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Correlation of Electron Microscopic and Secretory Response of Human Parathyroid Adenomas with Different Calcium Concentrations in Organ Culture

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Summary. Twelve parathyroid chief cell adenomas from patients with primary hyperparathyroidism were incubated in a tissue culture system in the presence of different calcium concentrations and for various time periods. The endocrine response of the tissue was examined electron microscopically and radioimmunologically.

After incubation in a medium of low calcium concentration the parathyroid adenomas showed ultrastructural signs of stimulation with proliferation of the hormone-synthesizing organelles. The development of the ultrastructural response could first be observed after four hours and increased up to several days. Radioimmunologically, an increase of the hormone secretion could be demonstrated.

Converse results were obtained after incubation of the tumor tissue under suppressive culture conditions.

To check for *de-novo* synthesis of the hormone released the tissue was incubated in a ^{75}Se-methionine-containing medium. This resulted in radioactivity of the secreted parathyroid hormone, indicating de novo synthesis in our culture system.

The biological potency of the released hormone was demonstrated by comparison of the PTH out of the medium with the international human MRC standard using two different radioassays.

Key words: Human parathyroid adenomas – Electron microscopy – Parathyroid hormone release – PTH radioimmunoassay – Pathophysiology of primary hyperparathyroidism.

Introduction

Ultrastructural and radioimmunological studies on parathyroid physiology have shown an inverse relationship between the calcium concentration of the extracel-

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lular fluid and the parathyroid hormone (PTH) secretion of normal human and animal parathyroid glands (Copp et al., 1961; Raisz, 1963; Care et al., 1966; Sherwood et al., 1966, 1968, 1970, 1971; Deftos et al., 1968; Hamilton and Cohn, 1969; Oldham et al., 1971; Martin et al., 1972; Black et al., 1973; Fujita et al., 1974; Feinblatt et al., 1975; Habener et al., 1975; Lee and Roth, 1975; Habener and Potts, 1976). This inverse correlation could be demonstrated in in-vivo and in-vitro experiments.

There are controversial data with regard to the autonomy of PTH secretion in primary hyperparathyroidism (PHPT). Adenoma tissue has often been said to be autonomous and therefore not sensitive to changes of the calcium concentration in the extracellular fluid (Rasmussen, 1968; Reiss et al., 1969; Buckle, 1968, 1970). After induction of severe hypercalcemia (Reiss et al., 1969; Buckle, 1970) or hypocalcemia (Buckle, 1968) in patients with parathyroid adenomas no change occurred in serum PTH concentrations. Other in vivo studies on hyperparathyroid patients produced contrary results: EDTA infusions (Potts et al., 1971; Wen Chen et al., 1972; Murray et al., 1972; Lockefeer et al., 1974) or phosphate infusions (Binswanger and Fischer, 1974) resulted in a decrease of serum calcium levels. In response to this there was an increase of serum PTH concentration exceeding the already raised serum PTH levels of the patients. Calcium chloride infusions, however, produced a sudden decrease of serum PTH (Murray et al., 1972; Wen Chen et al., 1972; Monchik et al., 1977). In vitro experiments (Chertow et al., 1977; Dietel et al., 1977; Birnbaumer et al., 1977) have supported the hypothesis that adenoma tissue is calcium sensitive.

The present study examines the in vitro influence of different calcium concentrations on the ultrasturcture and secretion of immunologically and biologically potent, de novo synthesized PTH from parathyroid adenomas. The study was performed in order to elucidate whether sensitivity of the tumor to extracellular calcium concentrations is evident. We also attempted to correlate ultrastructural changes and radioimmunological findings.

Materials and Methods

The adenoma tissue was obtained during operation from patients with PHPT. The diagnosis was established by histological evidence of a parathyroid chief cell or mixed cell adenoma and postoperative decrease of serum calcium. Twelve cases were examined.

The methods for preparation and incubation have been described in detail previously (Dietel et al., 1977). After transporting the tumor to the laboratory in cold medium the connective tissue was removed. Subsequently the tumor was cut into slices of about 1 mm^3. For culture medium Ham's F 10 (modified) was used with an addition of 10 ml fetal bovine serum and 0.5 ml glutamine (both from Flow Lab.) per 100 ml medium. The specimens were incubated at a temperature of 37° in an atmosphere of 95% air and 5% CO_2.

We used three calcium concentrations in the media: normal (1.2 mM, corresponding to the calcium concentrations of the extracellular fluid (Rasmussen, 1970)), low (0.6 mM) and high (2.6 mM). These calcium concentration in the media were controlled by atomic absorptions spectrophotometry. The magnesium concentration was always 0.83 mM.

The parathyroid adenoma tissue was examined electron microscopically immediately after surgery and after incubation in the different calcium concentrations. The periods of incubation of the specimens were 2 h, 4 h, 6 h, 1 day and then varied up to 26 days. The tissue was fixed in 3% glutaraldehyde in cacodylate buffer for two hours, then buffered with 0.1 M cacodylate

buffer, postfixed with 1.3% osmic acid and embedded in Epon. Ultrathin sections (400 Å) were cut on a Reichert OM U 2 ultramicrotome, stained with uranyl acetate and lead citrate and examined on a Siemens Elmiskop I.

For radioimmunological measurements of the PTH release the tissue was incubated first in medium of normal calcium concentration for two hours and subsequently changed to low or high calcium concentrations for another two hours. Control samples were cultured in normal calcium concentration for the whole test period to ensure the constancy of PTH secretion throughout this period of time. During the test the medium was changed hourly.

PTH release into medium with normal calcium concentration during the first hour was chosen as the reference value corresponding to 100% and the release during the following hours was expressed as percentage of the initial value.

To demonstrate a de novo synthesis in our tissue culture system the parathyroid adenoma tissue was incubated in a normal calcium concentration medium containing ^{75}Se-L-methionine (30 µCi/ml, Sqibb, 112 mCi/mg) for 2, 6 and 48 h. Specific activity of the labelled medium was calculated to be 1 µCi ^{75}Se/µmol L-methionine. After incubation PTH was separated out of the medium by gel filtration and subsequent rechromatography; the elution pattern of the rechromatographed medium was determined by measuring the ^{75}Se radioactivity of the fractions (Dorn and Montz, 1977). Molecular weight was estimated using the method of Wong and Lindall (1975).

