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Calcium redistribution, calcification and stone formation in the parotid gland during experimental stimulation and hypercalcaemia

Cytochemical and X-ray microanalytical investigations*

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Summary. Distribution and redistribution of intra- and pericellular calcium was investigated in the parotid gland of rats under secretory stimulation and hypercalcaemia. The effects of hypercalcaemia and secretory stimulation and of the combination of both were compared. Calcium content was determined by atomic absorption spectrometry. Calcium distribution within the tissue was demonstrated by light microscopical (GBHA) staining and electron microscopical (pyroantimonate method) cytochemistry in combination with X-ray microanalysis. Typical calcium depot sites were the basal and cellular membranes, the calcium buffer organelles (i.e. mitochondria) the secretory granules and the acinar lumina. After stimulation (by isoprenalin) a decrease of calcium-enriched secretory granules and a depletion of intracellular calcium buffer organelles occurred. During hypercalcaemia (induced by dihydrotachysterol), a calcium overloading of the cell membrane and intracellular buffer organelles without calcification was observed. Combined stimulation and hypercalcaemia induced an excessive calcium overloading of all intra- and extracellular calcium depots with excessive calcium release into the acinar lumina resulting in calcium phosphate aggregates and stone formation. Secretory stimulation and simultaneous hypercalcaemia exert potentiating effects on intracellular and intraluminal calcification proposing an importance for pathogenesis of human sialolithiasis.

Key words. Calcium redistribution – Calcification – Parotid gland – Cytochemistry – X-ray microanalysis

Calcium is involved in the regulation of cellular function of nearly all body cells. In the acinar cells of the parotid gland it contributes to regulation of secretion. Disturbances of cellular calcium metabolism and of extracellu-

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lar calcium homeostasis may induce pathological calcifications of the acinar cells and acinar lumina as is well-known in the sialolithiasis accompanying sialadenosis. In investigations of Seifert [12, 19, 20] and Selye [21] it was shown that combined secretory stimulation of acinar cells and drug-induced hypercalcaemia were able to bring about such alterations. Intracellular calcium distribution and its redistribution under the influence of hypercalcaemia and secretory stimulation are unknown. Therefore, in the present experimental study we investigated the influence of secretory stimulation and hypercalcaemia as single effectors or in combination on the intraacinar and intracellular calcium distribution. Calcium distribution at the tissue and cellular level of the parotid acini was demonstrated by morphological, cytochemical and X-ray microanalytical methods.

Material and methods

Four experimental groups, each containing ten five weeks old female Wistar-rats (80–150 g), were formed and treated for four days as described below. Animals of all groups were fed with standard laboratory food and tap water ad libitum.

The first group (9 animals) served as a control and received twice daily intraperitoneal injections of 2.5 ml 0.9% saline.

The second group (7 animals) treated twice daily with 25 mg N-isopropyl-noradrenalinsulfat = isoprenalin (Aludrin®, Boehringer, Ingelheim, FRG) i.p. This drug being a betasympathicomimeticum should stimulate the secretion of the parotid gland.

The third group (10 animals) received twice daily injections of 2.5 ml 0.9% saline i.p. and, in addition, two doses of dihydrotachysterol (A.T. 10®) at the first (1 mg) and the third (0.5 mg) experimental day. A.T. 10 (kindly provided by Fa. Bayer, Leverkusen, FRG) is a potent hypercalcaemic vitamin D derivate and was given by stomach tube.

The fourth experimental group (10 animals) was treated with twice daily intraperitoneal injections, each containing 25 mg Aludrin® and, in addition, with oral dihydrotachysterol doses (1 mg at the first, 0.5 mg at the third experimental day). All animals were sacrificed after four days.

After killing of the animals in ether anaesthaesia the parotid glands were removed and processed further for chemical determination of the calcium content, for conventional light microscopy, for cytochemical investigations and – in cases of some animals in each group – for electron microscopy and X-ray microanalysis.

For *chemical determination* of calcium contents, tissue specimens were dryed, weighed and hydrolysed in concentrated HNO_3 and 70% $HClO_4$ (14). The total calcium contents of the specimens were measured after dilution in a $SrCl_2$-containing solution applying a Perkin-Elmer atomic absorption spectrometer. The values obtained were related to the dry weights of the specimens.

For *cytochemical demonstration* of the non-mineralized and mineralized calcium in unfixed tissue specimens, we cut cryostat sections. These were stained with glyoxal-bis(2-hydroxyanil) (=GBHA) according to the procedure described before [18]. Thickness was constant in all sections (20 µm).

Maximum staining intensity in the parotid acini was measured using a scanning microdensitometer (Fa. Vickers M 85, York, UK) applying a wavelength of 527 nm. The diameter of the scanning spot was 1 µm, the diameter of the measuring field was 200 µm. A tissue-free area within the same slide served as a blank standard.

