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Early events of secretory granule formation in the rat parotid acinar cell under the influence of isoproterenol. An ultrastructural and lectin cytochemical study

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SUMMARY

The events involved in the maturation process of acinar secretory granules of rat parotid gland were investigated ultrastructurally and cytochemically by using a battery of four lectins [*Triticum vulgaris* agglutinin (WGA), *Ulex europaeus* agglutinin I (UEA-I), G*lycine max* agglutinin (SBA), *Arachys hypogaea* agglutinin (PNA)]. In order to facilitate the study, parotid glands were chronically stimulated with isoproterenol to induce secretion. Specimens were embedded in the Lowicryl K4M resin.

The *trans*-Golgi network (TGN) derived secretory granules, which we refer to as immature secretory granules, were found to be intermediate structures in the biogenesis process of the secretory granules in the rat parotid acinar cell. These early structures do not seem to be the immediate precursor of the mature secretory granules: in fact, a subsequent interaction process between these early immature granule forms and TGN elements seems to occur, leading, finally, to the mature granules. These findings could explain the origin of the polymorphic subpopulations of the secretory granules in the normal acinar cells of the rat parotid gland.

The lectin staining patterns were characteristic of each lectin. Immature and mature secretory granules were labelled with WGA, SBA, PNA, and lightly with UEA-I. *Cis* and intermediate cisternae of the Golgi apparatus were labelled with WGA, and *trans* cisternae with WGA and SBA.

INTRODUCTION

It is well known that the processing and transport of exportable proteins in parotid acinar cells, as in many other cell types, follow the conventional pathway, from rough endoplasmic reticulum to secretory granules, through the Golgi apparatus (Castle *et al.*, 1972; Hand, 1971).

Through specific mechanisms, secretory proteins destined to regulate secretion are selected from the mixed content of the *trans*-Golgi network (TGN) (Griffiths and Simons, 1986; Ikonen and Simons, 1998; Orci *et al.*, 1987), and conveyed to immature secretory granules where their concentration and packaging take place (Jamieson and Palade, 1967). Subsequently, these forming granules are converted to mature granules, specialized intracellular organelles that act as a storage pool for secretory proteins (Arvan and Castle, 1998). The formation of the secretory granule is a highly specialized and reg-

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ulated event, and, from its early phases, is energy, temperature and cytosol dependent, involving small GTP-binding proteins, heterotrimeric G proteins, inositol phospholipid metabolism, protein phosphorylation (Austin and Shields, 1996; Cockcroft, 1999; Ling *et al.*, 1998; Oashi and Huttner, 1994; Wieland and Harter, 1999).

Several studies have reported the existence of more than one population of mature secretory granules in parotid acinar cells in terms of their different matrix electron density configuration (Hand, 1972; Pinkstaff, 1980, 1993; Simson *et al.*, 1974; Tandler and Phillips, 1993).

Such a polymorphic picture is generally accepted to be a direct reflection of the segregation of many of the major constituents of granules in specific areas of their matrix, as demonstrated by several immunocytochemical studies in mammalian salivary glands which showed a characteristic heterogeneous compartmentalization within the granule for different molecules, such as α-amylase, agglutinin and proline-rich proteins (Mansouri *et al.*, 1992; Matsuura and Hand, 1991; Marchetti *et al.*, 2000; Staneva-Dobrovski, 1997; Takano *et al.*, 1991, 1993; Vugman and Hand, 1995).

Chronic treatment with the β-adrenergic agonist isoproterenol induces rapid cell proliferation in the salivary gland, causing an extensive exocytosis of secretory granules (Barka, 1965; Schneyer, 1962); and isoproterenol inducible secretory proteins increases after 8-10 days of treatment (Matsuura and Hand, 1991; Mehansho and Carlson, 1983). As isoproterenol acts as an inducer of secretion in the salivary gland, it facilitates the study of the different morphological steps of secretory granule formation.

It may be presumed that the normal sorting machinery of secretory proteins by TGN is not disturbed by isoproterenol treatment, since this drug does not alter the stacked structure of this organelle. It is know that the preservation of the stacked structure of the Golgi apparatus is an essential prerequisite for the normal sorting machinery function (Yamashima and Tamaki, 1996).

