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Immunoelectron microscopy of amylase in the human parotid gland

Ultrastructural localization by use of both the protein A-gold and the biotin-avidin-gold technique*

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Summary. Specimens of the human parotid gland were studied by immuno-electron microscopy for the presence of amylase. Both the protein A-gold technique and the biotin-avidin-gold technique were used on the same specimens. Different fixations were tried. Amylase was detected in the zymogen granules in high amounts. This enzyme could even be seen in glutaraldehyde fixed and routinely embedded material. The subcellular localization of this enzyme opens a new field of functional morphological studies and studies in special tumours including acinic cell carcinomas.

Key words: Immunoelectron microscopy – Protein A-gold-technique – Biotin-avidin system – Amylase – Parotid gland

The first specific detection of amylase in the parotid gland was obtained histochemically by the starch substrate film method (Smith and Frommer 1972). By immunofluorescence, amylase was demonstrated in acinar cells of the human parotid gland (Kraus and Mestecky 1971). The staining of the acinar cells was confirmed by Korsud and Brandtzaeg (1982) who showed that intercalated duct epithelium, in contrast, did not contain this enzyme. Caselitz et al. (1983) using the immunoperoxidase technique observed amylase in acinic cells of the parotid gland and in acinic carcinomas.

The light microscopical distribution of amylase displayed a granular appearance so that it seemed worthwhile to undertake a study in man at the subcellular level. In keeping with the RER-Golgy-zymogen granule secretory pathway of the cells synthesizing proteins (Palade 1975) immunocy-

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tochemical techniques localized amylase in the subcellular compartments of the rat pancreatic acinar cell (Roth et al. 1978; Bendayan et al. 1980). Tanaka et al. (1981) detected immunoreactive amylase in the acinar secretory granules of rat parotid gland.

The colloidal gold staining methods (introduced by Fauck and Taylor 1971) are highly efficient immunocytochemical techniques for the localization of antigens at the electron microscope level (De Mey 1983). Because of their high electron density and small size gold particles offer easy detection (increased resolution) and clear identification of the ultrastructure.

Protein A, produced by most strains of Staphylococcus aureus binds to the Fc portion of IgG molecules from several mammalian species and can be labelled with colloidal gold (Roth 1982).

The avidin-biotin complex which possess a high affinity and stability provides a sensitive method to localize antigens in tissues (Heitzmann and Richards 1974; Bayer et al. 1979; Hsu et al. 1981). Egg white glycoprotein avidin has an extraordinary affinity for the vitamin biotin. The EABA (endogenous avidin-binding activity) suppression technique is thought to saturate the unoccupied binding sites of avidin with monovalent free biotin in tissues rich in biotin-containing enzymes (Wood and Warnke 1981). Colloidal gold can be conjugated to avidin.

To our knowledge, no immunoelectron microscopy of amylase in human salivary glands has been undertaken, there is in particular no study using both the protein A-gold and the biotin-avidin-gold technique. We tried to localize α-amylase in acinar cells of human parotid glands at the ultrastructural level applying both methods.

Material and methods

Reagents. Antiserum to alpha-amylase was raised in rabbits using purified pancreatic α-amylase (Dr. Grenner, Marburg). It showed cross-reactivity of salivary amylase as detected by the Ouchterlony-test. Protein A-gold with a particle diameter of 20–25 nm was purchased from E-Y Laboratories (San Mateo, USA) biotin-labelled goat anti-rabbit IgG from TAGO (Burlingame, USA) and avidin-gold with a particle diameter of 20–25 nm from E-Y Laboratories (San Mateo, USA).

Preparation of tissue. Small pieces of parotid glands were fixed in different solutions and at different concentrations: 1%, 2% and 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) and 2% paraformaldehyde in PBS buffer (pH 7.4). In most cases, primary fixation with glutaraldehyde was followed by incubation in 2% osmium tetroxide for 120 min. Specimens were washed in buffer and embedded in Epon 812 after dehydration. Sections (450–1,300 Å thick) were cut with Reichert-Jung OmU4-ULTRACUT and mounted on 300-mesh nickel grids.