For electron microscopy, tissue was fixed in 2.5% glutaraldehyde dissolved in 0.1 molar sodium cacodylate buffer (pH 7.4). The tissue was postfixed in buffered 1% OsO_4. After dehydration

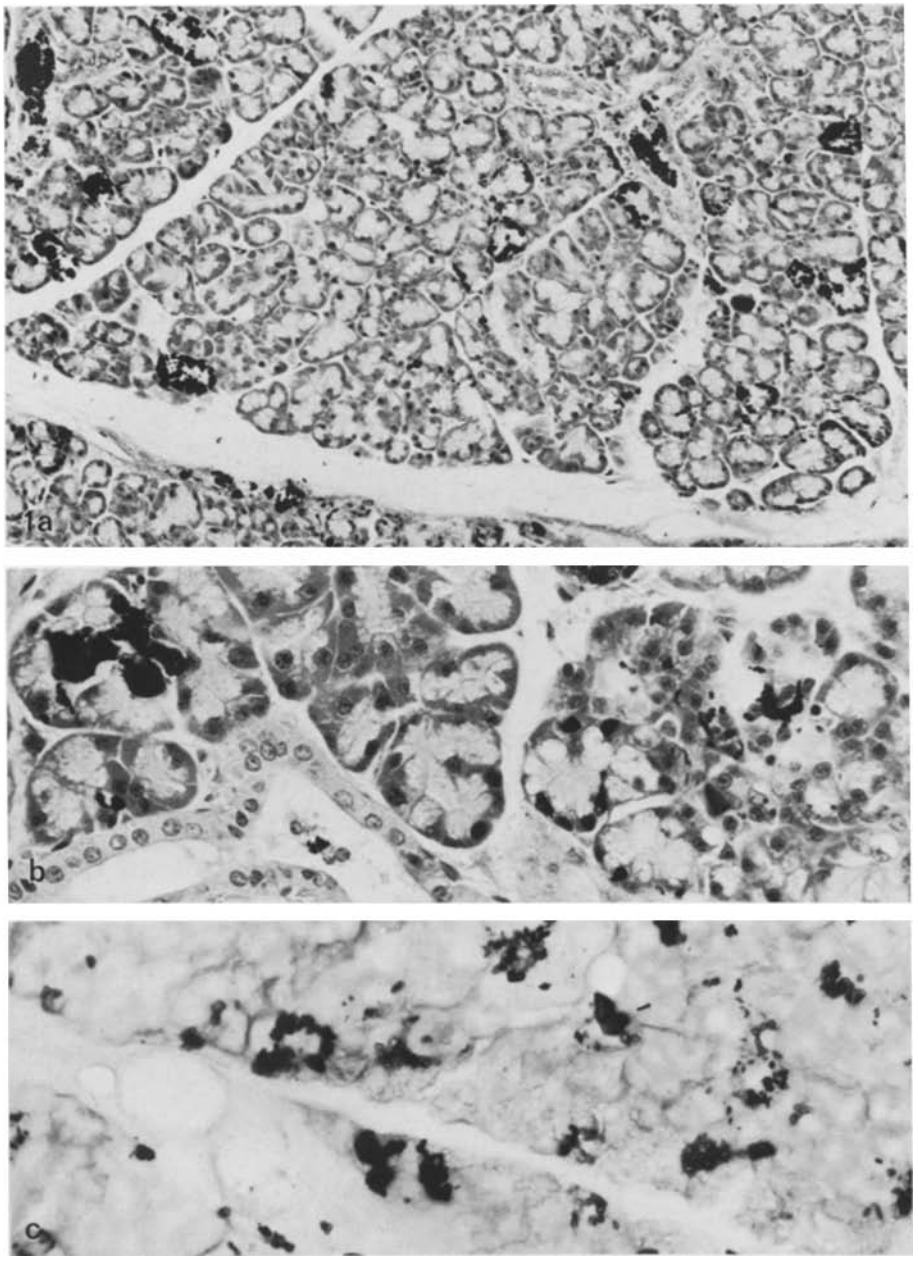

Fig. 1a–c. Parotid gland, rat. Treatment with isoprenalin and dihydrotachysterol during 4 days. **a** Black staining of acinar and intraductal calcifications. (Kossa, ×150). **b** Calcification of acinar cells and intraluminar material (Kossa, ×375). **c** Moderate diffuse calcium staining in acinar cells with focal dark staining of calcified deposits (calcium staining with GBHA, no counter staining) (×150)

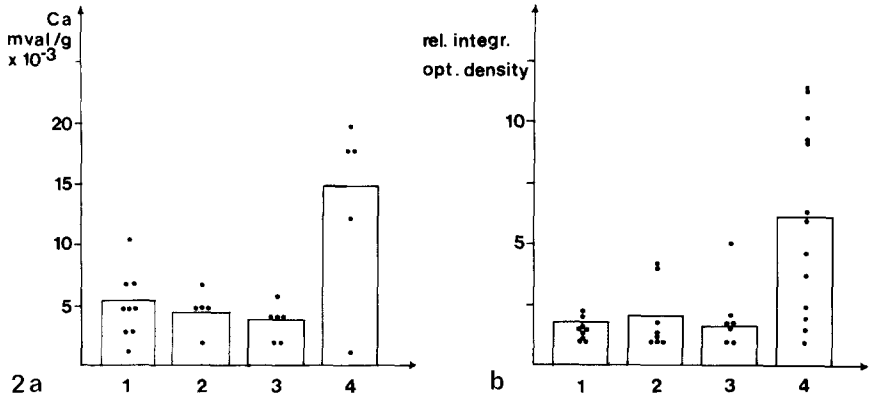

Fig. 2a, b. Calcium content of parotid gland determined by atomic absorption spectrometry. The calcium content is expressed as mval calcium per g tissue dry weight. **b** Intensity of calcium staining by the GBHA reaction in parotid acinar cells. The staining intensity is measured by microdensitometry. The bars in **a** and **b** represent the different experimental groups: 1 = untreated controls, 2 = secretory stimulation (IPR), 3 = hypercalcaemia (DHT), 4 = hypercalcaemia and secretory stimulation (DHT and IPR)