Since secretory granules of salivary glands contain a large number of glycoproteins (Bennick, 1982; Carlson, 1988; D'Amico *et al.*, 1999; Miao *et al.*, 1995; Wallach *et al.*, 1975), we have employed a series of different lectins in order to gain further insights into the secretory granule maturation process.

In the present study we examined the maturation process of the secretory granules in parotid acinar cell of isoproterenol-treated rats. The TGN area and the immature secretory granules were particularly examined both ultrastructurally and cytochemically.

MATERIALS AND METHODS

Reagents

d-l-Isoproterenol, lectin-gold complexes (WGA, UEA-I, SBA, PNA, 10 nm gold), inhibitory carbohydrates (N-acetylglucosamine, neuraminic acid, α-L-fucose, N-acetylgalactosamine, galactose), bovine serum albumin (BSA), and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Glycine was obtained from Merck (Darmstadt, Germany) and Lowicryl K4M resin from Polyscience (Warrington, PA, USA).

Treatment of animals

Male Wistar rats, weighing 150-200 g were used. The animals, divided in two groups (six animals per group), received twice daily intraperitoneal injections for ten days, respectively with: a) saline, and b) isoproterenol 25 mg/Kg body weight. Twenty-four hours following the last isoproterenol injection, and after the same period of fasting, parotid glands were removed under anesthesia with pentobarbital (approximately 1 ml of 50 mg/ml of anesthetic).

Tissue preparation

The parotid glands were fixed by immersion in 1% glutaraldheyde and 4% para- formaldehyde in 0.1M phosphate buffered saline (PBS), pH 7.4, for two h at 4°C. After rinsing with PBS and incubation in 0.15 M glycine in PBS for 1 hr at room temperature to block free aldehyde groups, samples were embedded in Lowicryl K4M resin by stepwise lowering of the temperature down to -35°C during ethanol dehydration. The resin was polymerised under ultraviolet light for 24 hrs at –35°C, and then continued for 48 hrs at room temperature. Ultrathin sections were cut with a diamond knife and mounted on nickel grids coated with formvar.

Cytochemical staining

Labelling with lectin-gold complexes was carried out as a direct technique. Each step was performed in a moist chamber at room temperature

by floating the grids on drops of the different reagents. After treatment with 0.5% BSA in PBS for 10 min, the grids were incubated for 1 h at room temperature with the lectin-gold complexes (diluted 1:10 with buffer containing 0.1M PBS plus 0.5% BSA and 0.05 %Tween 20). After washing with PBS and distilled water, the grids were counterstained with 2% aqueous uranyl acetate (5 min) and Reynolds lead citrate (5 min) and, then analysed using a Hitachi H-600 transmission electron microscope.

The specificity of lectin binding sites was controlled by performing the incubation of each lectin with the corresponding inhibitory sugar (0.1 M) before application to the sections for 1 h at room temperature. Such control stainings essentially eliminated the lectin binding.

RESULTS

Fig. 1 shows two vicinal acinar cells of untreated rat parotid gland. Each cell contains a distinct morphological subpopulation of secretory granules. In general, we observed two main morphological types of mature granule matrix: one type with patches of dense material producing a flecked configuration, and the other type with dense rims, with or without dense cores, producing the so-called target granules.

The treatment with isoproterenol did not induce any observable changes in the ultrastructure of acinar cells, except for the Golgi apparatus and secretory granules. The Golgi apparatus, althought it preserved a well-defined cis-trans pattern, showed "hypertrophy". In treated cells, the secretory granules appeared larger, lighter and uniform in their electron density in comparison with the normal ones; the immature granules were found to be different from the mature ones because of their smaller size and lower electron density. Immature secretory granules seemed to originate as dilations of saccules at TGN extremities (Fig. 2a). Most of the granules were observed to be in close apposition to protrusions from TGN, which budded and penetrated into their matrix (Fig. 2b); subsequentely, such protrusions were completely engulfed by the forming granules (Fig. 2c).