Cytochemical labelling. All reactions were done at room temperature. The antisera were diluted with PBS containing 1% bovine serum albumin (PBS/BSA) pH 7,4. Some grids were floated, sections down, on a large droplet of 10% H_2O_2 for 10 min (etching). Thorough washing of the grids in PBS, was followed by incubation in normal goat serum (NGS) 1:30 for 30 min.

The sections on the grid were then transferred into large droplets of the anti-amylase antiserum (dilution of 1:20 or 1:50) for 2 h. They were "jet washed" by a mild spray of PBS and PBS/BSA.

In the avidin-biotin technique biotin-labelled goat anti-rabbit IgG diluted to 1:10 were applied to grids for 1 h and "jet washed" with PBS and PBS/BSA.

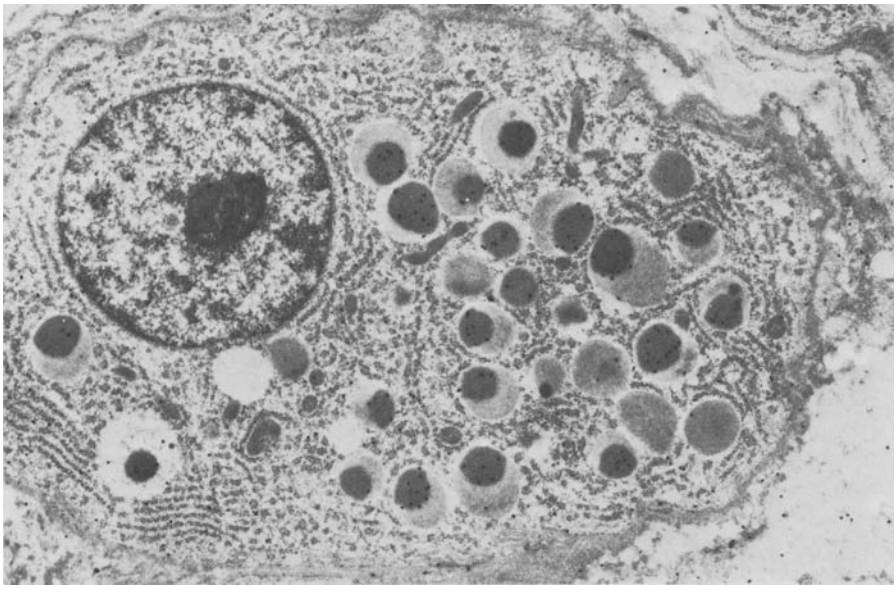

Fig. 1. Parotid gland. Gold particles in the zymogen granules, indicating the presence of amylase. Protein A-gold technique. Fixation 3% glutaraldehyde. Concentration of the primary antibody 1:20. × 6,800

The grids were incubated in the gold-labelled solutions for 1 h. Protein A-Gold and Avidin-Gold were diluted to 1:5 or 1:10. This incubation was followed by thorough washing in large volumes of PBS/BSA, PBS and finally distilled water.

Sections were then stained with 3% aqueous uranyl acetate (30–45 min) and lead citrate (2 min). They were viewed with a Zeiss electron microscope 9S2.

Control preparations included replacement of the primary antiserum by normal rabbit serum and PBS.

Results

The different approaches to immunoelectron microscopical procedures (e.g. etching) are dealt with in the discussion. We obtained the best results under the following conditions: Glutaraldehyde fixation preserved the ultrastructure and enough antigenicity of the amylase (Figs. 1–3 and Figs. 5, 6). Paraformaldehyde showed a poorer ultrastructural preservation, but the antigenicity was not better (Fig. 4). Although most of the sections were postfixed with osmium tetroxide, there was still enough antigenicity left to detect amylase in the zymogen granules (Figs. 1–3 and Fig. 6).

The granule matrix, appearing to have withdrawn from the margin, possessed a uniformly dense and compact core (Figs. 1–3 and Fig. 6). Some secretory granules were rather pleomorphic (Fig. 6). Without osmium postfixation the matrix was more electron lucent and inhomogeneous (Fig. 5).

The best results were obtained in sections of 600 to 800 Å thickness. The figures shown were obtained from sections which were not etched.