in graded ascending concentrations of ethanol the tissue was further processed by propylene oxide and embedded in EPON 812. Ultrathin sections were stained by uranyl acetate and lead citrate. The specimens were examined using a PHILIPS (Eindhoven, Netherlands) EM 400 electron microscope. In addition, the non-mineralized and mineralized calcium was demonstrated at the ultrastructural level. For this purpose parotid tissue was fixed for 2 h in a solution of 2.5% glutaraldehyde and 2.0% potassium pyroantimonate (pH 7.5). Tissue was postfixed for one hour in a solution of 1% OsO_4 and 2% potassium pyroantimonate (pH 7.2). After dehydration in a graded series of ethanol concentrations the tissue was embedded via propylene oxide in EPON 812 as described above. Unstained 1,000 Å thick sections were analysed by an energy dispersive X-ray microanalyzer (Fa. ORTEC 6030, Oak Ridge, TN, USA) combined with a PHILIPS EM 400 electron microscope. The measuring spot (diameter 0.1 μm) was brought under electron microscopical control into position resp. of the basolateral cell membrane, the mitochondria, the secretory granules and the acinar lumen. The electron beam in the area of the measuring spots is exciting the specimen to emit X-rays which are collected and analysed for their energy. The X-ray energy is characteristic for the different elements, the intensity of the X-rays reflects the amount of the element in the excited specimen volume (Fig. 7).

The measuring conditions were identical in all measurements. The calcium content of the analysed area in the specimen was compared with the calcium content of a calcium standard analysed before. The proportion of the calcium contents in the specimen and the standard is expressed as the k-ratio. A calcium content in the specimen identical to that of the calcium standard results in k-ratio of 1.0. Applying the iterative SPRINT program (Fa. ORTEC) the overlapping peaks of calcium and antimony were deconvoluted. The EPON 812 used for tissue embedding reveals a constant chlorine content. The determination of the chlorine amount in a tissue-free part of the section could therefore be used as an internal standard for calculation of a correction factor for compensation of section thickness variations.

Results

Light microscopically the glands of the stimulated groups (group 2) revealed swelling of the acinar cells with enlarged loosely structured nuclei. Inflammatory infiltrates were absent. PAS staining revealed a lack of intracellular secretory granules. The few remaining granules showed reduced staining.

