first evidence for the implication of the pituitary gland in the control of extracellular calcium was obtained in the toad *Xenopus laevis*, one of the few permanently aquatic anurans. Removal of the pituitary gland induced hypocalcemia that could be partially corrected by extracts of the anterior lobe (Shapiro and Zwarenstein, 1933). These observations were confirmed afterward.

In the mainly aquatic salamanders and newts, there is more evidence for a role in calcium control of the pituitary gland, although its importance is variable and related to the presence or absence of the parathyroid glands. In red-spotted newts, which possess parathyroids, removal of the pituitary gland had no significant effect on plasma calcium, whereas parathyroidectomy resulted in marked hypocalcemia (Wittle, 1984). In some other newt species removal of either the parathyroids or the pituitary gland caused hypocalcemia (Oguro and Uchiyama, 1975; Matsuda *et al.*, 1991). Conversely, in the giant water salamander the parathyroidectomy had no effect on plasma calcium whereas significant hypocalcemia followed the removal of the pituitary gland (Oguro, 1973). In some urodeles that are lacking parathyroid glands, removal of the pituitary gland produced hypocalcemia (Oguro and Uchiyama, 1975; Oguro *et al.*, 1978). That PRL was the active hypophyseal principle was indicated by studies showing that the hypocalcemia following removal of the pituitary gland can be compensated by injections of preparations of mammalian PRL (Oguro *et al.*, 1978; Pang, 1981b). In *Cynops pyrrhogaster*, a newt species that exhibited a transient hypocalcemia after removal of its parathyroid glands, the recovery following this operation could be blocked by hypophysectomy (Oguro *et al.*, 1978) and by injection of an antiserum against homologous prolactin (Matsuda *et al.*, 1990). Thus, in the amphibians some species seem to depend on the parathyroid glands for their hypercalcemic control, some on the pituitary gland, and others on both. However, the function of prolactin in this group in the control of calcium homeostasis of the extracellular fluid still has to be defined. Studies on the importance of the extracellular Ca^{2+} level on prolactin secretion in amphibians are badly needed.

In fish the evidence for a role of PRL in calcium metabolism is more complete than in amphibians. Removal of the pituitary gland in freshwater fish frequently, although not invariably, leads to hypocalcemia. The reported difference in the responses may be related to the species studied and to the calcium concentration of the ambient water (see review by Wendelaar Bonga and Pang, 1989). The reduced calcium levels caused by removal of the pituitary gland of killifish from low-calcium freshwater

could be corrected by pituitary homogenates or mammalian prolactin (Pang, 1981a), an observation that was confirmed for other species. Treatment with mammalian as well as teleost PRL induced hypercalcemia in intact freshwater fish, although negative results were also reported (Wendelaar Bonga and Pang, 1989). Studies by Flik *et al.* (1984, 1989a) showed that cells specialized for ion transport in the gills of several fish species contain a specific Ca^{2+}-ATPase activity that is the driving force for branchial calcium uptake from the water and that is stimulated by PRL. Administration of PRL increased the calcium density of the skeleton in tilapia (Wendelaar Bonga and Flik, 1982), and thus the hypercalcemic effects of PRL are unlikely to be produced in a PTH-like fashion, i.e., by stimulating bone calcium release.

Pituitary factors other than PRL that were reported to increase plasma calcium levels in fish are ACTH and hypercalcin, the hypercalcemic factor postulated by Parsons *et al.* (1978) to explain the fast-acting hypercalcemic effects of pituitary extracts of cod and eel. These reports need confirmation, however (Wendelaar Bonga and Pang, 1989).

2. *Control of PRL Secretion*

a. *Calcium and Magnesium Concentration of the Water.* The calcium status of a fish is dependent on the calcium concentration of the ambient water to such an extent that fish and water have even been considered as an "open system" in terms of calcium exchange. Whether this is defendable or not, changes in water calcium concentration are reflected, although often only transiently, in changes in calcium concentration in the blood plasma and in the bony compartment, in particular the scales. For several freshwater teleost species an inverse relationship was established between the water calcium concentration and PRL cell activity (Wendelaar Bonga and Pang, 1989). Species differences do exist, however. Whereas in the African cichlid *Oreochromis mossambicus* PRL secretion could be suppressed almost completely by high water calcium concentrations (Wendelaar Bonga *et al.*, 1985), in European eels only a modest decrease occurred after a similar treatment (Olivereau and Olivereau, 1983). In seawater a relationship between the water calcium concentration and PRL cell activity is questionable for most species. Whereas in sticklebacks the PRL cells became clearly activated after reduction of the high calcium concentration typical for seawater, only moderate effects or no effects at all were reported for other species (Wendelaar Bonga and Pang, 1989) and circulating PRL levels were not influenced by reducing the calcium concentration of the ambient seawater (Nicoll *et al.*, 1981; Hirano, 1986). Thus, a specific relationship between the water calcium concentra-

tion and PRL cell activity was only demonstrated for a few teleost species, and was not found for fish in general. Changes in water magnesium levels were also reported to influence prolactin secretion. In sticklebacks and tilapia, fish with PRL cells responsive to changes in water calcium, PRL secretion could be suppressed by high water magnesium levels, although magnesium ions were less effective than calcium ions in these species (Wendelaar Bonga, 1978; Wendelaar Bonga *et al.*, 1985).

b. *Extracellular Calcium.* In their studies of tilapia PRL cells *in vitro*, Grau and Helms (1989, 1990) found that PRL release was independent of the extracellular Ca^{2+} concentration, although a basal extracellular calcium concentration was required. MacDonald and McKeown (1983) examined the Ca^{2+} dependency of PRL cells of coho salmon *in vitro* and established a 3-fold rise when Ca^{2+} increased from 0 to 1.3 m*M*, which is close to plasma Ca^{2+} in salmon, and a decrease at higher concentrations. The authors suggested that PRL might have some role in homeostatic Ca^{2+} regulation. This suggestion was not supported by *in vivo* observations on the relationship between prolactin cell activity and plasma total or free calcium. Although during experimental manipulation of water calcium concentration in fish an inverse relationship between changes in plasma calcium and PRL cell activity was reported, a clear dependency of PRL on plasma calcium, whether total or ionic calcium, was not established for any one species (Olivereau *et al.*, 1982; Wendelaar Bonga *et al.*, 1985, 1988). Strongly reduced or increased Ca^{2+} levels are inhibitory for endocrine cells in general, and do not allow conclusions with respect to prolactin's function in fish. Osmolarity has more pronounced effects on PRL release *in vitro* than Ca^{2+}, but the physiological significance of this phenomenon is also unclear because there is some discrepancy with *in vivo* observations (Wendelaar Bonga and Pang, 1989).

c. *Hormones and Neurotransmitters.* Control of PRL cells seems to be dominated by hypothalamic centers, as is usual for pituitary cells. Teleost PRL cells *in vitro* respond to TRH with increased secretion, whereas dopamine, somatostatin, and vasoactive intestinal peptides are inhibitory. The response of the PRL cells to these factors *in vivo* may be modulated by the sensitivity of the cells to, e.g., osmolarity of the extracellular fluid, cortisol, or estrogens (Nishioka *et al.*, 1988; Grau and Helms, 1989; Wendelaar Bonga and Pang, 1989). No clear relationship with calcium regulatory hormones can be deduced from literature on the control of PRL secretion (Wendelaar Bonga and Pang, 1989).