Immunoreactive PTH was determined by radioimmunoassay as described previously (Altenähr et al., 1977). The materials used were highly purified bovine PTH (Nordmeyer et al., 1976) for standards and for radioiodination (Hunter and Greenwood, 1962) with ^{125}I 10 Ci/mg (Behring-Werke, Marburg) and an antiporcine PTH antiserum from rabbit in a final concentration of 1:10000. With this antiserum cross reaction and superimposable dilution curves were found with bovine PTH highly purified, 1450 MRC units/mg protein (Nordmeyer et al., 1976) total medium from human parathyroid adenoma tissue and serum of patients with secondary renal hyperparathyroidism. There was no reaction with the human 1–34 synthetic fragment (Beckmann, Palo Alto) up to a concentration of 100 ng/tube, suggesting specifity of binding to the COOH terminal fragment of the PTH molecule. The non-specific binding effect was measured with the culture medium batches from each experiment. The range of the standard curves was 0.2–3.0 ng bPTH/100 ml medium. The intraassay coefficient of variation was 3.4% and the interassay coefficient of variation was 8.4%.

For characterization of the PTH in the culture medium the gel-filtrated material was measured by the two site coated tube PTH radioassay[1] (Hesch, et al., 1975) and by the labelled antibody membrane assay[1] (LAMA) (McIntosh and Hesch, 1975, 1976; Chansel et al., 1977). This was done by reference to the biological potent international human MRC standard 75/549 (Medical Research Council, Mill Hill, London). The biological potency of the MRC standard was checked by McIntosh and Hesch (1975) using the method of Albano et al. (1974).

Results

Since the chief cells of normal rat, bovine and human parathyroid glands pass through a secretory cycle (Roth and Raisz, 1966), one will find some inactive cells in stimulated parathyroids and active ones in suppressed glands. Therefore the examination of large areas of glandular tissue is necessary for morphological appraisal of endocrine activity. This is even more important in parathyroid adenomas, because their cytological variability is greater than that of normal parathyroid glands (Altenähr, 1972). We therefore paid special attention to the relationship between active and inactive chief cells in several slides, as well as to the ultrastructural criteria of individual cells.

[1] The examinations of the biological potency were kindly performed by R.D. Hesch and H. Jüppner, University of Hanover, Fed. Rep. of Germany