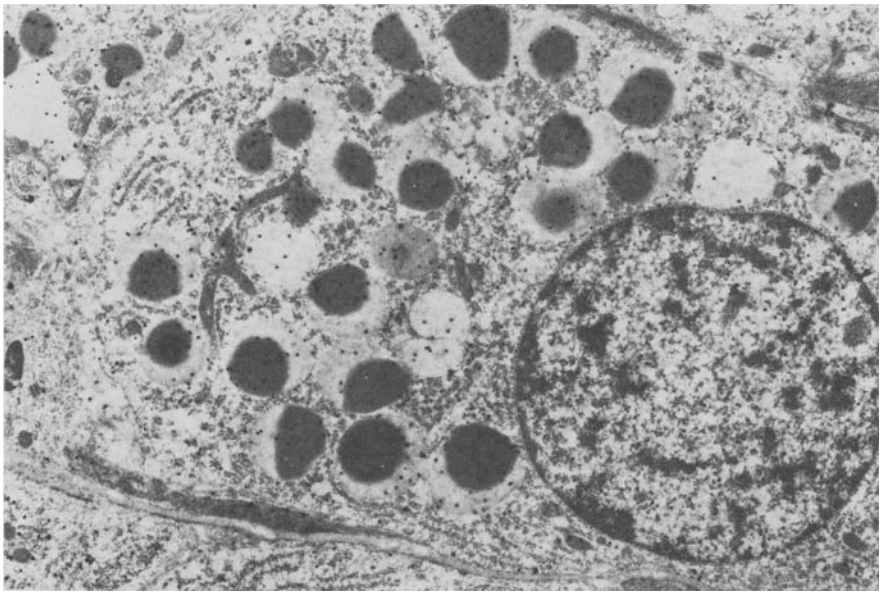

Fig. 2. Parotid gland. Gold particles in the zymogen granules, indicating the presence of amylase. Compare to Fig. 1 (Protein A-gold). Biotin-avidin-gold technique. Fixation 3% glutaraldehyde. Concentration of the primary antibody 1:25. × 7,700

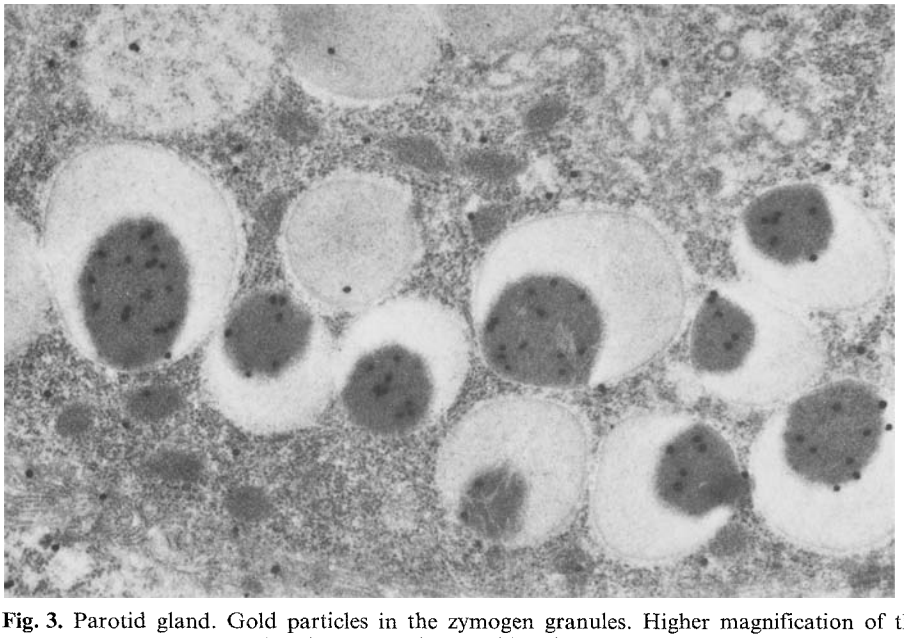

Fig. 3. Parotid gland. Gold particles in the zymogen granules. Higher magnification of the same specimen as shown in Fig. 1. Protein A-gold technique. Fixation 3% glutaraldehyde. × 17,000