B. Calcitonin

1. Introduction

a. *Aquatic Amphibians.* In the aquatic amphibians, i.e., some adult anurans, anuran tadpoles, and the urodeles, the function of CT seems comparable to that of the more terrestrial amphibians (see Section II,B,1). After surgical removal of the ultimobranchial bodies of the frog *Rana pipiens* (Robertson, 1970) or bullfrog tadpoles (Sasayama and Oguro, 1976), hypercalcemia developed when the animals were exposed to calcium-enriched water. The hypercalcemia could be reduced or prevented by transplantation of ultimobranchial tissue or by injection of salmon CT (Sasayama, 1978). These data are comparable with a role of CT in suppressing hypercalcemia in these animals.

b. *Bony Fishes.* The physiological function of CT in fish is still under debate, partially as a result of the relative scarcity of the experimental data when compared with the higher vertebrates, and partially as a result of the inconsistency of many of these data. In conformity with the first studies on CT in mammals and birds, several investigators tried to demonstrate a hypocalcemic effect of CT in fish (Dacke, 1979). The results were variable. No effect on plasma total calcium was observed after injection of mammalian CT preparations or synthetic salmon CT in many different freshwater or seawater fish (Dacke, 1979; Copp and Kline, 1989). Infusion of Japanese eels with a homologous antiserum did not influence plasma Ca^{2+} levels (Hirano *et al.*, 1981). Conversely, hypocalcemia following CT treatment was reported for freshwater European eel (Chan *et al.*, 1968; Lopez *et al.*, 1976), sticklebacks adapted to low-calcium freshwater (Wendelaar Bonga, 1981; only the ultrafiltrable calcium fraction was reduced), and for mudskippers that had been made hypercalcemic by taking them out of the water (Fenwick and Lam, 1988). Hypophosphatemia accompanied the reduction in plasma calcium in European eels (Chan *et al.*, 1968) and hyperphosphatemia was reported for mudskippers kept in, as well as out, of the water (Fenwick and Lam, 1988). The absence of an effect in most experiments may not only have been caused by the absence of any hypocalcemic potency of the CT preparations used, but also by the effective counteraction of hypercalcemic control mechanisms. In this respect it is of interest that in sticklebacks CT injections stimulated prolactin cell activity (Wendelaar Bonga, 1980). Prolactin has hypercalcemic effects in this species. This would indicate that CT has at least some calcium-lowering activity in fish; this was supported by the results of removal of the ultimobranchial glands. Partial ultimobranchialectomy in European and Ameri-

can eels led to transient, although moderate, hypercalcemia, which became more pronounced when the fish were exposed to high calcium water (Lopez *et al.*, 1976; Fenwick, 1978). The transient nature of the effect might have been caused by regeneration of the gland or by increased secretion of stanniocalcin: the corpuscles of Stannius became enlarged after ultimobranchialectomy (see Section III,C). With respect to plasma calcium levels the effect of ultimobranchialectomy was to some extent similar to that of removal of the Stannius bodies. The latter operation led to marked but transient hypercalcemia (Fenwick, 1974; Hanssen *et al.*, 1989) and was followed by gradual hypertrophy and hyperplasia of the ultimobranchial gland (Lopez *et al.*, 1968). We conclude that CT probably has some hypocalcemic activity in fish. This potency is certainly less than that of stanniocalcin, the hormone of the Stannius bodies. This was illustrated by the observation on different species that the hypercalcemia following removal of the Stannius bodies activated the ultimobranchial cells but still remained excessive for at least 4–6 weeks. It could be rapidly reduced by the hormone of the Stannius bodies, stanniocalcin (see Section III,C). Although CT may have hypocalcemic effects under some conditions, it is doubtful whether it has a hypocalcemic function in fish. The doses necessary to demonstrate an effect on plasma calcium are high and nonphysiological.

In another important aspect the effect of CT is comparable to that described for higher vertebrates: activation of osteoblasts and promotion of bone mineral deposition has been reported after injection of salmon CT in tilapia (Wendelaar Bonga and Lammers, 1982). It is very possible that CT has a role in skeleton protection during periods of high calcium demand, such as during ovarian growth (see below).

c. *Elasmobranchs.* In contrast to the observations on bony fishes, salmon CT injections produced hypercalcemia in three sharks and rays (Glowacki *et al.*, 1985).

2. *Control of CT Secretion*

a. *Environmental and Extracellular Ca^{2+}. i. Aquatic amphibians.* Only a few studies prevail on the control of CT secretion in the aquatic vertebrates. In bullfrog tadpoles, the ultimobranchial bodies became hyperactive when the ambient water was enriched with calcium. Since ultimobranchialectomy led to hypercalcemia under these conditions, the conclusion is indicated that the C cells responded with enhanced secretion to a rise in extracellular calcium (Robertson, 1970, 1971b).

ii. Bony fishes. Data are available only on teleost fish. When euryhaline fish are transferred from freshwater to seawater, usually a mild

hypercalcemia develops. If CT would operate as a hypocalcemic or anti-hypercalcemic hormone, one would expect that the ultimobranchials are highly active under the latter condition. However, the results of such experiments are contradictory. Pang (1971) could not find histological evidence for increased cellular activity in killifish from seawater as compared to freshwater. A similar observation was reported by Peignoux-Deville *et al.* (1975) for European eels. Hirano *et al.* (1981) could not find any difference in plasma CT levels between freshwater and seawater Japanese eels, and in salmon migrating from seawater to freshwater, no clear relationship between plasma CT and plasma calcium was found (Watts *et al.*, 1975). Conversely, a study on Japanese eels transferred to seawater (Orimo *et al.*, 1972) showed that plasma CT levels increased rapidly after transfer. Suryawanshi and Mahajan (1976) reported for catfish that hypercalcemia caused by calcium enrichment of the water induced hypertrophy of the ultimobranchial cells. The rise in plasma calcium in these fish was relatively high. Transfer of sticklebacks from normal seawater to low-calcium seawater, a procedure followed by a substantial drop in plasma calcium, was followed by regression of the ultimobranchial cells (Wendelaar Bonga, 1980). The extreme hypercalcemia that developed after removal of the Stannius bodies led to hyperactivity of the ultimobranchials (Lopez *et al.*, 1968; Wendelaar Bonga and Greven, 1978). Ultimobranchial hyperactivity was also reported for parrotfish, which ingest extremely large amounts of calcium feeding on coral (Fontaine *et al.*, see Peignoux-Deville *et al.*, 1975). One might conclude that the glands only respond with increased CT secretion to marked hypercalcemia. This could explain why CT levels were not notably changed during migration, when plasma calcium levels do hardly change, but were affected after abrupt transfer to high- or low-calcium water, which is usually associated with transient plasma calcium imbalance. Our tentative conclusion is further supported by the results of the only *in vitro* study known to us on fish ultimobranchial cells, which showed that cultured trout C cells were far less responsive to changes in extracellular Ca^{2+} than the C cells of the terrestrial vertebrates (Roos *et al.*, 1974), and also less responsive than the cells of the Stannius bodies (see Section III,C). Thus the available data, albeit limited, on the control of CT secretion in fish do not indicate a dominant role for CT in the homeostatic control of extracellular calcium, although some effect cannot be excluded under extreme conditions.

b. *Hormones. i. Pituitary gland.* Removal of the pituitary gland of Japanese eels did not influence circulating CT levels, although the animals developed a significant hypocalcemia (Hirano *et al.*, 1981).

ii. Estrogens. In fish there is a relationship between gonadal activity and plasma CT levels comparable to that of the terrestrial vertebrates. The ultimobranchial glands show histological signs of increased secretory activity during natural or experimentally induced sexual maturation, in particular in female fish (Peignoux-Deville *et al.*, 1975). Yamauchi *et al.* (1978) reported significantly increased CT levels in male eels during spawning. Serum calcium was unchanged. In another study on eels these authors determined immunoreactive CT during ovarian maturation induced in eels by chum salmon pituitary homogenate. Serum CT levels increased parallel with the gonadosomatic index, and peaked during ovulation. No changes in serum calcium levels were observed (Yamauchi *et al.*, 1978). Watts *et al.* (1975) determined circulating CT levels in male and female sockeye salmon during their spawning migration from the sea to freshwater. Plasma CT was higher in female fish and increased during migration until spawning. Afterward it decreased, below the concentration in males. Björnsson *et al.* (1986) and Fouchereau-Peron *et al.* (1990) determined plasma CT levels in rainbow trout during the yearly sexual cycle. In female fish the CT levels were elevated during the 3 months of the reproductive period. They increased until ovulation and decreased sharply thereafter. The authors could not find a close correlation between circulating CT levels and free or protein-bound calcium concentrations. In male fish, plasma CT levels were constant throughout the year (Björnsson *et al.*, 1986), or fluctuated in relation to the gonadosomatic index (Fouchereau-Peron *et al.*, 1990). In female fish, protein-bound calcium increased during the period of oogenesis. Free calcium was unchanged. It was concluded that free plasma calcium is not the primary controlling factor for CT secretion, because of the lack of a clear correlation between CT levels and plasma free calcium (Björnsson *et al.*, 1986). Recently Björnsson *et al.* (1989) showed that injections of 17β-estradiol increased plasma CT levels in several salmonid species. They considered a reproductive role for CT in fish more likely than a calcium regulatory role (Björnsson *et al.*, 1989). Direct evidence for a reproductive role is still missing, however.

iii. Gastrointestinal hormones. In their study on cultured trout ultimobranchial cells Roos *et al.* (1974) reported that pancreozymin had a marked stimulatory effect on CT release. However, relatively high concentrations were required (around 10^{-4} *M*).

c. *Second Messengers.* Roos *et al.* (1974) found that dibutyryl cAMP stimulates CT release by cultured trout ultimobranchial cells.