The reaction to the lectin WGA, used to detect N-acetylglucosamine and N-acetylneuraminic acid

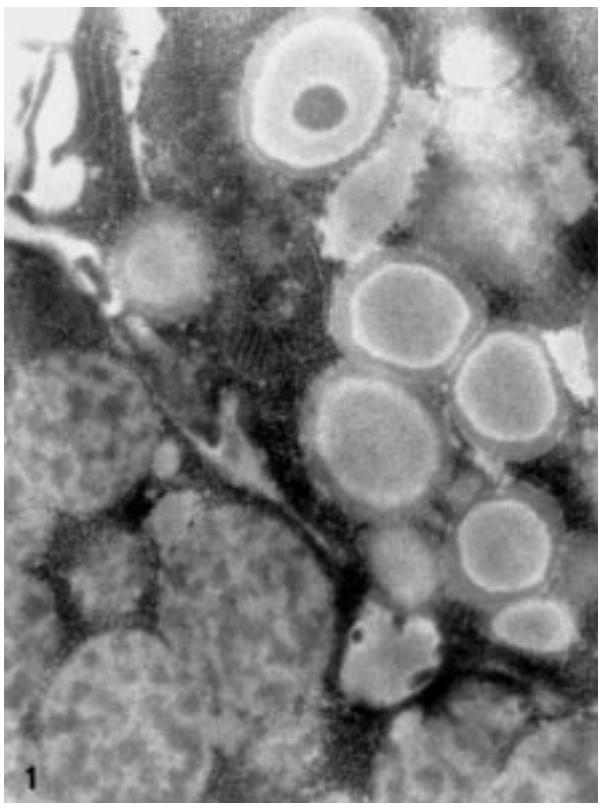


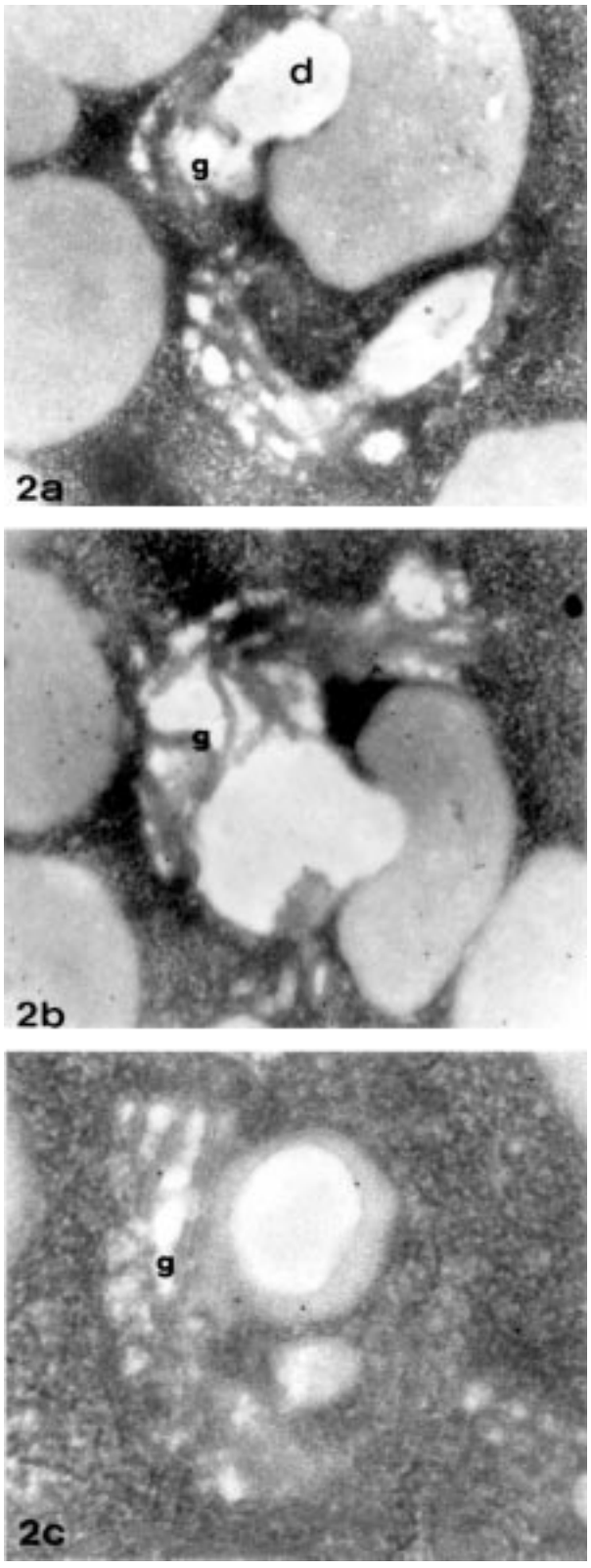
Fig. 1 - Acinar cells of normal rat parotid gland. Two morphological subpopulations of mature secretory granules are seen (x 24,500).

residues, was observed in immature granules. The reaction was localized mostly over the capping electrondense outer zone of granules. Several gold particles were found over each compartment of the Golgi stack (Fig. 3a).