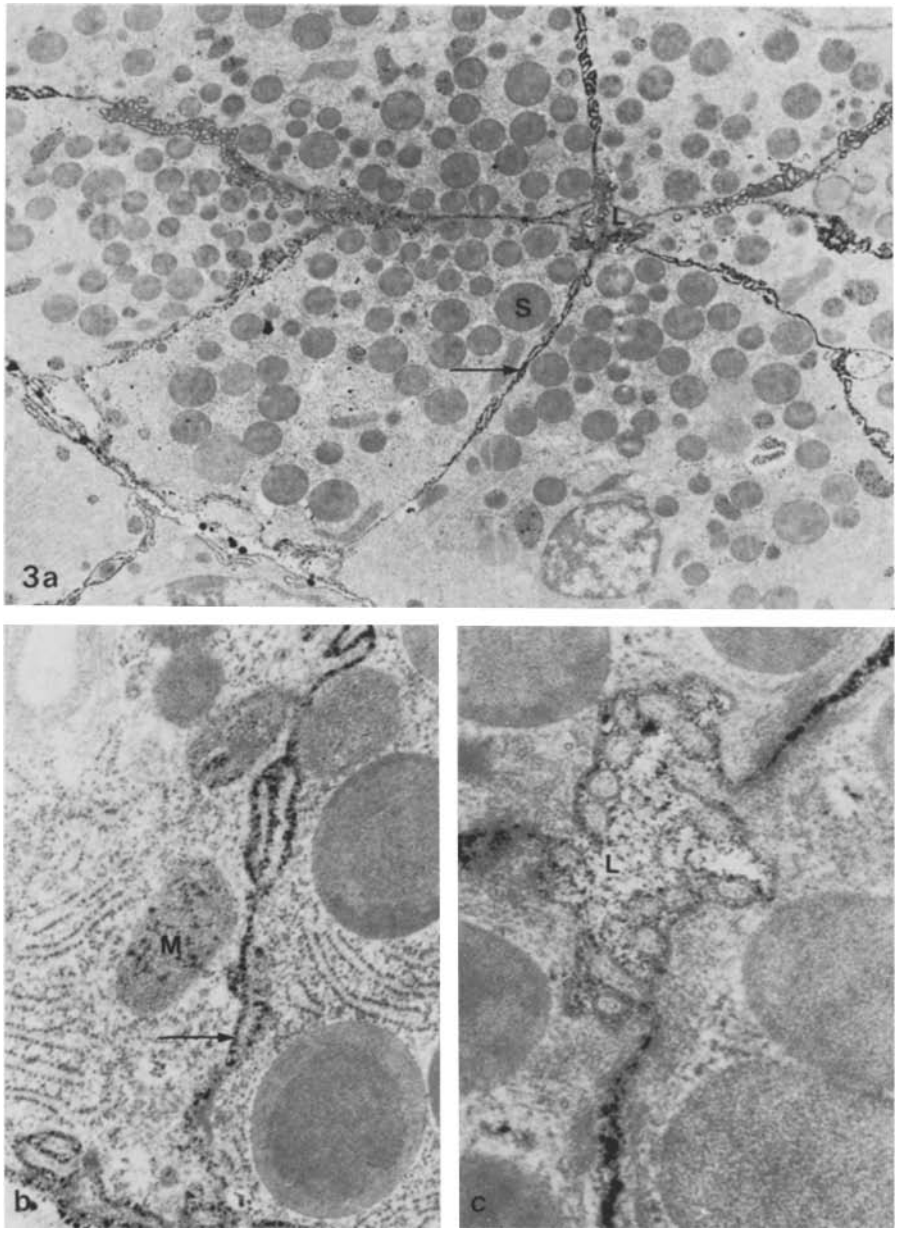

Fig. 3a, b. Parotid acinar cells, rat. Untreated controls. Cytochemical demonstration of calcium distribution by black granular reaction product, moderately filled calcium depots at the cell membrane (*arrow*), within mitochondria (*M*) and in the acinar lumen (*L*). Lack of reaction product in the secretory granules (*S*). Pyroantimonate reaction. (**a** ×6,100, **b** ×28,300, **c** ×42,000)

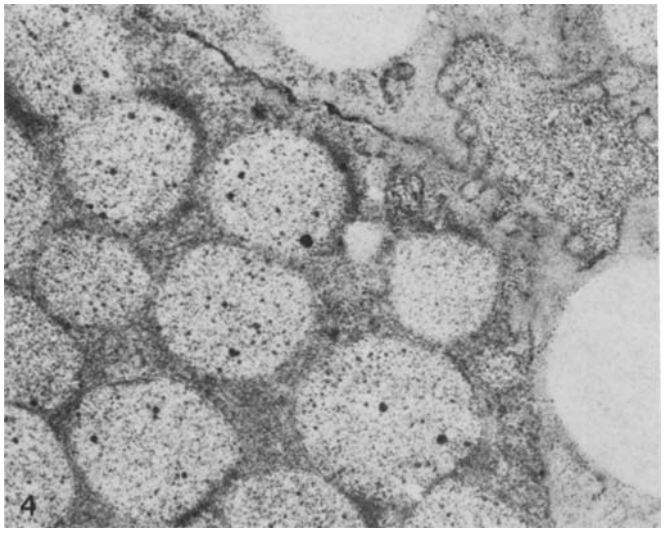

Fig. 4. Parotid acinar cell, rat. Secretory stimulation. Increased calcium impregnation of the loosely structured secretory granules. Pyroantimonate reaction. (× 22,700)

In some parts necroses of individual acinar cells or total acini were found. The Kossa staining was negative in group 2 indicating absence of calcifications within the tissue. In the hypercalcaemic group (group 3) the haematoxylin eosin and PAS staining showed a picture similar to that of the control group (group 1). In the Kossa staining of group 3 rare focal calcifications could be found exclusively in the vessel walls. Calcifications in the acini were totally lacking. In the hypercalcaemic and stimulated group (group 4) light microscopy showed the signs of marked stimulation. Enlargement of the acini, swelling of the acinar cells with enlarged nuclei and secretory degranulation of the cells with only rare swollen and lightly stained secretory granules in the apical part of the cells could be seen. Some acinar cells and acini were necrotic. Some parts of the acinar lumina and ducts were filled with PAS-positive material. In the Kossa stain, profuse calcification of individual acinar cells or of total acini was detected. In the lumina of some acini and ducts stone-like calcium concrements were present (Fig. 1a and 1b). In some areas calcification of intraglandular vessels and of necrotic cells were seen.

The chemical determinations of the calcium content in the parotid tissue resulted in mean values which showed insignificant differences in groups 1–3 (Fig. 2a). In contrast, the glands of the hypercalcaemic and stimulated group (group 4) showed a three times higher calcium content than the other groups (Fig. 2a).

The microdensitometric determination of the calcium content in GBHA stained sections showed results comparable to those of the chemical measurements. The cytochemical content of mineralized and non-mineralized calcium was nearly identical in groups 1–3. In the hypercalcaemic and stimu-