C. Stanniocalcin

1. Introduction

The corpuscles of Stannius (CS) are found exclusively in teleostean and holostean fishes. They produce a hormone (stanniocalcin) that seems unique for these animals and that prevents hypercalcemia. The glands are associated with the kidney. In most fishes one pair is found. Higher numbers are present in more primitive teleosteans, whereas several hundreds have been described in the holostean *Amia calva*. The cytophysiology of these glands has been reviewed by Krishnamurthy and Bern (1971), Wendelaar Bonga and Pang (1986), and Hirano (1989). Briefly, the bodies contain either one or two structurally different types of gland cells (type-1 and type-2 cells), both showing the ultrastructure of protein-producing endocrine cells. They may produce different hormones or may represent different phases of the same cell type, although each responds differently to experimental treatments. In typical marine fish so far only type-1 cells have been described, whereas two types occur in freshwater fish or euryhaline fish spending part of their life cycle in freshwater. The type-1 cells predominate and are rounded with extensive granular endoplasmic reticulum and large secretory granules. The small granules are present in the type-2 cells, which are slender, often with cytoplasmic extensions.

The CS are well vascularized. The blood vessels form an intricate capillary network that is drained by veins that join the renal portal system. The CS are richly innervated by autonomic nerves that penetrate the gland along with the blood vessels. Small nerves follow the venules and arterioles running through the connective tissue septa that separate the lobes of gland cells. No synaptic contacts with the gland cells have been reported, and the nerves and axons seem to end at a distance from these cells. The larger nerves may be associated with ganglionlike groups of neurons at the periphery of, or outside, the glands (Wendelaar Bonga and Pang, 1986). In a detailed study on trout CS, Unsicker *et al.* (1977) demonstrated the presence in the nerves of considerable amounts of catecholamines, 5-hydroxytryptamine, and acetylcholine. Although nervous control of secretion of the CS cannot be excluded, most authors consider it likely that the nerves are mainly controlling the blood flow through the glands. Removal of the glands (stanniectomy) leads to marked hypercalcemia (Fig. 11; Hirano, 1989). The hypercalcemia is caused by increased Ca^{2+} influx from the water (So and Fenwick, 1979; Milet *et al.*, 1979). It persists for 5 to 7 weeks (Fenwick, 1978; Hanssen *et al.*, 1989), which indicates that endogenous calcitonin, probably secreted at higher levels in

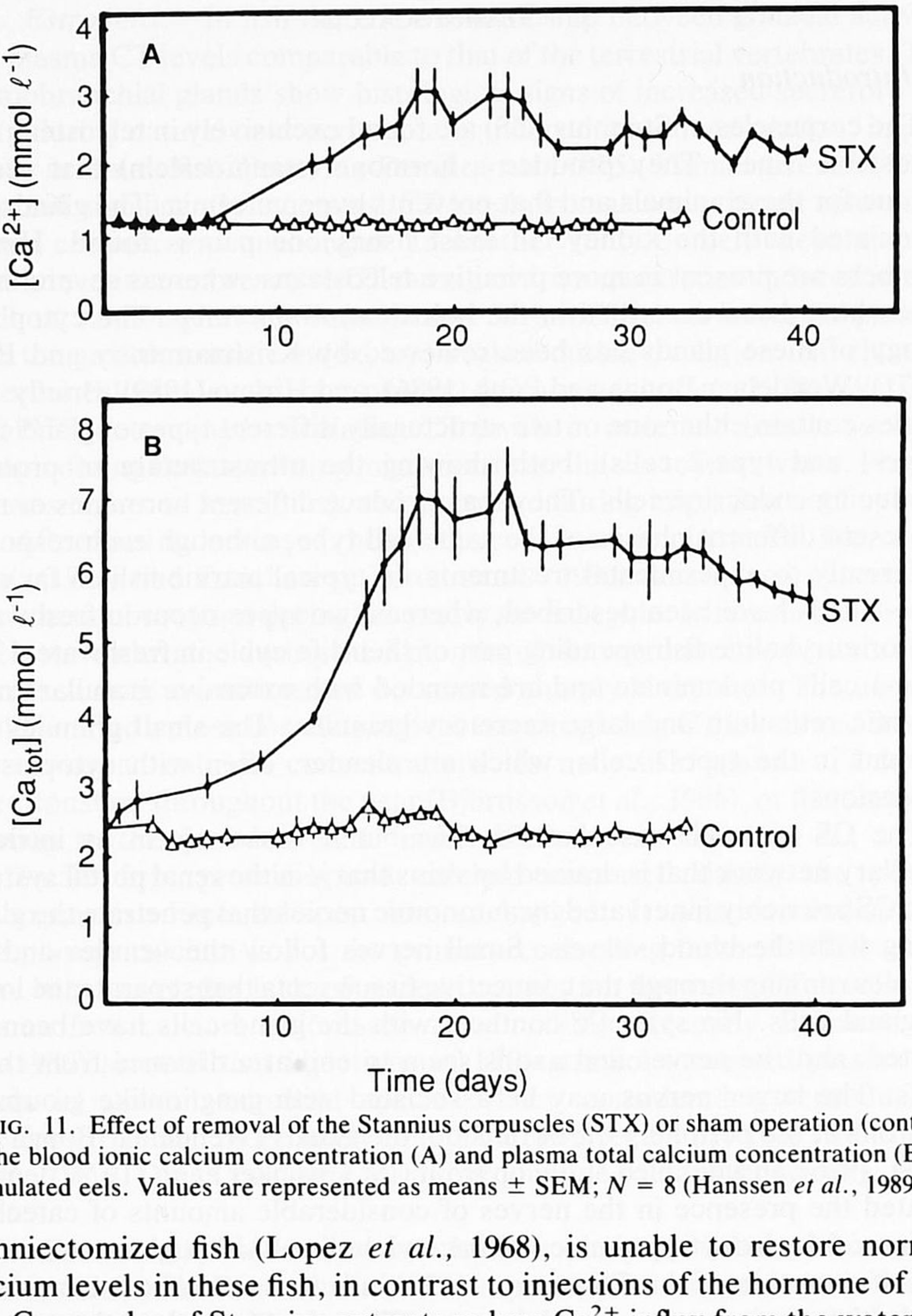

FIG. 11. Effect of removal of the Stannius corpuscles (STX) or sham operation (control) on the blood ionic calcium concentration (A) and plasma total calcium concentration (B) in cannulated eels. Values are represented as means ± SEM; $N = 8$ (Hanssen *et al.*, 1989).

stanniectomized fish (Lopez *et al.*, 1968), is unable to restore normal calcium levels in these fish, in contrast to injections of the hormone of the CS. Corpuscles of Stannius extracts reduce Ca^{2+} influx from the water by acting on the gills (So and Fenwick, 1979; Milet *et al.*, 1979) and on the gut (Takagi *et al.*, 1985).