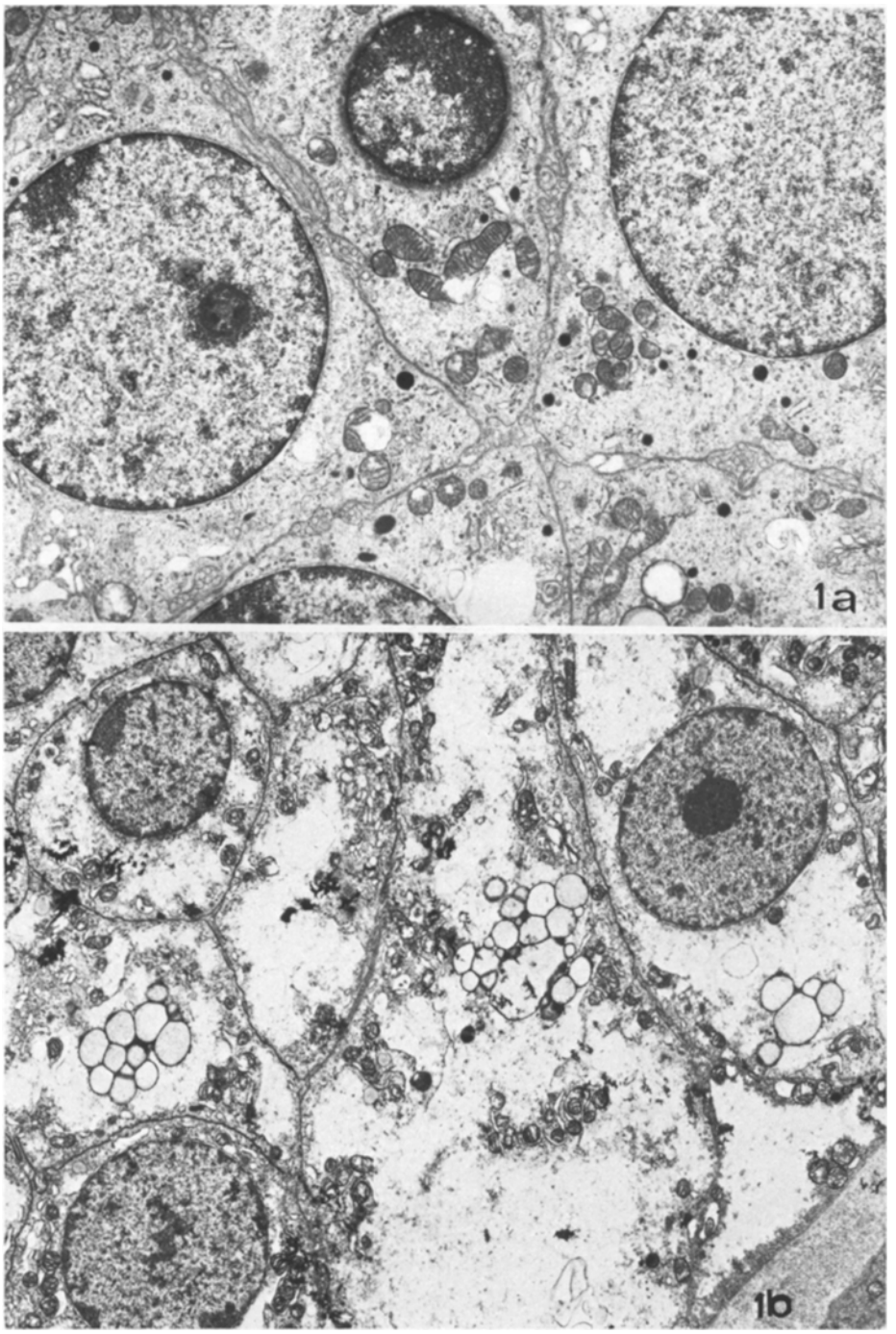
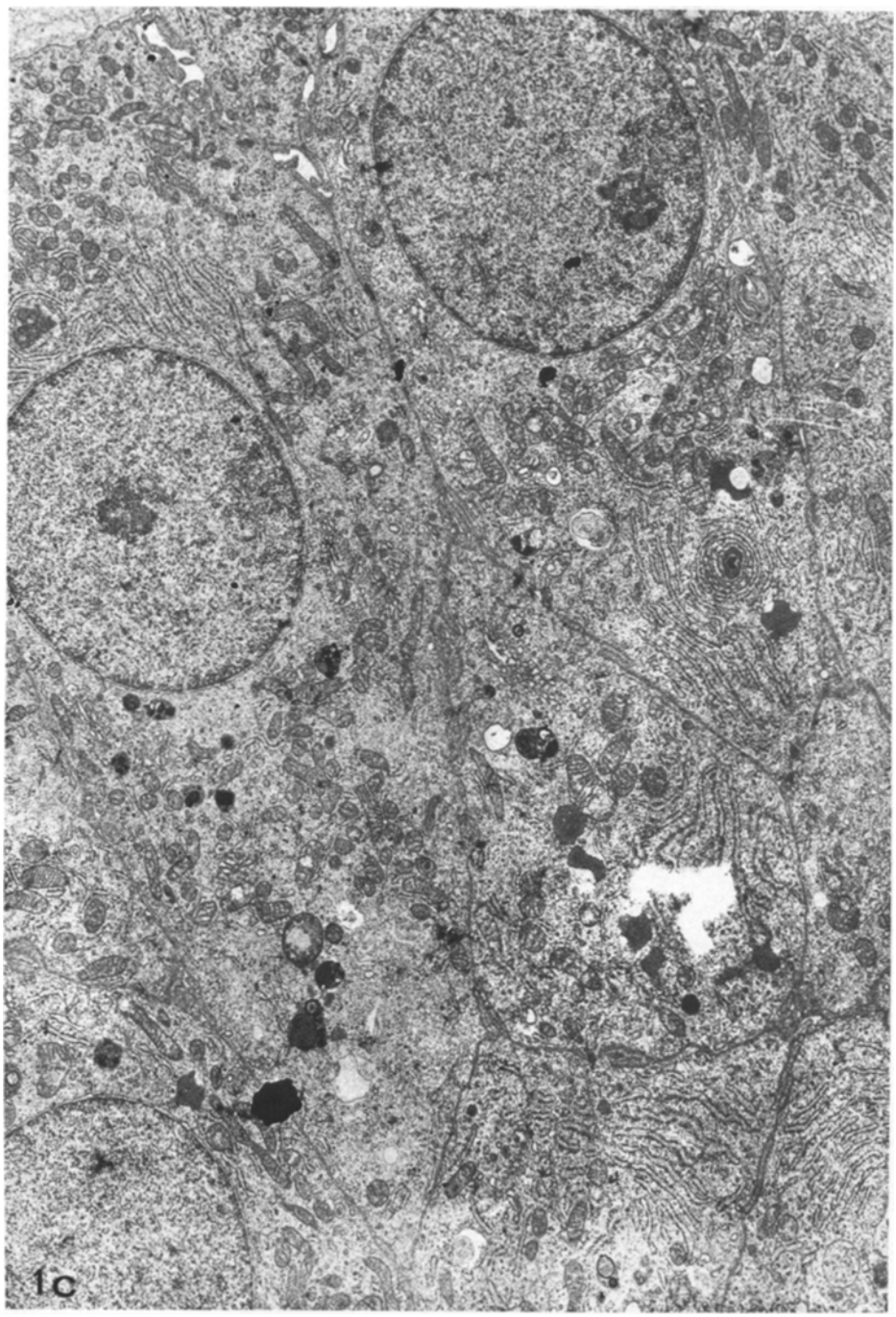
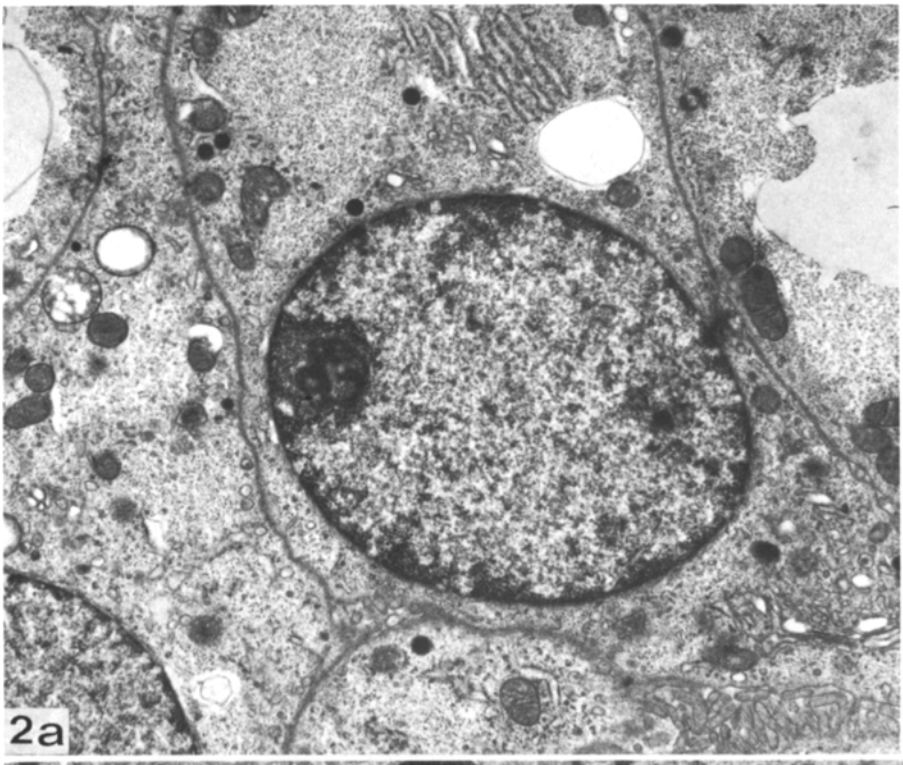