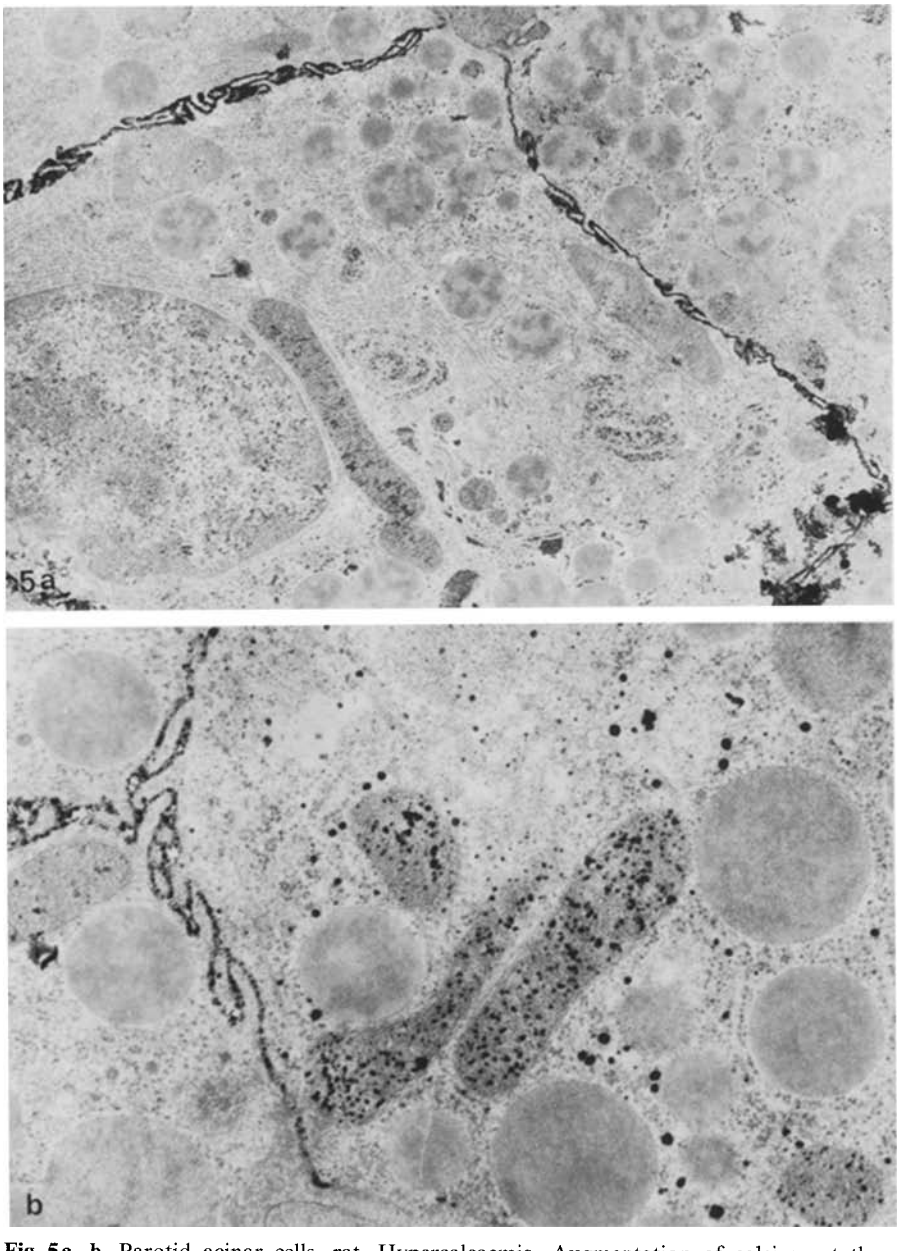

Fig. 5a, b. Parotid acinar cells, rat. Hypercalcaemia. Augmentation of calcium at the cell membrane and within mitochondria. Normal secretory granules and acinar lumina. Pyroantimonate reaction. (**a** ×10,000, **b** ×27,300)