2. Identity, Biosynthesis, and Action of Stanniocalcin

There has been some discussion on the identity of the active principle of the CS. Pang *et al.* (1973) characterized it as a heat stable product of high

molecular weight and called it hypocalcin. A small glycoprotein with a molecular weight between 3000 and 4000 kD was isolated from salmon by Ma and Copp (1978) and was named teleocalcin. In the CS of European eels a product was identified that disappeared from the gland after calcium infusion. It cross reacted with an antiserum against PTH and therefore the name parathyrin of the CS was suggested (Milet *et al.*, 1980; Lopez *et al.*, 1984). More recently Butkus *et al.* (1987, 1989) established the primary structure of the main secretory protein of the CS of Australian eels from the cDNA sequence. It appeared to be a preprotein of 263 amino acids: a 17 amino acid signal peptide, a prosegment of 15 amino acids, and the mature form of the hormone consisting of 231 amino acids. The N terminal 1-20 fragment seems to contain most biological activity, since it inhibits Ca^{2+} uptake in fingerling trout. The N-terminal sequence of eel proved to be almost identical to the N terminal of hypocalcemic glycoproteins isolated from salmon and trout (Wagner *et al.*, 1986; Lafeber *et al.*, 1988a). It had no true homology with PTH. Most authors agreed on stanniocalcin as the new name of the hormone (Flik *et al.*, 1989b), a glycoprotein with a molecular radius that seems to vary slightly between species and that probably occurs in a labile dimeric form. In salmon the monomeric form of the hormone is a 26-kDa product, with a 32-kDa precursor (Wagner *et al.*, 1988); in trout and goldfish these values are 28 and 32 kDa, and in eels 30 and 34 kDa, respectively (Flik *et al.*, 1989b).

Stanniocalcin appeared to be a relatively fast-acting hormone with the capacity to reduce effectively experimentally increased plasma calcium levels, by inhibiting the Ca^{2+} influx from the water (Wagner *et al.*, 1986; Lafeber and Perry, 1988; Hanssen *et al.*, 1989; Perry *et al.*, 1989), and leaving the Ca^{2+} efflux unaffected (Lafeber *et al.*, 1988c; Fig. 12). As indicated earlier by using CS extracts (Bailey and Fenwick, 1975) the purified hormone specifically reduced the ionized calcium fraction (Lafeber *et al.*, 1988b; Hanssen *et al.*, 1989). A model for the mode of action of stanniocalcin is presented in Fig. 13.

3. Control of Stanniocalcin Secretion

a. *Calcium Concentration of the Water.* Although fish are able to regulate their plasma calcium concentration efficiently, there is a close relationship between the calcium concentration of the ambient water and the secretory activity of the gland cells of the CS, in particular the predominant type-1 cells. As established with light and electron microscopy, these cells become activated upon transfer of fish from freshwater, with moderate to low Ca^{2+} levels (generally below 1 m*M*), to seawater (full strength seawater contains about 10 m*M* Ca^{2+}). This has been demonstrated for, e.g., goldfish, killifish, sticklebacks, and trout (Wendelaar

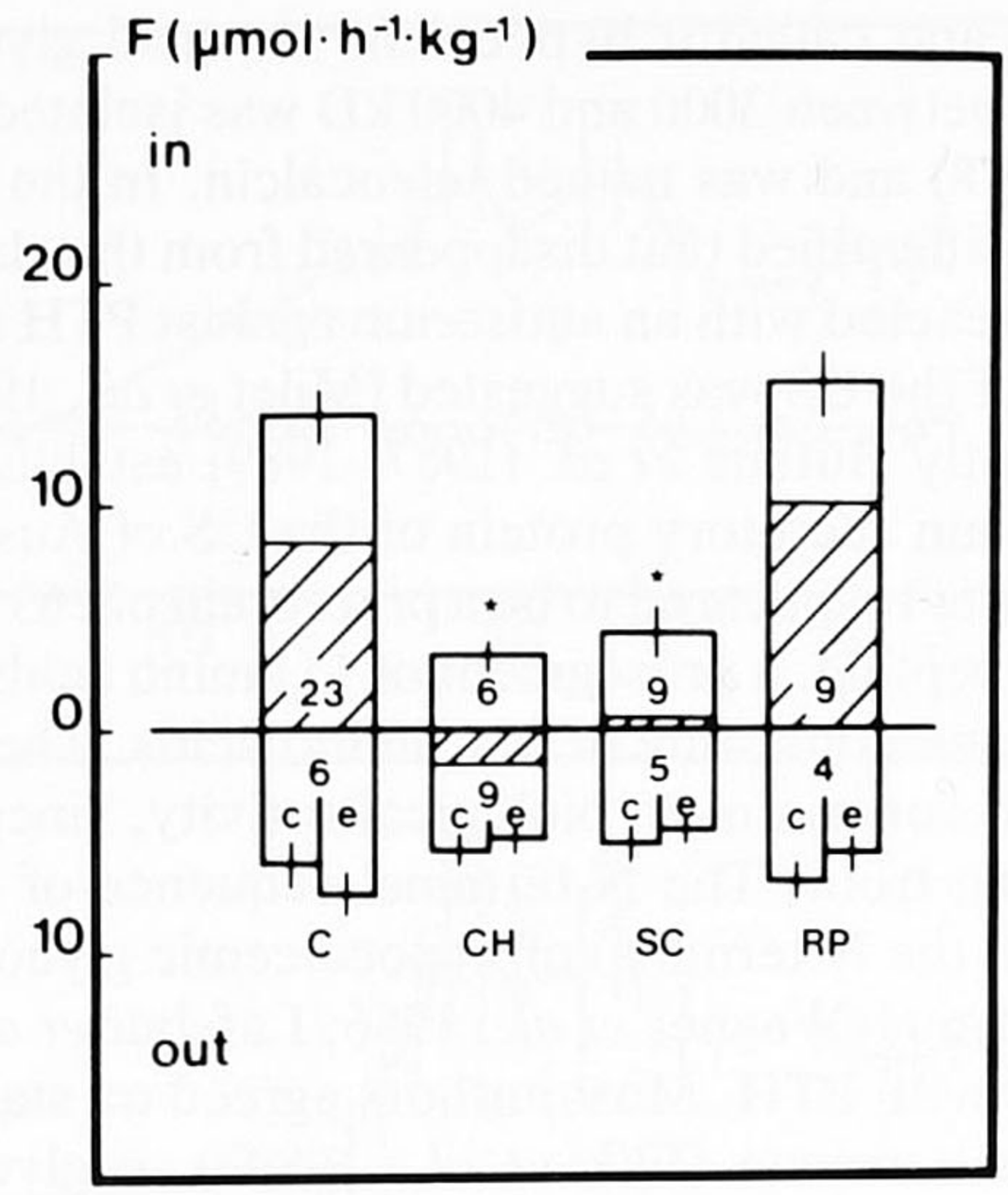

FIG. 12. Effects of Stannius corpuscles crude homogenate (CH), purified stanniocalcin (SC), and residue proteins (RP) on Ca^{2+} influx and efflux across the gills in trout. Control (C), sham injected fish; hatched bars: net flux; *, significantly different from controls; means ± SE; numbers of fish per group are indicated in the bars (Lafeber *et al.*, 1988c).