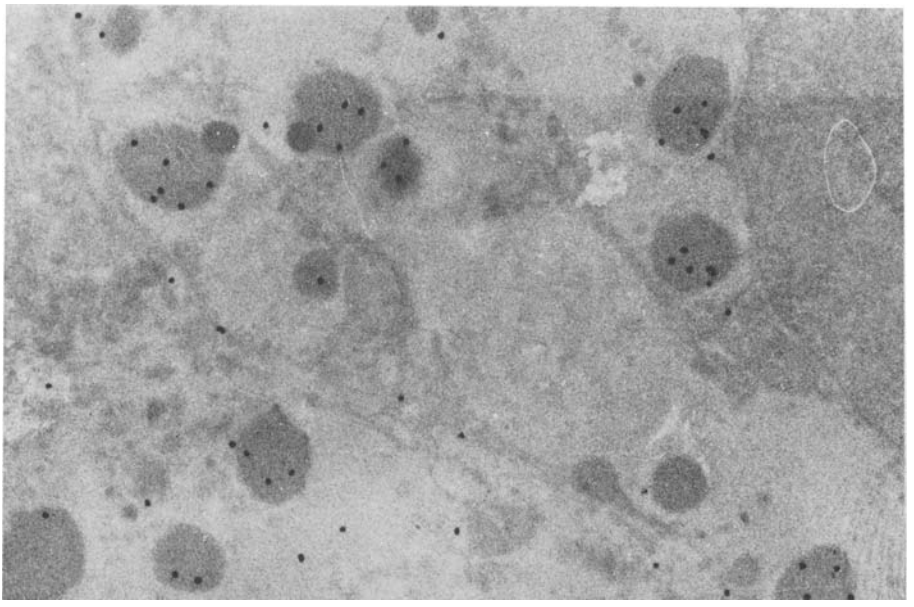

Fig. 4. Parotid gland. Gold particles in the zymogen granules. Poor preservation of the cellular organelles. Protein A-gold technique. Fixation 2% paraformaldehyde. Concentration of the primary antibody 1:25. ×13,800

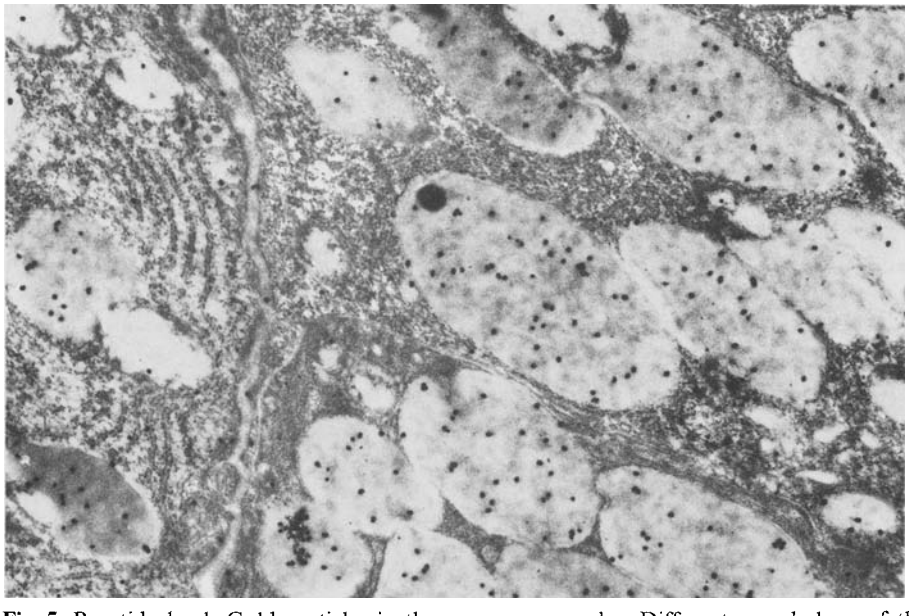

Fig. 5. Parotid gland. Gold particles in the zymogen granules. Different morphology of the granules because of no osmium fixation. Biotin-avidin-gold technique. Fixation 3% glutaraldehyde. Concentration of the primary antibody 1:25. ×14,000