Fig. 1. a Postoperative human parathyroid adenoma with a distinct interdigitation of the cell membranes, some hormone synthesizing organelles, some peripherally concentrated secretory granules and few lipid vacuoles. (Compare Fig. 1 b and c). (×7,000) **b** The same adenoma as Figure 1 a after incubation in high calcium medium for one and a half days. The cytoplasm is more



electron-lucent, the cellmembranes are straighter, secretory granules are not visible, and the number of lipid vacuoles and complex lipid bodies is increased (× 3,000). **c** The same adenoma as Figure 1a after incubation in low calcium medium for 28 days, with the signs of strong stimulation: extensive proliferation of the granular endoplasmic reticulum is seen (× 4,800)



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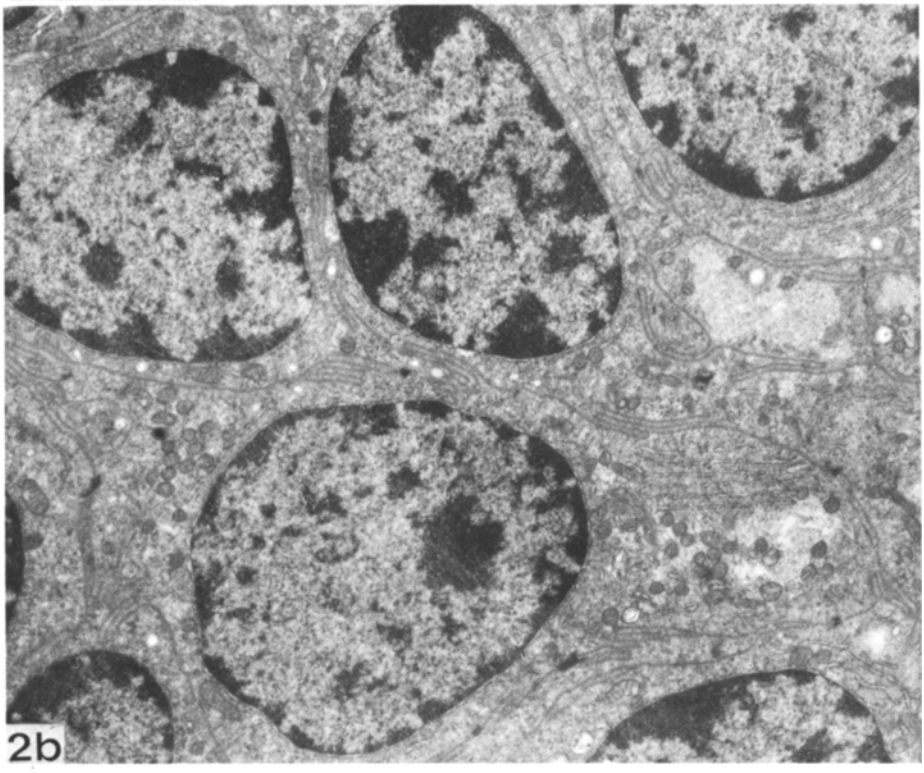

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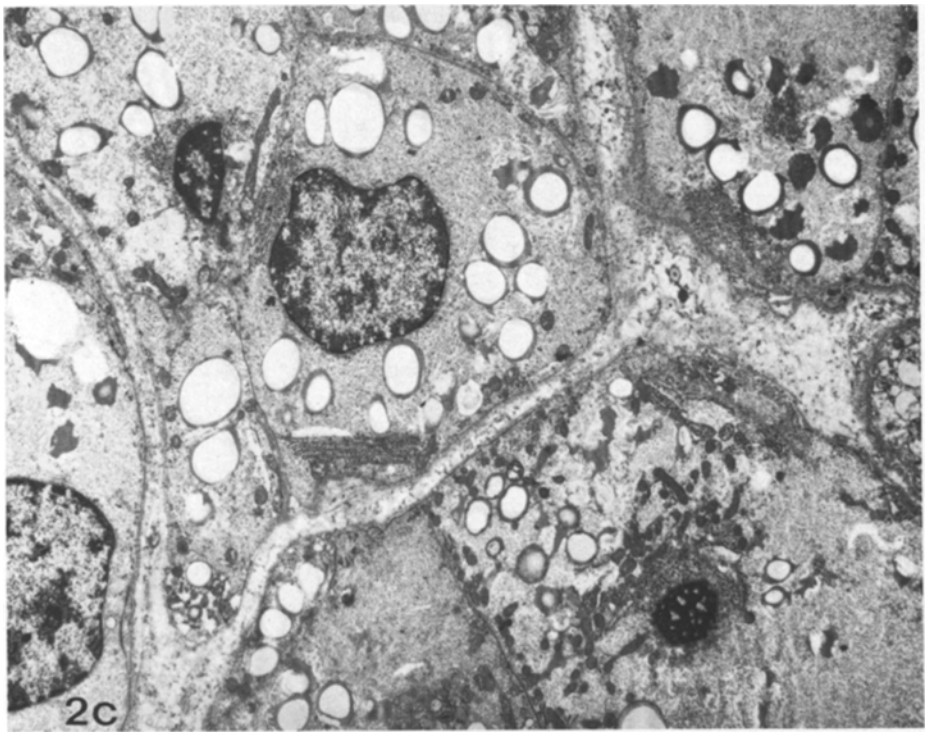

Fig. 2. a Human parathyroid adenoma after incubation in normal calcium medium for four days. (Compare Fig. 2b and c). (×3,200). **b** The same adenoma as Figure 2a. After incubation for three days in low calcium medium the cytoplasm appears more electron-dense, the number of hormone synthesizing organelles, the tortuosity and interdigitation of the plasmalemma are increased, while the lipid has disappeared (×4,800). **c** The same adenoma as Figure 2a after incubation in high calcium medium for six days. As indicators of suppression, large lipid vacuoles and straight cell membranes are visible. The nuclei show an irregular shape and peripherally concentrated heterochromatin (×2,500)

Ultrastructural Response of Parathyroid Adenomas to Low Calcium Concentration

Compared with postoperative material and control incubations in normal calcium concentrated medium (1.2 mM Ca^{++}), tissue cultured in the presence of low calcium (0.6 mM Ca^{++}) concentration showed the first ultrastructural changes between 4 and 6 h. An increase of the number of highly active chief cells could be observed up to several days (Fig. 1a and c, Fig. 2a and b).