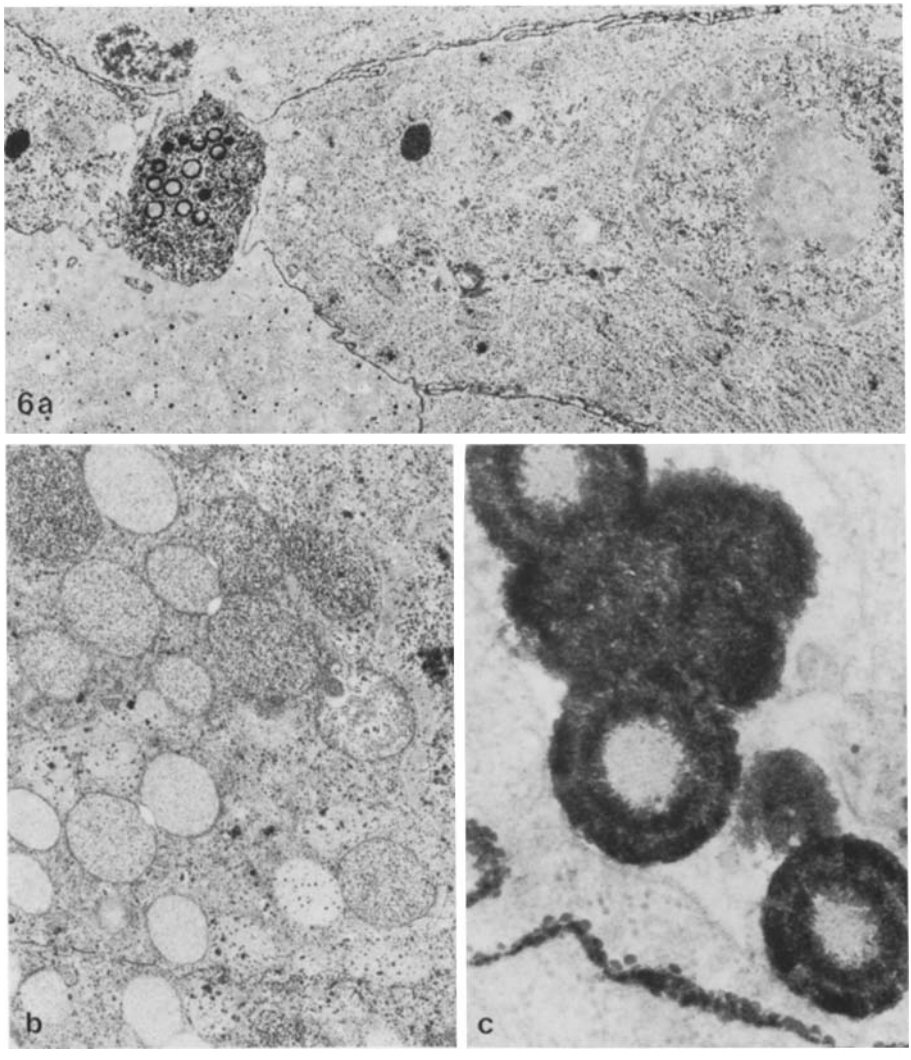

Fig. 6a–c. Parotid acinar cells, rat. Hypercalcemia and secretory stimulation. **a** Excessive calcium overloading of the acinar cells and particularly of the acinar lumen. **b** Intense calcium impregnation of secretory granules. **c** Round lamellated stone-like calcifications in the acinar lumen. Pyroantimonate reaction. (**a** ×5,500, **b** ×9,000, **c** ×59,200)

lated group (group 4) individual acini developed a fourfold increase of stainable calcium, while the calcium content of the other acini was in the range of those of the control group (group 1) (Fig. 1c and 2b).

Ultracytochemical demonstration of calcium in the parotid gland revealed a characteristic ultrastructural calcium distribution pattern, which developed characteristical alterations during stimulation and hypercalcaemia. Typical stainable calcium pools in the acinar cells were the basolateral cell membrane