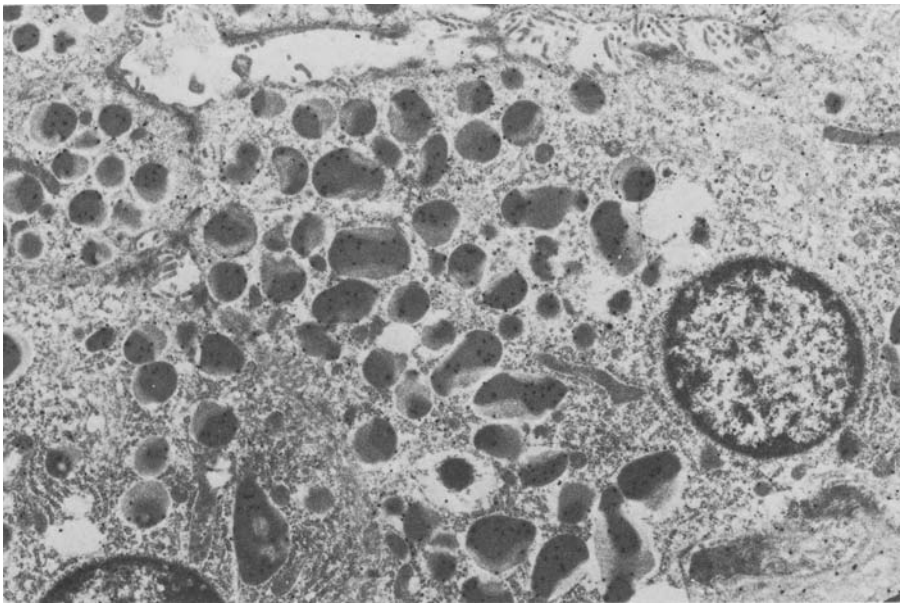

Fig. 6. Parotid gland. Gold particles in the zymogen granules. Pleomorphic aspect of the zymogen granules. Preparation as in Fig. 1, same specimen. × 7,400

Gold particles were concentrated in the zymogen granules over the dense core. There was an unstained halo around the positive core (Figs. 1–3). The acinar lumen, the rough endoplasmatic reticulum and the remaining cytoplasm displayed a less intense labelling. The granules of sections without osmium postfixation contained more gold particles (Fig. 5). Background staining over mitochondria, nucleus and connective tissue was low. The control experiments showed very few gold particles over the different cellular organelles.

Discussion

Immunoelectron microscopy has to resolve several problems; those of ultrastructural integrity, diffusion of labelled antibody throughout the tissues and preservation of antigenicity. For the detection of amylase in the post-embedding procedure we used, it was therefore necessary to find the best kind of fixative. We used material that was fixed in glutaraldehyde at different concentrations or in paraformaldehyde. The tissue was then embedded in Epon 812 with or without treatment of osmium tetroxide.

The different fixations showed a variable morphology of the granules. After the routine procedure involving fixation with glutaraldehyde followed by osmium tetroxide, the secretory granules are in general uniformly dense and compact, occasionally heterogeneity in granule matrix density shows

a bizonal or flecked configuration and the granule matrix sometimes appears withdrawn from the rim of the granule (Robinovitch et al. 1966; Simson et al. 1974). The decreased matrix density (loss of osmiophilia) following isoproterenol (Seifert 1962) suggest the loss of an osmiophilic, carbohydrate-containing lipid (Simson et al. 1974). Because it can cause dissociation of cellular macromolecules, osmium postfixation should be avoided when exploring the cytochemistry of cellular proteins and glycoproteins (Simson et al. 1978; Roth 1983). Prolonged primary fixation by osmium tetroxide causes rapid release of the soluble proteins from zymogen granules because osmium tetroxide apparently reacts with the granule membrane to increase its permeability (Amsterdam and Schramm 1966).

Paraformaldehyde indirect contrast to glutaraldehyde gives good immunoreactivity and poorly preserved ultrastructure. Elevating the pH at which paraformaldehyde fixation is carried out, improves ultrastructural preservation and reduces penetration (Eldred et al. 1983). In a post-embedding staining technique much of the antigenicity of intracellular proteins is destroyed as a result of dehydration and embedding with epoxy-based plastic resins (Roth 1982). Proper handling of the sections is necessary, i.e. dry sections and a dirty surface on the staining and washing solutions are to be avoided (Mollenhauer 1974). The effects of etching procedures with hydrogen peroxide are critically discussed by De Mey (1983). Etching agents should not remove the plastic; they enhance, however, the intensity of immunocytochemical staining and reduce membrane contrast (extraction of osmium) (Baskin et al. 1979). Large gold particles do not penetrate tissue sections and label solely the antigenic sites on the surface of the tissue sections (Bendayan 1982).