An extensive proliferation of organelles involved in the synthesis of proteohormone and in packing of secretory granules occurred. Compared to the adenoma tissue obtained from the fresh surgical specimen the number of free polyribosomes in the cytoplasm of the chief cells was raised; they were tightly aggregated in groups. Further, a marked increase of aggregated granular endo-

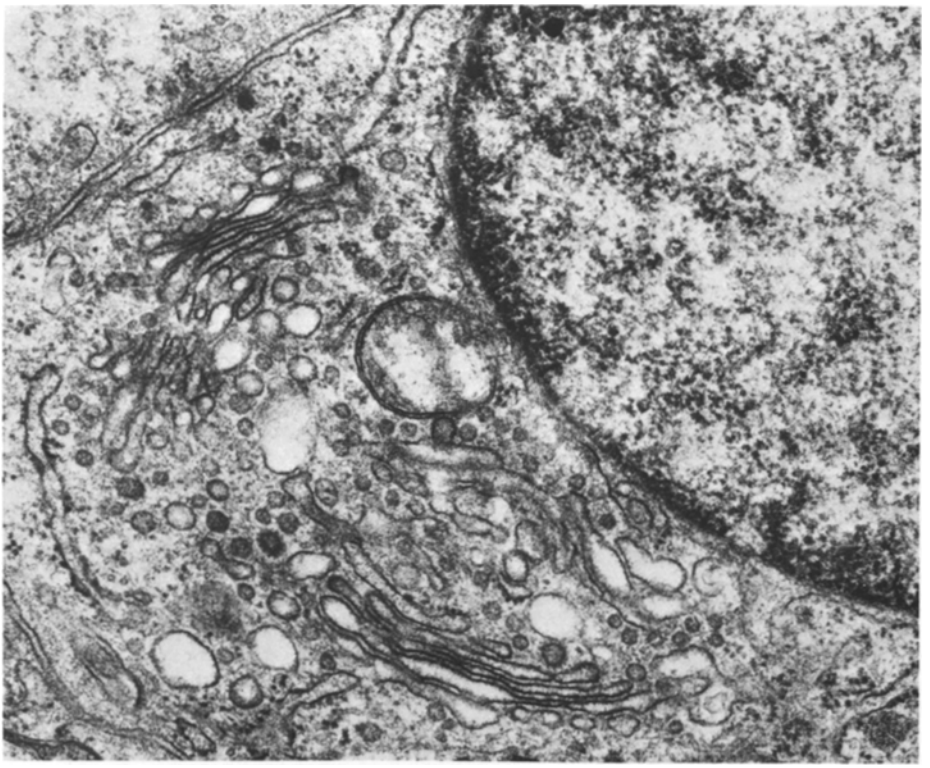

Fig. 3. High magnification of a stimulated cell of the adenoma of Figure 1a after incubation for six hours in low calcium medium. The signs of stimulation with large Golgi regions are already visible (× 14,000)

plasmic reticulum (GER) was observed (Fig. 1c) and the cisternae were arranged in flattened lamellar sacs, with numerous ribosomes closely attached on their outer surface. The Golgi apparatuses were large and composed of parallel or spiral arrays with flattened partly dumb-bell like sacs. Inside the membranes some moderately electron-dense material was observed (Fig. 3).

A change of the number of mature secretory granules was observed only after long-term cultures (several days). Under stimulating culture conditions the number of secretory granules per cell did not necessarily differ from control incubations, but comparing several slides of the control samples with low calcium incubated specimens revealed an increased number of secretory granules in the latter group. The secretory granules were often concentrated at the periphery of the cell, especially at the vascular pole (Fig. 4).

After short-term incubation there appeared to be no obvious change in number or shape of secretory granules. Emiocytotic secretion, with fusion of the membrane of secretory granules with the cell membrane, was rare and was only observed in this group of stimulated adenomas (Fig. 5a–c). Lipid bodies were less common than in control incubations, were smaller, and spread throughout the cytoplasm. Most of the stimulated cells did not contain

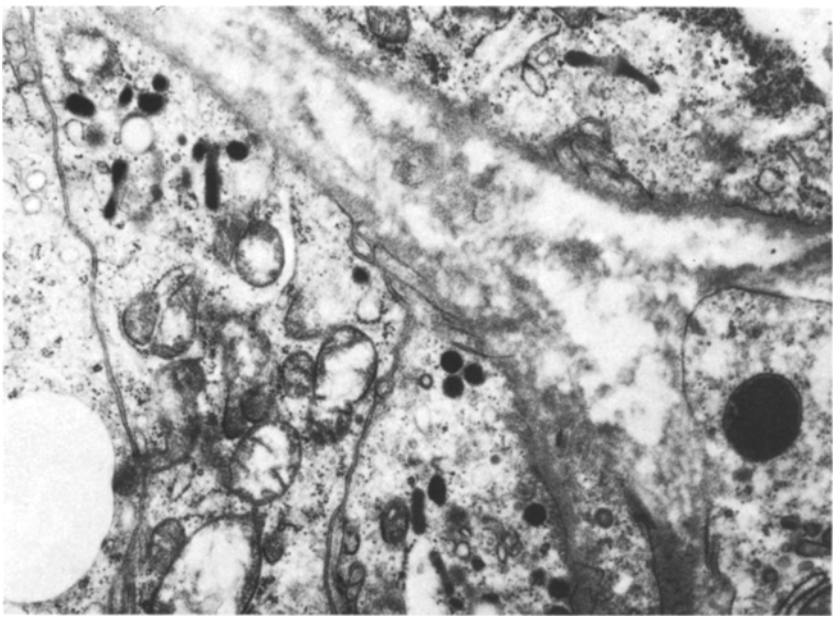

Fig. 4. Stimulated human parathyroid adenoma after an incubation period of ten days in low calcium medium. Secretory granules are numerous and concentrated at the vascular pool of the cell (×9,200)

any lipid. The number, shape and size of the mitochondria was not altered by the low calcium concentration.

The tortuosity of the cell membrane is a valuable indicator of secretory condition. It was markedly increased after stimulation, especially at the contact area of three or more adjacent cells where it extended as finger-shape infoldings in the occasionally widened intercellular space (Fig. 2b).