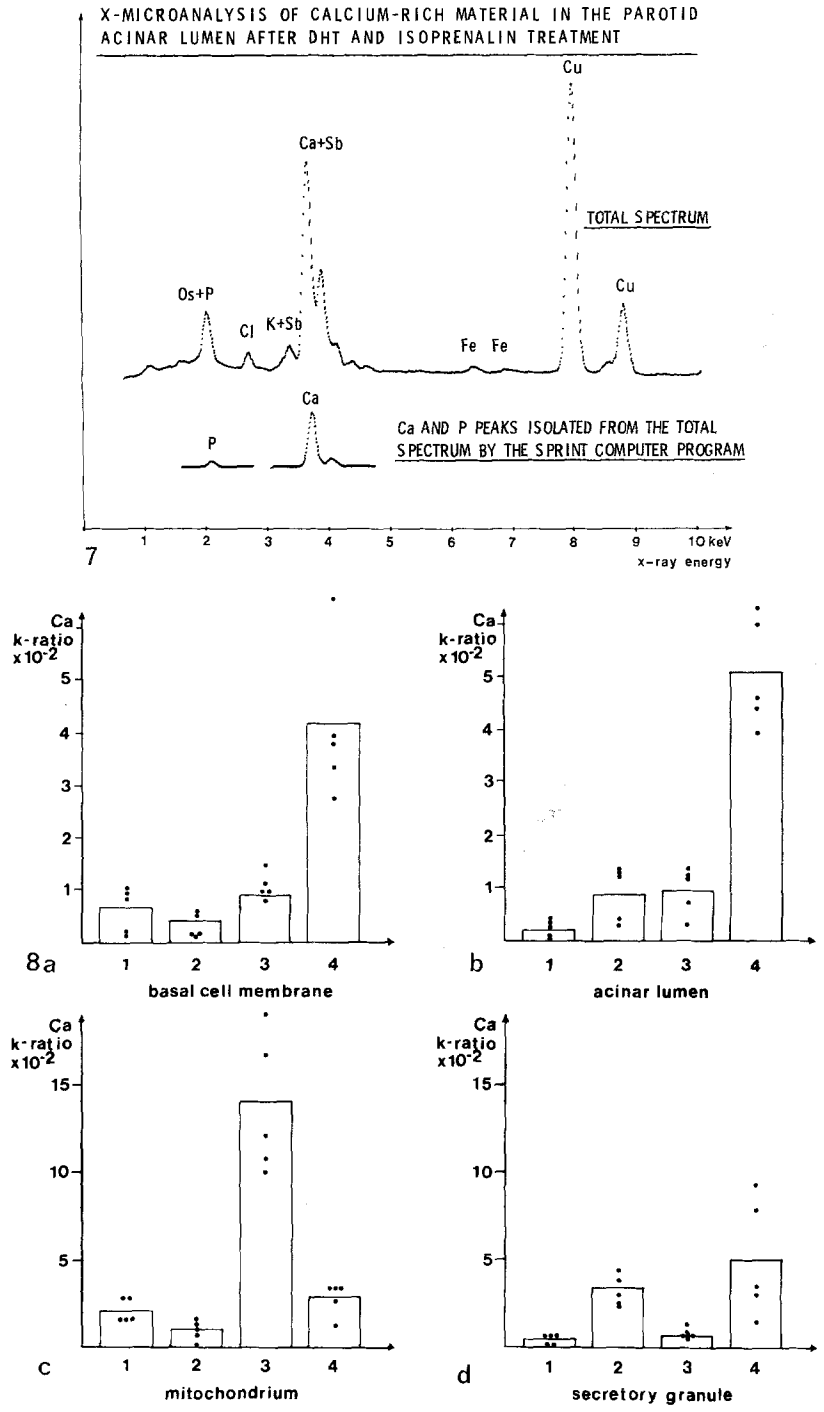

Fig. 7. Example of an X-ray microanalytical spectrum of an electron microscopic section of parotid tissue treated by the pyroantimonate reaction for the demonstration of calcium. The example spectrum was obtained in an intraluminar measuring field of a hypercalcaemic and stimulated gland. The calcium and phosphorus peaks at the bottom are calculated from the total spectrum by the SPRINT computer program

Fig. 8. X-ray microanalytical calcium content in different ultrastructural calcium depots in the parotid acini after cytochemical staining with the pyroantimonate reaction. The calcium content is expressed as the k-ratio (explanation see text). The bars represent the different experimental groups (see legend Fig. 2)

and the mitochondria (Fig. 3a). The acinar lumen showed a modest calcium content (Fig. 3c). By X-ray microanalysis, it was shown that the described black cytochemical reaction product really represents calcium. The most intense calcium specific X-ray emission in group 1 was found in the basolateral cell membrane and within the mitochondria (Fig. 3b).

In the stimulated group (group 2) conventional electron microscopy revealed secretory granules with a very loose internal structure (Fig. 4) and, compared to group 1, a significant augmentation and swelling of the endoplasmic reticulum and the Golgi apparatus indicating secretory activity. Ultracytochemically the pyroantimonate reaction showed an increased tingation of the secretory granules and acinar lumina. By X-ray microanalysis increased calcium depositions at these sites were found (Fig. 8b and d). The hypercalcaemic group (group 3) developed an increased pyroantimonate precipitation mainly in the mitochondria (Fig. 5b). The pyroantimonate staining of the acinar lumina was only indistinctly increased compared to the control group (Fig. 5a). X-ray microanalysis confirmed a distinct calcium accumulation within the mitochondria (Fig. 8c). Calcium content, in the acinar lumina and basolateral cell membranes was also augmented, but to a lesser extent (Fig. 8a and b). In the hypercalcaemic and stimulated group (group 4) an extreme pyroantimonate tingation of the basolateral cell membrane and the acinar lumen occured (Fig. 6a). The secretory granules contained several fine and delicate electron dense pyroantimonate deposits (Fig. 6b). The corresponding acinar lumina were densely filled with granular reaction product which in some parts impregnates homogeneous, concentrically lamellated round concrements resembling stone formations (Fig. 6c). In some areas homogeneous cloudy deposits could be observed.

X-ray microanalysis confirmed that these aggregates represent excessive calcium deposits. The presence of distinct phosphorus peaks beside the calcium peak in the coarse deposits indicates that these calcifications mainly consist of calcium phosphate. X-ray microanalysis also proved that basolateral cell membrane and acinar lumina were much more impregnated with calcium than in all other groups (Fig. 8a and b). The calcium content of the secretory granules was also augmented.

Discussion

The special role of calcium in the regulation of cellular secretory precesses is pointed out by many investigators [7–10, 12, 16–18, 21]. One of the early studies described calcium as a mediator of stimulus-secretion-coupling in example of the adrenal medulla [9]. In the mean time similar investigations have been elaborated for several excretory and endocrine glands [16–18]. Pathological calcifications of the salivary glands after pharmacological stimulation and hypercalcaemia have been described before using classical histological staining procedures [21]. In the mean time modern cytochemical and X-ray microanalytical methods for demonstration of cellular calcium have been developed [18]. So we extended the former experiments

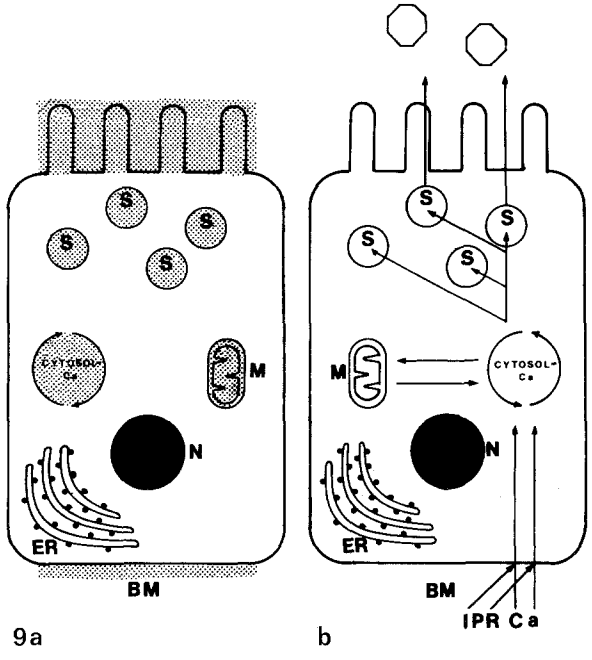

Fig. 9. a Schematic diagram of the cytochemically detectable cellular calcium depots (*dotted areas*) in the parotid acinar cell. **b** Schematic diagram of the proposed cellular calcium fluxes during stimulation and hypercalcaemia. *BM* = basement membrane, *ER* = endoplasmic reticulum, *M* = mitochondrium, *N* = nucleus, *S* = secretory granule