UEA-I, with its binding specificity for fucose, stained the maturing granules, mainly in their outer zone near the trans Golgi area. The Golgi stack showed no reaction to this lectin (Fig. 3b).

The SBA lectin-gold complexes, N-acetylgalactosamine probes, were found in the forming granules as well as mostly in trans cisternae of the Golgi stack (Fig. 3c).

Very weak staining for PNA, a lectin used to detect galactose-N-acetylgalactosamine dimers and galactose residues, was occasionally observed in the Golgi apparatus. Most Golgi apparatuses exhibited negative staining for this lectin. A discrete labelling was found in immature and mature secretory granules (Fig. 3d).



DISCUSSION

The ultrastructural picture of the mature secretory granule matrix of salivary glands, observed in the present work and generally consistent with previous studies (Mansouri *et al.*, 1992; Pinkstaff, 1980; Vugman and Hand, 1995), shows a striking polymorphism, according to the disposition of dense and light material within the granule.

Our observations suggest that such polymorphic subpopulations may originate at the TGN level. Dilations in the ends of cisternae at the TGN would form the immature granules. A frequent observation is that dilations at distal portions of the TGN would be gradually enveloped, with a capping process, by small immature forms, whose origin has not been localized by our study. Subsequentely, a complete enveloping process occurs, leading, finally, to the detachment of these new granules. The above-described process could explain the origin, though only partially, of the morphological subpopulation of secretory granules showing a dense rim, the so-called "target" granules.

Our results are consistent with those of Beaudoin *et al.* (1983), who supposed, from their cytochemical data, that the immature secretory granules of pancreatic acinar cells might not be the immediate precursors of the mature forms: in fact, only a subsequent interaction of the early immature granules with the TGN would give rise to the mature granules.

Thus, it is conceivable that the formation of secretory granules might also involve a fusion of vesicles originating elsewhere in the TGN area. Interestingly, the present study further supports the prospect, scarcely documented so far (Arvan and Castle, 1998; Urbe *et al.*, 1998), that the fusion of the secretory granules themselves may contribute to the maturation of secretory granules. Our study would support the possibility that the heterogeneous organization of the secretory granule matrix is due to the physical and temporal segregation of the constituents in specific matrix compartments. Such a differential distribution of some molecules

Fig. 2a-c - Photographic reconstruction of the probable secretory granule maturation process. A dilation (**d**) at the distal end of TGN elements is engulfed by a darker immature secretory granule (Fig. 2**a**, **b**). The complete engulfing process is seen in Fig. 2**c**. (**g**, Golgi apparatus; **a**: x 27,000; **b**: x25,000; **c**: x50,000).