Bonga and Pang, 1986). The CS were also activated in Ca-enriched freshwater (Wendelaar Bonga and Pang, 1986; Urasa and Wendelaar Bonga, 1987) but not in Ca-deficient seawater (Cohen *et al.*, 1975). Increased activity of the CS in seawater was further indicated by the high immunoreactive stanniocalcin levels as observed in the blood plasma of seawater eels.

b. *Extracellular Calcium.* Because changes in water Ca^{2+} concentration are frequently reflected in extracellular calcium levels, the effects of the external Ca^{2+} concentration on CS activity could well be mediated by extracellular calcium. This is in fact indicated by several reports demonstrating that increases in plasma calcium levels by injection of $CaCl_2$ result in degranulation of the gland cells of the CS. This was shown for goldfish, eel and, trout (Yamada *et al.*, 1982; Lopez *et al.*, 1984; Lafeber and Perry, 1988; Hanssen *et al.*, 1991). The degranulation was associated with the disappearance of stanniocalcin from the glands and stimulation of synthesis of the hormone (Flik *et al.*, 1989b), elevation of plasma stanniocalcin levels (Hanssen *et al.*, 1991), and inhibition of Ca^{2+} influx from the water (Hanssen *et al.*, 1989; Lafeber and Perry, 1988; Perry *et al.*, 1989). The hypercalcemia resulting from the injection of $CaCl_2$ was reduced rapidly in

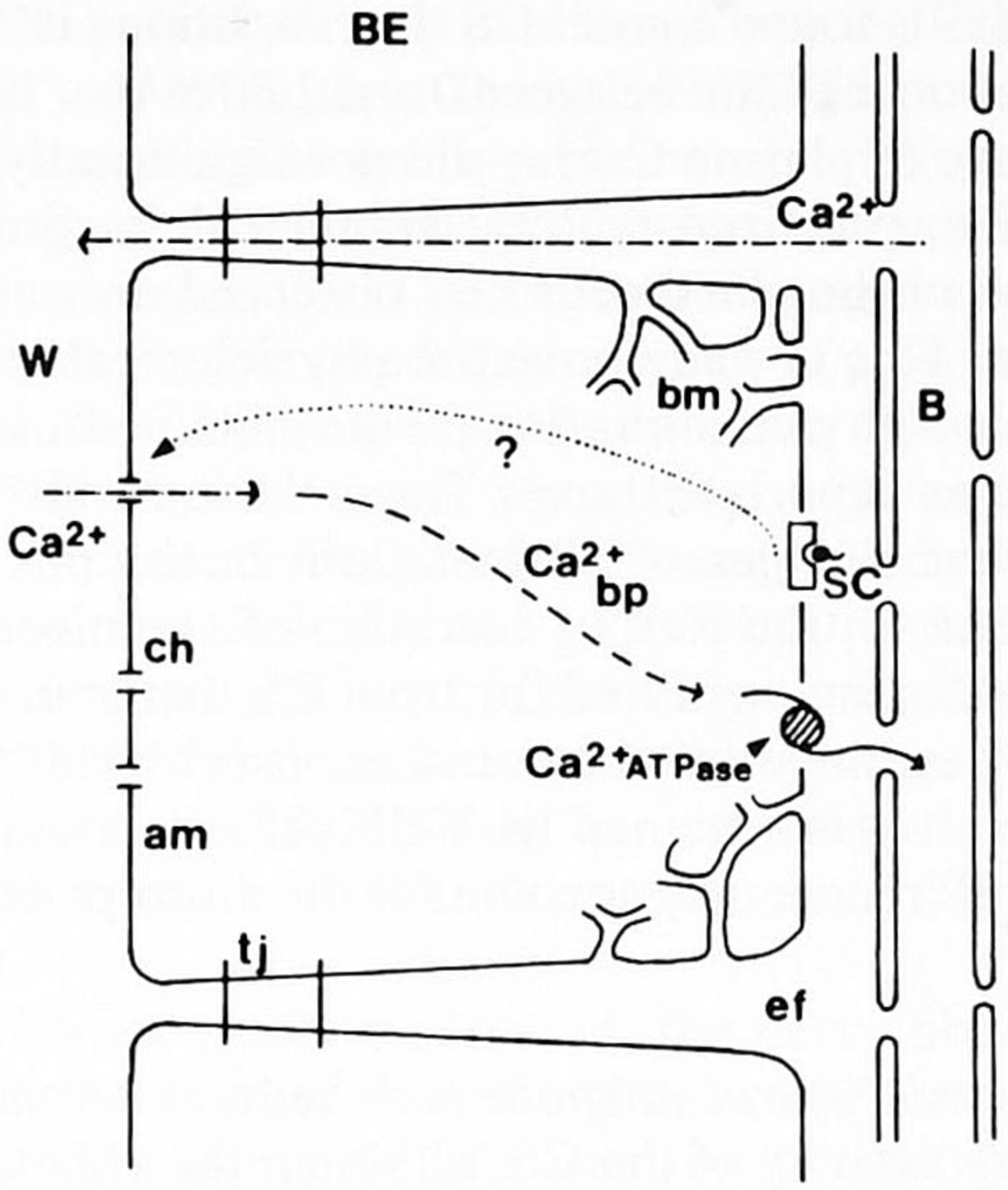

FIG. 13. Model for the action of stanniocalcin on calcium uptake by chloride cells in the bronchial epithelium (BE), based on observations of eels and trout. Ca^{2+} from the ambient water (W) enters the chloride cells in the gills passively through Ca^{2+} channels (ch) in the apical cell membrane (am). It is transported via Ca^{2+} binding proteins (Ca^{2+}_{bp}) and translocated across the basolateral cell membrane (bm) by Ca^{2+}-ATPase activity into the extracellular fluid (ef) and in the blood (B). Stanniocalcin (SC) blocks the Ca^{2+} entry into the chloride cells, an effect that may be mediated by an unknown second messenger; tj, tight junction (G. Flik, F. P. J. G. Lafeber, P. K. T. Pang, S. F. Perry, and S. E. Wendelaar Bonga, unpublished).

intact fish. When the CS were removed, the $CaCl_2$-induced hypercalcemia persisted, demonstrating that the restoration of preinjection plasma Ca^{2+} levels was affected by the CS and not by the ultimobranchial bodies (Perry *et al.*, 1989).

The above data show that there is a negative feedback relationship between plasma ionic calcium and stanniocalcin secretion by the CS. The question presents itself whether the plasma Ca^{2+} concentration influences the stanniocalcin secretion directly, at the level of the gland cells, or indirectly, i.e., via normal or neural pathways. This problem has been studied by analysis of the effects of varying Ca^{2+} concentrations on CS secretion *in vitro*. Aida *et al.* (1980) showed that incubation of salmon CS in high-Ca^{2+} media (3 and 6 m*M* Ca^{2+}) led to degranulation of the gland cells. At a concentration of 1.5 m*M* the upper end of the physiological range of plasma Ca^{2+} degranulation was absent, although it could be induced by the Ca^{2+} ionophore calimycin. In line with these results,

Hanssen *et al.* (1991) found for eel CS that variations in Ca^{2+} concentration of the incubation medium between 0 and 2.00 m*M*— thus including the physiological range of plasma Ca^{2+}—did not significantly affect the basal release of newly synthesized or total stanniocalcin. Stimulated release and degranulation of the gland cells was observed only at concentrations of 2.5 and higher. This is well above the physiological plasma Ca^{2+} concentrations. Such high concentrations were obtained experimentally by $CaCl_2$ injections, as reported above. Thus, these results do not support the hypothesis that changes of plasma Ca^{2+} in the physiological range have a direct effect on the rate of secretion of stanniocalcin. However, Wagner *et al.* (1989) demonstrated for trout CS that stanniocalcin release is concentration-dependently stimulated *in vitro* by Ca^{2+} between 1 and 2.5 m*M*. This result was obtained with CS cells in primary culture. This fact, or species difference, may account for the discrepancy between these observations.