Desmosomes were demonstrable, but no changes were apparently induced by a low calcium medium.

Ultrastructural Response of Parathyroid Adenomas to High Calcium Concentration

Adenomas cultured in high calcium medium were mainly composed of chief cells showing the cytological signs of suppression. Their cell volume was moderately reduced compared with cells incubated in normal calcium concentration, this was also seen in light microscopic observations. The cytoplasm was less electron-dense and the cytoplasmic content of organelles concerned with hormone synthesis and secretion was strikingly decreased (Fig. 1a, b; Fig. 2a, c). The cells contained inconspicuous GER and Golgi apparatuses, loosely dispersed throughout the cytoplasm. The membranes of the GER appeared to have a reduced density of ribosomes.

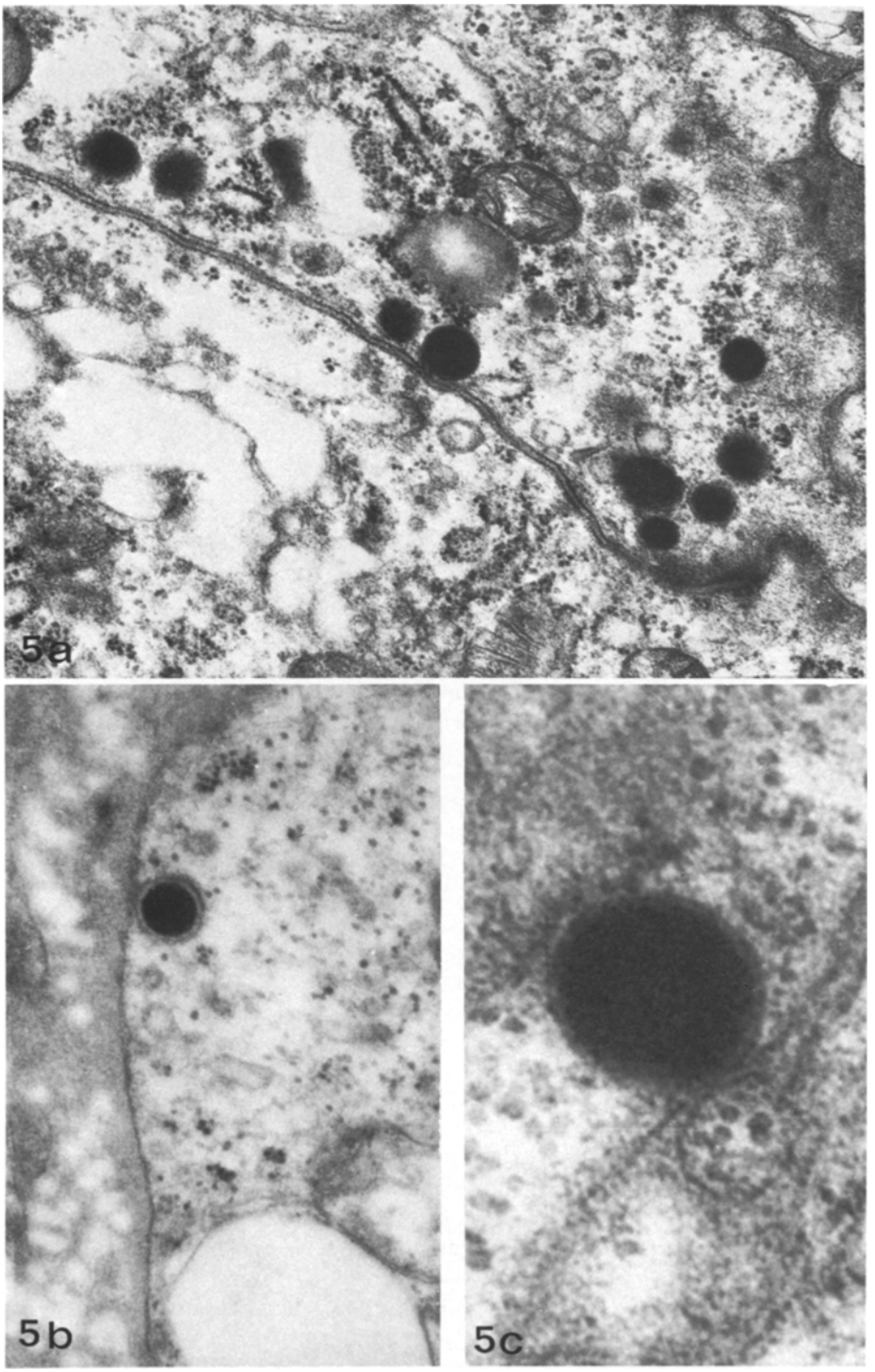

Fig. 5a–c. Secretory granules fusing with the plasmalemma indicating emiocytotic secretion under tissue culture conditions. Incubation time was between 3 and 6 days in low calcium. (**a** ×30,000; **b** ×28,000; **c** ×69,000)