in order to show the intracellular calcium distribution and redistribution between the different cell compartments after secretory stimulation and hypercalcaemia. In addition to subjective semiquantitative evaluation of the cytochemical reaction product, X-ray microanalysis enabled us to elaborate objective quantitative data about the calcium redistribution. The results showed that the combination of secretory stimulation and hypercalcaemia induces an intracellular shift of calcium. This was shown much less clearly by chemical methods than by the calcium specific X-ray microanalysis in combination with the pyroantimonate method [18].

Confirming the findings of Simson [24, 25] we found that the cytochemically detectable calcium in non-stimulated acinar cells is mainly located at the basolateral cell membrane and within the mitochondria. The calcium content of mitochondria increases with hypercalcaemia and decreases with secretory stimulation. Mitochondria may, therefore, – as already shown for other types of cells [16–18, 25] – be regarded as intracellular buffer organelles. The cellular calcium depots in the non-stimulated acinar cells are shown schematically (Fig. 9a). Sympathetic stimulation induces a dyschylia resembling a sialadenosis caused by stimulation of beta-sympathetic receptors in the parenchyma of the gland [4–6, 13]. This stimulation may be neural or pharmacological, i.e. by Aludrin® [19, 10].

The findings mentioned above indicate that sympathetic stimulation has the following effects on calcium homeostasis of the acinar cell:

1) Increased calcium loading of the secretory granules.
2) Increased release of secretory granules from the acinar cell.
3) Reduced calcium loading of the intracellular calcium buffer organelles.

Presumably, sympathetic stimulation generates an increased calcium influx into the acinar cells. The calcium-enriched secretory granules, however, are released to a higher extend resulting in a calcium depletion of the typical cellular calcium storage organelles like the mitochondria. Similar calcium redistribution under the influence of secretory stimulation had been found by Schäfer and Klöppel [16, 18] in the stimulated pancreatic B-cells.

The experiments of Seifert [12, 19, 20] and Selye [21] had shown that sympathetic secretory stimulation of the salivary glands and simultaneous hypercalcaemia induce calcification of the glands. The present study describes the intra- and pericellular calcium redistribution related to this phenomenon. The increased amount of affluent calcium during hypercalcaemia may pass through the excited cell membrane of the acinar cells after isoprenalin to a greater extent than in the unstimulated cells (Fig. 9b). Subsequently, calcium enters the secretory granules in a greater quantity and is secreted at particularly high levels into the acinar lumina. Hypercalcaemia not only elevates the calcium concentration in the acinar lumen but also elevates cellular secretion rate [11]. At present, the importance of this finding in relation to the formation of salivary stones is not clear. In the presented experimental model the vitamin D derivant dihydrotachysterol (DHT) presumably acts via its hypercalcaemic effect on general calcium homeostasis. Besides that, it may be important that recent data in the literature describe the presence of vitamin D receptors in the parotid gland [15]. Whether these receptors are mediating a direct effect of applicated DHT on the secretory process, remains to be elucidated.

Sialolithiasis may be caused by a siladenitis. In the present experiment no signs of sialadenitis as a cause of the parotid calcifications and the observed dyschylia were found. The described necrosis of individual acinar cells with inflammatory reactions can be attributed to the Aludrin® effect [23].

After secretory stimulation and hypercalcaemia calcium-rich material within the acinar lumina transforms into calcified aggregates. Under the influence of decreased pH of the secretory material a smaller fraction of the intraluminal calcium is bound to proteins. Within the range of 0–10 mmol/l increasing calcium concentrations induce a proportional increase of the calcium fraction bound to mucoids. Increment of the calcium excretion exceeding this range does not induce an augmented mucoid binding of calcium [26]. This means that, while calcium concentrations are exceeding 10 mmol/l, an increasing amount of calcium ions is available for precipitation to calcium phosphate resp. calcium carbonate. Analysis of salivary stones of patients revealed the predominance of calcium phosphates [22]. In agreement with that, our present X-ray microanalytical data of the calcified deposits in the acinar lumina showed distinct phosphorus peaks besides the calcium peaks. These calcifications are originating as a consequence of *calcium supersaturation* of the saliva or in relationship to crystal nucleation by cellular debris. Chemical analysis of the salivary stones revealed the presence of calcium-acid phospholipid-phosphate complexes with

large amounts of sphingolipids, which are present in plasma membranes of acinar cells [2].

The pathogenesis of sialolithiasis is variable. Important factors may be inflammation or dyschylia combined with disturbances of electrolyte secretion. The analysis of salivary stones, former studies and the present investigation are pointing at the special role of calcium in this phenomenon. The combination of secretory stimulation and disturbances of the general calcium homeostasis induces a particularly augmented calcium excretion of the parotid acinar cells. This calcium is aggregated in the acinar lumen. The described alterations of the cellular calcium distribution and their consequences may be factors in the pathogenesis of the clinically important process of sialolithiasis.

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