c. *Hormones and Neurotransmitters.* Several hormones may influence the secretory activity of the CS, although the effects may be mainly indirect. The evidence is exclusively based on histological observations. No relationship between thyroid glands and CS has been demonstrated. The pituitary gland may be implicated in the control of CS activity, but the observations are inconclusive. After hypophysectomy, no effect, reduced granulation, or hypertrophy and hyperplasia have been reported (Hirano, 1989). Ovine prolactin caused hypertrophy of the CS as well as hypercalcemia, indicating that the effect of prolactin was mediated by plasma calcium (Olivereau and Olivereau, 1978). The absence of direct effects of prolactin has been substantiated by the finding that ovine prolactin had no direct effects on stanniocalcin secretion of trout CS in primary culture. Direct effects of neither salmon growth hormone nor salmon gonadotropic hormone could be demonstrated (Wagner *et al.*, 1989). Removal of the adrenocortical homologue of freshwater eels resulted in regression of the CS, whereas after the same operation in seawater eels the glands showed hypertrophy. These effects were also most likely mediated by plasma calcium, since after the operation the freshwater eels became hypocalcemic and the seawater eels hypercalcemic (Hanke *et al.*, 1967; Olivereau and Olivereau, 1968). Injection of mammalian calcitonin stimulated the type-1 cells of the CS of sticklebacks (Wendelaar Bonga, 1980). Removal of the ultimobranchial glands in eels led to regression of the CS (Chan, 1972), similar to the removal of the ovaries in sexually mature female catfish (Pandey, 1988). The latter effect could be prevented by estradiol injections. These injections stimulated the CS in intact fish (Pandey, 1988). In sexually mature female fish, the secretory activity of the CS is generally

elevated during the period of rapid ovarian growth (Subhedar and Prasado Rao, 1979; Urasa and Wendelaar Bonga, 1985). Then the liver produces calcium phospholipoproteins (yolk proteins or vitellogenins) under the influence of estrogens. These yolk proteins are secreted into the blood, transported to the ovaries, and incorporated in growing oocytes. This process causes markedly elevated plasma levels of protein-bound calcium. The activation of the CS during reproduction may be a response to these high protein-bound calcium levels since they may tend to increase the plasma ionic calcium concentration (Urasa and Wendelaar Bonga, 1985). Multiple injections of vitamin D_3 and $1,25(OH)_2D_3$ induced hyperactivity of the CS in catfish (Srivastav *et al.*, 1985; Srivastav and Srivastav, 1988). This effect probably is also indirect because vitamin D_3 and several of its metabolites, including $1,25(OH)_2D_3$, cause hypercalcemia in these fish (Swarup and Srivastav, 1982; Srivastav *et al.*, 1985; Srivastav and Srivastav, 1988).

Although the CS are richly innervated, the nerve fibers are associated with the blood vessels rather than the gland cells, and neural control of the secretory activity seems therefore not indicated. However, recently R. G. J. M. Hanssen (unpublished) showed that the acetylcholine agonist carbachol stimulates stanniocalcin synthesis and release by eel CS *in vitro*. The effect was dose related and could be blocked by the atropine. This observation opens the possibility that the secretory activity of the CS is modulated or even controlled by the nervous system.

4. Second Messengers

Only a few studies have dealt with extracellular signal transduction in the CS cells. Aida *et al.* (1980) showed that the calcium ionophore A23187 caused degranulation of the CS of coho salmon if some extracellular Ca^{2+} was present, thereby mimicking the effects of high extracellular Ca^{2+}. This observation was confirmed by Wagner *et al.* (1989) for rainbow trout CS cells in primary culture. EDTA or cobalt chloride blocked the effect of high calcium on hormone release. These observations indicate that Ca^{2+} entry into the cell, possibly via voltage-gated Ca^{2+} channels, can initiate stanniocalcin release. However, the voltage-gated channel blockers verapamil and nifedipine gave inconclusive results. The authors concluded that either the binding sites for these compounds were unrelated to voltage-dependent Ca^{2+} channels or more than one type of Ca^{2+} channel was present in the trout CS cells (Wagner *et al.*, 1989). Different concentrations of dibutyryl cyclic AMP were ineffective in changing STC release *in vitro* from the CS of the cichlid fish *Oreochromis mossambicus* (S. E. Wendelaar Bonga, unpublished).

IV. Conclusions

A. Parathyroid Hormone

Parathyroid hormone cells have at least three properties that make these cells unique among the endocrine cells, and optimally equipped for the homeostatic control of extracellular Ca^{2+}. First is the intimate inverse relationship between PTH release and the extracellular Ca^{2+} level that enables these cells to respond rapidly and efficiently to very small changes in Ca^{2+}. This property of the cells is based on the extreme sensitivity of a Ca^{2+}-receptor mechanism on the outer cell membrane. Although in the last 10 years there has been much progress in our understanding of the functioning of this mechanism, we are still waiting for its definite identification. Dihydropyridine-sensitive Ca^{2+} channels coupled to guanine nucleotide regulatory proteins are good candidates.

The relationship between intracellular Ca^{2+} and PTH release represents another unique feature of the PTH cells: it is the reverse of what is found in other gland cells in that an increase in cytoplasmic Ca^{2+} concentration is associated with inhibition of hormone release. The studies on permeabilized cells have revealed the complexity of the relationship between intracellular Ca^{2+} and PTH secretion. Cytoplasmic Ca^{2+} is certainly not the common denominator of stimulus secretion coupling. At least the inhibitory effect of high extracellular Ca^{2+} on PTH release may be mediated by local fluxes across the outer cell membrane rather than by an increase in cytoplasmic Ca^{2+}. Most stimulatory agonists of PTH release increase cAMP levels. However, for at least the stimulatory actions of low extracellular Ca^{2+}—the dominating factor in the control of PTH release—and of other cations a rise in cAMP is not a prerequisite. Regulation of protein kinase C by diacyl glycerol could be the common key mechanism for stimulus-secretion coupling in the PTH cells.

The third unique property of the PTH cells is their capacity to increase hormone release without adapting the rate of hormone synthesis during the first hours of stimulation. This phenomenon is effected by maintaining a high rate of PTH synthesis combined with inactivation of up to 90% of the newly found products under unstimulated conditions. Although uneconomical, this capacity enables the PTH cells to respond more readily and efficiently to a persistent release-promoting stimulus than other gland cells. Other cells, after depletion of their hormone stores, have to reactivate hormone synthesis if not gene translation and processing of gene products. The process of control by degradation of the output of the PTH cells bears only a slight resemblance to the process of crinophagy that has been reported for PRL cells (Farquhar, 1976). In the latter cells lysosomal

breakdown of secretory granules occurs after sudden inhibiton of hormone release and synthesis, whereas in the PTH cells synthesis proceeds unabated for at least several hours. Only more prolonged action of inhibitory or stimulatory agonists leads to adjustment of the rate of gene transcription and eventually of cell size and numbers. Thus, typical for the PTH cells are the high basal rate of hormone synthesis and the delay of at least several hours between the start of the stimulus and the onset of changes at the level of gene transcription.

In addition to extracellular Ca^{2+}, many hormones and neurotransmitters have the capacity to influence PTH release. With few exceptions, the physiological significance of the many nonionic PTH agonists is still poorly defined. For hormones such as calcitonin, glucagon, secretin, and cortisol the concentrations required to evoke a response seem too high to be of physiological importance. A potentiating role of these hormones when acting in concert with other factors cannot be excluded. Growth hormone, PRL, and estrogens, although implicated in the control of Ca^{2+}-dependent processes such as growth, placental calcium transfer, and egg formation seem to have mainly indirect effects on PTH secretion. These hormones promote the transfer of calcium via the blood to their respective target organs, and this transport will affect the parathyroid glands. Since growth hormone and PRL are capable of changing vitamin D hydroxylase activity of the kidneys (Wendelaar Bonga and Pang, 1989), there is a fair possibility that these hormones also influence PTH secretion via $1,25(OH)_2D_3$. To date this steroid seems to be the only serious candidate for a direct and physiologically important modulator of PTH secretion. It would be of interest, however, to examine the effects of PRL and growth hormone on the setpoint for Ca^{2+} of the PTH cells. Given the dominant position of extracellular Ca^{2+} in the control of PTH secretion, what then might be the role of these hormones? The close relationship between Ca^{2+} and PTH release seems to leave at most a modulating influence to these factors, at least for short-term regulation. Nevertheless, modulation may have important physiological functions in the long term, and several possibilities present themselves such as:

1. *Adjustment of calcium homeostasis to specific requirements of different life stages*. Fetal and newborn mammals maintain their extracellular calcium at a higher level than older animals. This may be connected to the rapid growth and calcification of the skeleton during early life. There is good evidence now, although limited to a few species, that the high extracellular calcium level is associated with a higher setpoint for Ca^{2+} and thus of lower sensitivity to Ca^{2+} of the PTH cells than in older animals. This suggests that the setpoint is a physiological variable that is under hormonal

or nervous control. Setpoint regulation needs more attention also, because of the recent report that the setpoint *in vitro* may be lower in individual isolated cells than in a cell population (Fitzpatrick and Leong, 1990). It should further be examined whether the setpoint is changed during other periods of high calcium transfer through the body circulation, in particular during the egg-laying cycle in birds and reptiles, and during ovarian growth in amphibians. Hormones to be considered for this type of adjustment of PTH secretion are growth hormone, PRL, and estrogens, possibly via stimulation of $1,25(OH)_2D_3$ synthesis, as mentioned above.