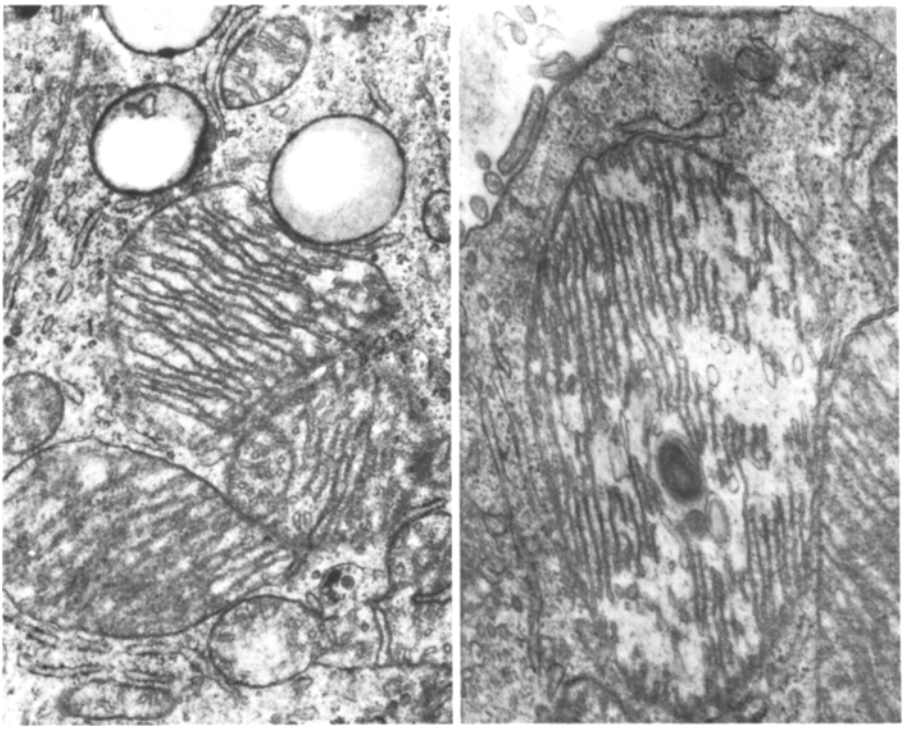

Fig. 6. High magnification of some giant mitochondria after incubation for 12 days in high calcium medium. Some lipid vacuoles are visible (× 16,000)

Most of the chief cells were completely free of secretory granules (Fig. 1b). However even under the influence of high calcium concentration, single chief cells contained some well developed hormone synthesizing organelles and appeared to be in the active phase of protein synthesis.

The cell content of lysosomal and lipid bodies was obviously different in the high calium group when compared with the control incubations and especially when compared with the low calcium treated specimens. Small lipid vacuoles were markedly more numerous and often aggregated as complex lipid bodies (Fig. 2c). Electron-dense lysosomal bodies were also more frequent in the high calcium group.

The mitochondria were as numerous as in control samples but often larger. Sometimes giant mitochondria were seen (Fig. 6), closely packed with cristae showing an electron-lucent matrix.

The cell membranes appeared to be relatively straight, with only few interdigitations. The cells contained abundant glycogen. Nuclei were sometimes of an irregular shape, with densely clumped and peripherally concentrated heterochromatin.

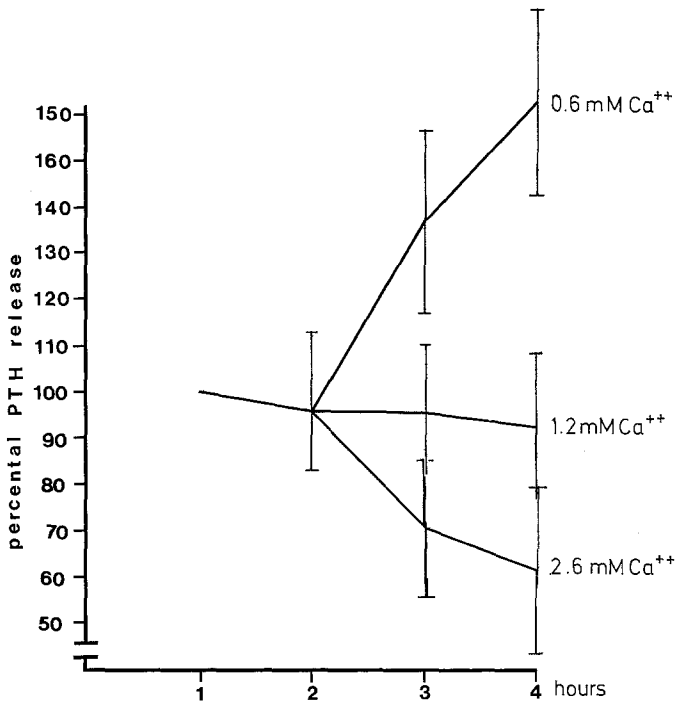

Fig. 7. Release of immunoreactive PTH at different calcium concentrations in the culture medium. Number of adenomas $N=12$. Significant differences between 2nd and 4th hour values ($P<0.005$). The values are expressed as percentage of the first hour reference value. Each point represents mean $\pm$ SD

Secretory Response of Parathyroid Adenomas to Different Calcium Concentrations

The range of absolute amount of iPTH released into the culture medium within one hour, measured by the radioimmunoassay, was with few exceptions between 20–300 ng/ml.

Hormone release from the control cultures, in which the calcium concentration was constant at 1.2 mM Ca^{++} during the test, was also constant (Fig. 7), with a small fall from 100% (first hour reference value) to 92% after 4 h. In response to the low calcium medium the iPTH concentration of the culture medium rose rapidly. There was an increase from 100% (first hour reference value) to 137% after 1 h up to 163% after 2 h in low calcium concentration. (Fig. 7). Changes in the opposite direction could be measured in high calcium concentration. The hormone concentration diminished from 100% to 87% after 1 h down to 61% after 2 h incubation in high calcium (Fig. 7).

Incorporation of ^{75}Se-Methionine into Newly Synthesized PTH and Determination of Biological Potency

After incubation of the parathyroid gland adenoma tissue in a medium containing ^{75}Se-methionine this medium was chromatographed twice in order to separate PTH. By measuring the radioactivity of the rechromatographed medium an elution pattern with four peaks of radioactivity could be obtained. Peak two had a molecular weight of 10000–12000 and the highest immunoreactivity. This protein fraction seemed therefore mainly to represent intact human PTH (Dorn and Montz, 1977). Labelled methionine had been incorporated into the PTH molecule indicating *de-novo* synthesis in our tissue culture system. In the two-site coated tube radioassay, which probably measured structurally intact 1–84 PTH (Hesch et al., 1975) only peak two reacted nearly as well as the MRC standard 75/549. In the receptor assay (labelled antibody membrane assay, LAMA; McIntosh and Hesch, 1975) the membrane binding of peak two was also high at about 25% less than the MRC standard 75/549.

As the MRC standard is biologically potent (McIntosh and Hesch, 1975) and its dilution curves with these two assays were nearly parallel to those of peak two, it is very probable that peak two fraction was also biologically potent.

Discussion

The present study reports radioimmunological PTH measurements and ultrastructural findings from 12 human parathyroid adenomas exposed to different calcium concentrations in the extracellular fluid in vitro. The only similar report is published by Chertow et al., 1977 about four adenomas. In addition to this we performed long term cultures for ultrastructural analyses.

Although parathyroid adenoma tumor cells are less homogenous than those of normal parathyroid glands (Altenähr, 1972) ultrastructural changes in response to different calcium concentrations in our in vitro system were established.