From the results at the light microscopical level we could localize amylase in the zymogen granules of the acinar cells of human parotid gland by the protein A-gold and avidin-gold technique. This observation correlates well with findings in animal glands. Interestingly, amylase was detected in sections which were fixed in glutaraldehyde postfixed in osmium tetroxide and which were not etched. Obviously, amylase was stable enough for this treatment. Parallel treatment with paraformaldehyde without osmium fixation showed similar results. The ultrastructural preservation was poorer than in the material fixed in glutaraldehyde. When we compared the protein A-gold and the biotin-avidin-gold technique we observed similar results concerning the staining intensity.

Intracellular localization of amylase was detected by a combination of electron microscopy, radioautography and cell fractionation procedures. Palade (1975) analysed the secretory process of protein synthesis in the pancreatic exocrine cell on a subcellular level. This accepted model of a RER-Golgi-zymogen granule secretory pathway (cisternal packaging-exocytosis theory) may be adopted for the parotid gland (Seifert 1962). The "equilibrium" theory proposes a steady-state equilibrium between the enzyme contents of the cytoplasm and the other compartments (transport by the movement of molecules through membranes) (Rothman 1975).

Immunocytochemical procedures allow us to localize antigens on a mo-

lecular level. By using the protein A-gold technique for detection of amylase in the rat pancreas (Roth et al. 1978; Bendayan et al. 1980) and in the rat parotid gland (Tanaka et al. 1981) numerous gold particles were present over the zymogen granules, the Golgi region, the acinar lumen and less intensively in the remaining cytoplasm. Bendayan (1982) demonstrated the co-localisation of carboxypeptidase B or chymotrypsinogen and amylase in the same secretory granules by double immunocytochemical labelling. Tanaka et al. (1981) used an antiserum against purified rat parotid amylase in concentrations of 1:1,500 to 1:2,500 and detected gold particles in the rough endoplasmatic reticulum and the Golgi complex only with high concentrations of antiserum.

Our study, which was done on human material, showed that amylase was primarily localized in the secretory granules and to a lesser extent in the endoplasmatic reticulum. The antiserum to pancreatic amylase seems to be characterized by a relatively low affinity to parotid amylase because high concentrations (1:25 to 1:50) proved useful.

The zymogen granules are a special feature of some secretory cells, and there have been debates about their composition. Classic zymogen granules are membrane-limited and measured 0.5–1.5 μm in diameter. Because of the granule heterogeneity (for example following fixation or isoproterenol) it is some-times difficult to distinguish serous granules from mucin garanules solely by fine structural examination (Erlandson 1981). Simson et al. (1974) provided evidence that the acinar secretory granules of the rat salivary gland could contain proteins, carboxhydrates and lipoidal material. In the submandibular gland Nieuw Amerongen et al. (1982) suggest the presence of seromucous acinar granules. By immunoelectron microscopy further cytochemical differentiation is possible: Tanaka et al. (1981) showed some secretory granules of the rat parotid gland with an amylase-rich shell and an amylase-poor core, and renin was localized in the submandibular glands of SWR/J mice by Tanaka et al. (1980). A glycoprotein, designated GP-2, is considered a membrane-associated secretory protein of the rat pancreas (Geuze et al. 1981). Kraehenbuhl et al. (1977) conclude that all bovine pancreatic exocrine cells, and all zymogen granules and Golgi cisternae in each cell, are qualitative alike with regard to their complement of the five secretory proteins (trypsinogen, chymotrypsinogen A, carboxypeptidase A, RNase and DNase).

It will be interesting to study acinic cell carcinomas because two basic cell types are present in this tumour (Hirtzler et al. 1969; Erlandson 1972; Ghadially 1980): serous-like cells containing zymogen granules and ductular cells resembling the pluripotential intercalated duct cells. Immunoelectron microscopy could supply further evidence to the histogenetic concept of origin from the reserve cell of the intercalated duct (Batsakis et al. 1977). Our observations showed that immunoelectron microscopy is a valuable tool to follow ultrastructural changes in the cell which cannot be analysed by ultrastructure alone. This opens a new field of functional analysis in pathological conditions (e.g. stimulation, suppression, neoplastic transformation).

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