2. *Control of daily and annual cycles in extracellular calcium levels.* Daily cycles have been reported for most vertebrate groups, and annual cycles have been established in some lower vertebrates (Fig. 8). These cycles run parallel with changes in PTH levels or in other parameters for parathyroid gland activity. At least for the daily rhythmicity an endogenous origin is likely. Both the daily and annual cycles indicate that extracellular calcium is not rigidly controlled at a fixed level, and that most likely parathyroid secretion is under autonomic control of the PTH cells for the short term only. Hormones that might be involved in this type of modulation of PTH secretion are not known.

3. *Adjustment to calcium and phosphate shortage.* PTH release in response to short-term hypocalcemia seems an adequate response to restore normocalcemia. However, high and prolonged release rate evoked by chronic hypocalcemia might be uneconomical and injurious to the skeleton. Chronic hypocalcemia may be caused by dietary calcium shortage or by a high rate of bone mineralization, placental calcium transfer, or egg formation. It would lead to progressive demineralization of the bone and to phosphaturia. Under these conditions adjustment of PTH release to total body calcium and phosphate balance is necessary to limit the potentially injurious side effects of PTH action. The main hormone regulating total body calcium and phosphate balance, $1,25(OH)_2D_3$, could be involved in this type of adjustment of PTH secretion.

The understanding of the significance of the role of $1,25(OH)_2D_3$ in PTH control has been complicated by the apparently biphasic action on the parathyroid glands. The short-term inhibitory effects reported in early literature are likely indirect and mediated by the elevation of extracellular Ca^{2+} following administration of the steroid. Its receptor-mediated inhibitory effect on PTH gene transcription, although indicated by histological observations of cellular inactivation, has long escaped the attention of biochemists, probably because it is a long-term phenomenon. Both the short-term and long-term effects occur at physiological concentrations. Total body calcium and phosphate balance are controlled by $1,25(OH)_2D_3$

through its stimulating effect on intestinal calcium and phosphate uptake. We tentatively conclude that during hypocalcemia caused by intense skeletal calcification, placental calcium transfer, or egg formation, the potentially harmful effects of PTH on the skeleton are reduced or prevented by enhanced formation of $1,25(OH)_2D_3$. This steroid subsequently stimulates intestinal calcium uptake and suppresses PTH synthesis. The formation of $1,25(OH)_2D_3$ in the kidneys may be promoted by one or more of the hormones known to influence this process: growth hormone during growth, PRL during placental calcium transfer, estrogens during egg formation, and PTH during hypocalcemia in general.

B. Prolactin

In the terrestrial vertebrates the evidence for the involvement of PRL in calcium metabolism is limited to calcium handling during gestation and lactation in mammals. It would be of interest to investigate whether PRL would have a comparable function in the promotion of calcium from parent to offspring in the lower vertebrates. The available data indicate that only estrogens are involved. In the aquatic vertebrates the role of PRL in calcium regulation is definitely more comprehensive, although still not well defined and often confusing because of species-related differences. The data on amphibians show that PRL has a higher hypercalcemic potency in animals lacking parathyroid glands and with a more aquatic lifestyle. This would be interpreted as indicating that PRL has a function more or less equivalent to PTH in these animals. However, as will be discussed in the section on stanniocalcin, there is a fundamental difference in calcium metabolism between terrestrial vertebrates and fishes, and this may also apply to the lower amphibians and the purely aquatic amphibian larvae. For these animals the water might be an additional alternative source of calcium, as it is in fish. Any definite conclusion with respect to the role of PRL in the homeostatic control of calcium in the lower amphibians is premature because of the scarcity of data.

For several fish species it has now been established that PRL has hypercalcemic actions, and in this respect the hormone might be compared with PTH. However, there are several differences with the latter hormone. First, PRL raises plasma Ca^{2+} by stimulating the uptake of Ca^{2+} from the water and not from the bone. Second, PRL is certainly not primarily implicated in the control of Ca^{2+} homeostasis of the extracellular fluid. The control of PRL secretion is not dominated by changes in extracellular Ca^{2+}, but by hypothalamic factors and, possibly, factors such as plasma osmolarity. As has been concluded in recent reviews on PRL in fish, its main function seems to be the control of the integumental permeability to

water and ions (Hirano, 1986; Wendelaar Bonga and Pang, 1989). The involvement of prolactin in calcium metabolism may be derived from this function, because calcium is important for structural integrity and permeability control of cellular membranes.

In the absence of other known serious candidates for a PTH-like homeostatic function in fish one could tentatively consider the possiblity that a true hypercalcemic homeostatic hormone is missing. Such a function might be dispensable in these animals for reasons discussed in the section on stanniocalcin.

C. Calcitonin

Whereas the regulation of PTH secretion is fully consistent with a homeostatic function of the hormone in the control of extracellular Ca^{2+}, several arguments point against a comparable antagonistic role for CT. First, the extracellular Ca^{2+} concentration is less prominent as a regulatory factor for CT secretion than it is for PTH secretion. The C cells are missing the exquisite sensitivity to changes in extracellular Ca^{2+} that characterizes the PTH cells, and the response is less prompt. Conversely, the involvement of hormones, in particular factors of gastric and intestinal origin and estrogens, is more prominent in the control of CT than of PTH. Second, whereas injection of PTH in adult animals, from mammals to amphibians, produces a dose-dependent hypercalcemia, exogenous CT usually decreases plasma calcium only mildly or not at all. The results are rather consistent from mammals to fish and indicate that CT is not essential for the minute-to-minute control of extracellular Ca^{2+}. This inference is further supported by the absence of serious consequences for plasma calcium following the removal of the C cells. Such observations have even been interpreted as a lack of hypocalcemic potency of CT. This interpretation seems untenable because there are many reports dealing with marked hypocalcemia after CT administration. In addition, in studies on the effect of exogenous CT on experimentally induced hypercalcemia the hormone almost invariably is effective in reducing plasma calcium levels to normal. An exception must be made for teleost fish (see below). How then can it be explained that CT proved to be ineffective in many experiments? Several explanations are plausible:

1. *The response of the bone cells*. Not only the properties of the hormone but also the condition of its main target organ, the bony tissue, determines the capacity of CT to reduce plasma calcium. Because the CT-induced hypocalcemia is mainly effected by the deposition, via cellular

interaction, of calcium and phosphate compounds, the effect of CT on plasma calcium is dependent on the presence of active bone-salt depositing cells as well as of adequate amounts of phosphate. In general, osteoblastic and osteocytic activity is highest in young animals and decreases with age. Only in female birds and female reptiles a particular type of metabolically highly active bony tissue develops during the egg-laying period. Interestingly, reports on marked hypocalcemia following CT administration are mainly restricted to young animals, from mammals to amphibians (reports on young fish are not available), and to egg-laying birds.

2. *Lack of adequate phosphate supplies*. There is evidence that the response to exogenous CT is enhanced by the concurrent injection of phosphate (Talmage *et al.*, 1981). The few reports of CT-induced hypocalcemia in fish include experiments with sticklebacks from low-calcium water (Wendelaar Bonga, 1980), a condition that is usually associated with hyperphosphatemia. The possibility of phosphate as a limiting factor for calcium deposition into the bone should receive more attention.

3. *Counteraction by PTH*. Any reduction of plasma Ca^{2+} by CT will be counteracted intensely by PTH, as soon as the Ca^{2+} level tends to fall below the setpoint for Ca^{2+} of the PTH cells.