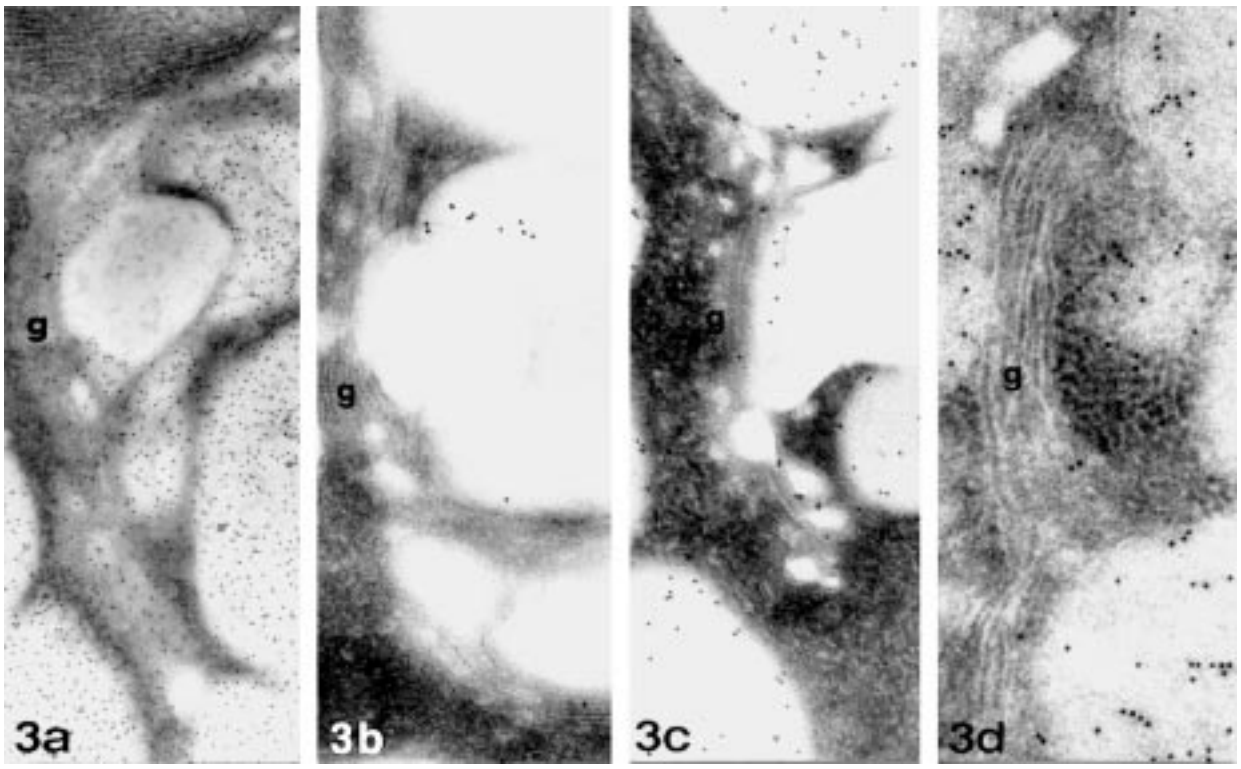


Fig. 3a-d - *WGA, UEA-I, SBA, and PNA stainings*. Golgi apparatuses exhibit different staining patterns according to the lectin used. WGA stains the outer zone of a forming granule in close apposition to the trans Golgi area, and each Golgi compartment shows labelling (Fig. 3**a**). UEA-I labels the immature granules, but no Golgi compartments (Fig. 3**b**), while the SBA reaction is mostly located in the trans cisternae as well as in the forming granule (Fig. 3**c**). PNA staining is observed over the granules,while the Golgi apparatus is not stained (Fig. 3**d**) (**g**, Golgi apparatus; **a**: x 28,000; **b**: x40,000; **c**: x 34,000; **d**: x 45,000).

within the granules may result from different and separate pathways which these molecules follow in entering the forming secretory granules. Several studies support the existence of more than one biosynthetic route in exocrine glands: Beaudoin *et al.* (1983) suggested that some pancreatic proteins may be directly transported from the rough endoplasmic reticulum to the forming secretory granules, thus bypassing the Golgi apparatus. In addition, Takano *et al.* (1991), have observed, in human parotid acinar cells, that amylase and agglutinin, two constituents of mature secretory granules, follow separate pathways between the Golgi apparatus and forming secretory granules. Takano *et al.* (1996) have demonstrated that some secretory proteins, such as peroxidase, are stored in the immature secretory granules even when the Golgi saccules are disrupted by monensin treatment, thus further confirming the possibility of some molecules bypassing the Golgi apparatus.

Our lectin cytochemical study would confirm the presence of different routes in the formation of secretory granules. In fact, although glycoprotein elaboration is considered to be located in the Golgi apparatus, PNA and UEA-I did not label the Golgi apparatus or the TGN protrusions, while the immature and mature secretory granules were stained with these lectins. A possible explanation is that the sugar determinants would be added after the forming granules and transport vesicles leave the Golgi apparatus. As stated previously by other authors (Jezernik and Pipan, 1986; Torrisi and Pinto da Silva, 1984), we can expect a participation in the granule maturation process by vesicles at the periphery of the Golgi apparatus. Such transport vesicles were not labelled in our study, probably owing to amounts of sugar residues in these vesicles too small to be detected by the technique employed.

Interestingly, the low electron density formations originating at the TGN and enveloped by forming

granules are not labelled by lectins. Probably, this process contributes in the formation of the granule for the non-glycosylated molecules, supporting our previous study (D'Amico *et al.*, 1999), in which lectins almost only marked the darker matrix portions of the normal acinar cell of rat parotid gland.

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