The main ultrastructural indicators of parathyroid stimulation or inhibition, evaluated by many in vivo and in vitro experiments on normal human and animal parathyroid tissue (Roth and Raisz, 1964, 1966; Capen and Young, 1967; Altenähr, 1970, 1972; Altenähr and Leonhardt, 1972; Fetter and Capen, 1970; Capen, 1971; Oldham et al., 1971; Roth, 1971; Black et al., 1973; Thiele et al., 1973; Roth and Capen, 1974; Chertow et al., 1975, 1977; Boquist and Lundgren, 1975) were also observed on human parathyroid gland adenoma tissue.

Under stimulatory conditions (low calcium medium) hormone synthesizing organelles (i.e. free ribosomes, granular endoplasmic reticulum, Golgi apparatus), the number of secretory granules and the tortuosity of the cell membrane increased, while the content of lipid decreased. Converse changes could be observed in response to suppressive culture conditions by a high calcium medium.

The radioimmunological measurements of secreted iPTH correlated well with the morphological results. All tumors investigated showed a secretory response to different calcium concentrations in the media for at least 2 h.

These results demonstrate the sensitivity of adenoma tissue to the stimulus of calcium and a response of iPTH secretion, as well as distinctive ultrastructural changes.

Chertow et al. (1977), Dietel et al. (1977) and Birnbaumer et al. (1977) reported similar in vitro results, whereas Sherwood et al. (1969, 1971) found calcium sensitivity only in two of twelve adenomas examined. Although the low and high calcium concentrations used in our experiments were not physiological, this study supports the thesis that there is no "functional autonomy" of parathyroid adenoma cells.

This thesis is further supported by several in vivo studies (Potts et al., 1971; Wen Chen et al., 1972; Murray et al., 1972; Lockefeer et al., 1974; Binswanger and Fischer, 1974; Monchik et al., 1977), where, after experimental changes of serum calcium levels of patients with primary hyperparathyroidism, an inverse relationship between serum calcium and serum iPTH concentrations was found.

There exist the contrasting results of Reiss et al. (1969) and Buckle (1969, 1970), who found no changes of serum iPTH in patients with PHPT after inducing hyper- or hypocalcemia. One possible explanation of this discrepancy is the following: if the tumor cells secrete immunoheterogenous fractions of the PTH molecule, the different secretory response measured by various investigators could be explained by a non-parallel increase of the released iPTH fractions, as identified by antibodies with different specifity. Otherwise, if the tumor cells increase the secretion of all fractions or of the intact hormone in parallel, each antibody should detect the increase of that fraction of the molecule for which it is specific. Differing half-lifes of the hormone fractions (Arnaud, 1973) could be a further point explaining the divergent results.

After incubation of the parathyroid adenoma tissue in a medium containing ^{75}Se-methionine the secreted iPTH was radioactive. The increased amount of hormone is thus a result of a de-novo synthesis and not a phenomen of "wash out" from necrotizing cells.

The molecular weight of peak two from the tissue culture medium and its strong reaction in the two-site coated tube radioassay indicated the presence of intact 1–84 PTH molecules. This and the specific receptor binding as found by LAMA, suggest a biological potency of peak two-PTH similar to that of the international human MRC standard 75/549, whose biological potency has been demonstrated (McIntosh and Hesch, 1975).

The time lapse between the secretory response measured radioimmunologically after 1 h and the first morphologically detectable changes after 4 h could be explained if stimulated cells initially deplete their hormone (phase I) and when stimulation is continous, increase their hormone synthesis (phase II) by an increase of intracellular organelles. Proliferation of the organelles, which is the morphological concomitant of stimulated hormone synthesis, needs a longer time interval. The period for the development of an obvious ultrastructural response to stimulation in our experiments on human parathyroid adenoma tissue is comparable with other in vitro studies on parathyroid glands from

normal animals: Roth and Raisz (1966) reported the first ultrastructural changes in rat parathyroid glands after an incubation time of 6 h, while Oldham et al. (1971) found the first ultrastructural changes in pig parathyroids after 2 h.

Other authors (Gittes and Radde, 1966; Targovnik et al., 1971; Sherwood et al., 1971; Blum et al., 1974; Habener et al., 1975; Mayer et al., 1976) have demonstrated an inability of complete suppression of PTH secretion in normal and adenoma tissue. The present study gives further evidence for a non-suppressible basal secretion of parathyroid adenoma tissue, as the calcium concentration of our high calcium medium was several fold higher than that ever occurring in vivo.

One possible hypothesis for pathophysiology of primary hyperparathyroidism is that the basal secretion of the enhanced mass of parathyroid tissue is a sufficient cause of overproduction of PTH and hypercalcemia (Mayer et al., 1976; Monchik et al., 1977). However, allthough adenoma tissue is sensitive to the calcium concentration in the extracellular fluid, the hypercalcemia in PHPT does not suppress all adenoma cells to that low basal secretion rate. This was demonstrated ultrastructurally, as the tissue taken postoperatively often showed cells with morphological signs of active protein synthesis, which could be suppressed by high calcium medium in in vitro incubations.

Another hypothesis suggested to explain the pathophysiology of PHPT is that the calcium sensitivity of the tumor cells is lower than that of normal tissue. So the pathological cells secret large quantities of PTH to produce a hypercalcemia, which is recognized as "normal" by these cells. This defective sensitivity could be related with any step of the endocrine mechanism of cellular hormone synthesis and/or release. Furthermore the overactivity of the tumor cells might be a hyperplasiogenic stimulus for the growth of the tissue (Roth and Raisz, 1964; Cohn and Hamilton, 1976). This is further supported by the evidence of a stimulating effect of low calcium medium on the cytoplasmic growth of normal animal parathyroid glands (Raisz, 1963).

In conclusion we suggest three points of general importance in the pathophysiology of primary hyperparathyroidism: i) there exist a non-suppressible basal secretion rate of PTH in tumor tissue, as already demonstrated in normal parathyroid glands, ii) there further exists some sensitivity of parathyroid adenoma tissue to calcium stimuli, iii) this sensitivity may be reduced when compared with normal parathyroids, resulting in a disturbed feedback mechanism but not in true autonomy.

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