Of the above three explanations, the last one most likely is of major importance. The calcium-lowering effects of CT during experimental hypercalcemia clearly show that the calcium-depositing capacity of the bone cells or the availability of circulating phosphate are in general not limiting, although they may modulate the intensity of the response to CT. Only in young animals, with highly active bone forming cells, the rate of CT-dependent calcium deposition may exceed the calcium-mobilizing capacity of PTH. In adult animals, the apparent predominance of PTH over CT in manipulating extracellular Ca^{2+} seems to warrant that stimulation of CT release may induce harmful hypocalcemia. This is indicated by the readily induced hypocalcemia by CT in parathyroidectomized animals.

If the hypocalcemic potency of CT cannot be denied, what then is the function of this hormone? The evidence points against a homeostatic function under steady state conditions of the animal. The extracellular Ca^{2+} level has a natural tendency to fall, as becomes clear after parathyroidectomy. Apparently, renal excretion and exchange of Ca^{2+} with the bone result in a negative balance for extracellular Ca^{2+}. Thus, when there is no net uptake from the environment, maintenance of extracellular Ca^{2+} homeostasis requires the continuous action of PTH, and not of a hypocalcemic hormone. Hypercalcemia may develop mainly during the episodic uptake of dietary calcium. Restoration of plasma Ca^{2+} needs an antihypercalcemic hormone. For the terrestrial vertebrates this clearly is CT, which

is in line with the postprandial surge in CT secretion that has frequently been reported.

Another process that may disturb calcium homeostasis is the female reproductive activity. This not only requires increased intestinal uptake of calcium, which is transported and stored temporarily in the skeleton or, in amphibians, in lime sacs. It further involves remobilization of calcium and transfer through the blood, either for placental transfer to the offspring or, in the submammalian vertebrates, for incorporation into egg shells and yolk protein. These proteins are transported from the liver to the ovarium. Part of the calcium seems to be loosely bound to these proteins and may represent an additional load for the plasma Ca^{2+} level. Thus, the high circulating CT levels reported for female mammals at the end of gestation, for birds during egg laying, and for fish during the period of ovarian growth can be explained as an antihypercalcemic response. That plasma Ca^{2+} levels are not, or only slightly, elevated under these conditions does not point against this interpretation, but indicates that also during periods of increased calcium transport via the circulation plasma Ca^{2+} is controlled efficiently. High CT secretion during female reproduction has been interpreted as a response aimed at protection of the skeleton against excessive demineralization. Antihypercalcemic and skeleton protective effects of CT are hard to separate because they represent interdependent aspects of CT action. We tentatively suggest, however, that the hypercalcemic stimulus predominates over any stimulus from the bony tissue in the control of CT secretion, since placental transfer or yolk formation proceed to the detriment of the skeleton when female animals are facing lack of exogenous calcium during gestation or ovarian growth. The importance of CT for controlling plasma Ca^{2+} following episodic intestinal calcium uptake and during female reproduction is reflected in the hormonal control of CT cell function: important stimulatory effects have been described for gastric and intestinal factors, in particular gastrin, and for estrogen. Marked species-specific differences have been reported for gastrin, varying from prominent direct stimulation at physiological concentrations as in pigs, to no effect at all in rats. The reports for estrogens are contradictory for mammals, but more consistent for the lower vertebrates, including fish. This is understandable since egg formation and ovarian growth are under estrogenic control, whereas placental transfer of calcium to the offspring is controlled by PRL and other hormones rather than estrogens. A specific but undefined function for CT during reproduction as has been suggested by others cannot be excluded, but in our opinion the assumption of such a function is not necessary to explain the present data on CT secretion in reproducing female vertebrates.

D. Stanniocalcin

The corpuscles of Stannius are now recognized as the source of an important hypocalcemic or, preferably, antihypercalcemic hormone of the holostean and teleostean fishes, and recently some consensus has been achieved about the identity of this hormone in teleosts. Caution is indicated, however, because the corpuscles have been studied in only very few of the more than 20,000 teleostean species. In these species the antihypercalcemic capacity of stanniocalcin clearly surpasses that of calcitonin, and its mode of action differs essentially from that of the latter. Stanniocalcin's main targets are not the bone but the entry routes of calcium: the intestine and, in particular, the gills. Thus, antihypercalcemic control in fish seems to be effected by reduction of calcium uptake from the environment and not by calcium deposition in the skeleton. Although some exchange of calcium and phosphate occurs between the skeleton—in particular the scales—and the extracellular fluid, fish bone seems to represent a phosphate store rather than a calcium store. At present we consider stanniocalcin the primary hormone for the homeostatic control of extracellular Ca^{2+} in fish, and in this respect it is equivalent to PTH in the terrestrial vertebrates. However, whereas PTH has a hypercalcemic function, stanniocalcin is antihypercalcemic. This difference reflects a fundamental difference in calcium economy between terrestrial and aquatic vertebrates: in the water, calcium is present as a virtually inexhaustible source that can be tapped almost instantaneously. Terrestrial animals are dependent for their calcium on intermittent and variable dietary uptake, and therefore have to rely on internal stores during times when external calcium supplies are insufficient or not available at all. In seawater, with an ionic calcium concentration about eight times that of the extracellular fluid, hypercalcemia is a continuous threat for fish. But also in freshwater, with Ca^{2+} concentrations similar to or up to ten times lower than that of the extracellular fluid, calcium can passively enter the body, across the outer apical membrane of specialized cells in the gills that are in close contact with the water. Even in very soft water the Ca^{2+} concentration is at least 100-fold higher than typical cytoplasmic Ca^{2+} levels. We consider this gradient the driving force for Ca^{2+} entry in fish. According to the model presented in Fig. 12, the passive entry of Ca^{2+} is under inhibitory control of stanniocalcin. Then it can be explained why extracellular Ca^{2+} increases to extreme values when the corpuscles of Stannius are removed. This rise contrasts with the fall in extracellular Ca^{2+} that follows removal of the source of the primary Ca^{2+} regulating hormone in the terrestrial vertebrates. The model further explains why the predominant homeostatic

regulator in fish could be an antihypercalcemic hormone rather than a hypercalcemic hormone, even in low-calcium fresh water.

Reduction of Ca^{2+} entry into the blood by stanniocalcin will not necessarily lead to reduction of plasma Ca^{2+}. This is effected as soon as the influx of Ca^{2+} falls below the losses of Ca^{2+} that occur through passive efflux across the gills or through renal excretion. In particular the hormonal control of Ca^{2+} excretion in fish needs more attention. Calcitonin might be involved (Fenwick, 1978). Because stanniocalcin seems to have no prominent effects on bone, the homeostatic regulation of Ca^{2+} in fish probably is less closely linked to phosphate metabolism than it is in the terrestrial vertebrates. Osteoclasts and encapsulated osteocytes are scarce or even absent in many fish, and this phenomenon indicates that the hormonal and skeletal mechanisms for the terrestrial type of control of Ca^{2+} homeostasis have developed during or after the water-to-land transition in the vertebrate evolution.

The control of stanniocalcin seems to be dominated by the Ca^{2+} concentration of the extracellular fluid. The cells producing this hormone do respond to changes in external Ca^{2+} *in vitro*, but whether or not the sensitivity of these cells to Ca^{2+} is sufficient to be of physiological importance still has to be demonstrated. A role for hormones and neural factors in the control of stanniocalcin secretion is indicated but its significance is unclear.

Given the indispensability of stanniocalcin for Ca^{2+} homeostasis in teleost fish, the question arises how extracellular Ca^{2+} is controlled in other groups of fish, such as lungfishes, elasmobranchs, and jawless fish in which no Stannius corpuscles have been found. Both latter groups have no calcified skeleton. Another question is how amphibian tadpoles and newts maintain Ca^{2+} homeostasis in the absence of stanniocalcin as well as parathyroid glands. Could calcitonin have a function comparable to stanniocalcin in these animals? Although in recent years there has been rapid progress in the understanding of calcium regulation in fish, these questions illustrate that our knowledge of aquatic vertebrates is still superficial and fragmentary. Because the development of the vertebrates started in the water, studies on fish and amphibians could provide data that are indispensable for our understanding of the evolution of calcium regulation in the higher vertebrates, including man.

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