Histol Histopath (1994) 9: 649-656

Histology and Histopathology

ZIO impregnation and cytochemical localization of thiamine pyrophosphatase and acid phosphatase activities in small granule-containing (SGC) cells of rat superior cervical ganglia

Y-P. Chau[1] and K-S. Lu[2]

[1]Department of Anatomy, National Yang-Ming Medical College and

[2]Department of Anatomy, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

Summary. Cytochemical relationship between Golgi complex and dense-cored granules (DCGs) of small granule-containing (SGC) cells in rat superior cervical ganglia was examined in electron microscopy by zinc-iodide-osmium tetroxide (ZIO) method and by enzyme cytochemistry for thiamine pyrophosphatase (TPPase) and acid phosphatase (ACPase). After ZIO impregnation, all the saccules of Golgi apparatus and some of tubular rough endoplasmic reticulum (rER) were stained. DCGs in periphery of SGC cells were not stained, but varying degrees of dense deposits occurred in the DCGs in vicinity of Golgi trans-saccules. Both TPPase and ACPase activities were localized in one or two stacked layers of saccules on the *trans* side of the Golgi complex. No reaction products were demonstrated in the DCGs. From these results, we suggest that the DCGs of SGC cells in rat superior cervical ganglia are derived from the Golgi complex, and that lysosomal cleavage of protein contents in the DCGs may occur in the *trans* Golgi saccules.

Key words: Cervical ganglia, ZIO impregnation, Thiamine pyrophosphatase, Acid phosphatase, Cytochemistry, Small granule-containing cells

Introduction

Previous studies with formaldehyde-induced fluorescence histochemistry (Eränkö and Härkönen, 1965; Grillo et al., 1974), conventional ultracytochemistry including chromaffin (Lever et al., 1977; Lu and Chau, 1992), uranaffin (Richards and DaPrada, 1977, 1980; Lu and Chau, 1992), and argentaffin reactions (Lu and Chau, 1992) and immunohistochemistry (Verhofstad et al., 1981; Sakai et al., 1989; Karhula et al., 1990) revealed an abundant storage of catecholamines, nucleotides and proteins in membrane-bound dense-cored granules (DCGs) of small-granule-containing (SGC) cells in sympathetic ganglia as well as in those of adrenal medulla (Winkler, 1977) and carotid body (Alfes et al., 1977). Moreover, the Golgi complex contained various phosphatase activities and was actively involved in granule formation in secretory cells (Broadwell and Oliver, 1981; Goldfischer, 1982). A parallel event to protein-nucleotide-neurotransmitter complexes forming within dense-cored vesicles in the Golgi region of adrenochromaffin cells (Alfes et al., 1977; Kobayashi et al., 1978) or glomus cells in carotid bodies (Böck, 1980; Fujita et al., 1988) is expected to occur in the SGC cells of sympathetic ganglia. Although most of the recent studies have been focused on the chemical contents and the possible functional roles of SGC cells, Hsiao and Lu (1982) reported on cytoplasmic granule formation and the role of Golgi complex of SGC cells in rat coeliac-superior mesenteric ganglion complex with special reference to ZIO impregnation. In addition, the significance of DCVs has not been previously indicated and the phosphatase activities of SGC cells has never been studied, thus we employed zinc-iodide-osmiun tetroxide (ZIO) impregnation and enzyme cytochemistry for acid phosphatase (ACPase) and thiamine pyrophosphatase (TPPase) activities in rat superior cervical ganglia, which contain most numerous SGC cells in rat, to investigate the cytochemical relationships of Golgi complex and membrane bound DCVs in the SGC cells.

Offprint requests to: Dr. Yat-Pang Chau, Ph.D., Department of Anatomy, National Yang-Ming Medical College, 155, 2nd Sec., Li-Nung Street, Shih-Pai, Taipei, 11221, Taiwan, Republic of China

Materials and methods

Adult Long-Evans strain rats weighing 200-300 g of both sexes were used in the present study. Under anaesthesia (sodium pentobarbital, 50 mg/kg i.p.), the animals were sacrificed by cardiovascular perfusion

through the left ventricle of the heart with a fixative containing 2% paraformaldehyde, 2% glutaraldehyde in 0.067M cacodylate buffer (pH 7.4). At completion of perfusion, superior cervical ganglia were removed and fixed in the same perfusate overnight (4 °C). After several rinses in buffer, the ganglia were embedded in 7% agar and cut into 5 µm-thick sections with an Oxford vibratome. Areas containing SIF cells in 50 µm sections were identified by fluorescence microscopy, dissected out under a stereomicroscope and rinsed several times in cacodylate buffer for further processing.

For *conventional electron microscopy*, the trimmed 50 µm-thick sections containing SIF cells were post-fixed in 1% osmium tetroxide, dehydrated in alcohol and embedded in Epon-Araldite mixture.

For *ZIO impregnation study*, the trimmed 50 µm-thick sections were immersed in a ZIO mixture for 18 h at 4 °C, dehydrated in alcohol and embedded in Epon-Araldite mixture. The ZIO mixture was prepared as follows (Akert and Sandri, 1968): solution A was 2% aqueous OsO_4, solution B consisted of 15 g of zinc powder to which 5 g of iodine crystal and 200 ml of double-distilled water were added, and 6-8 ml of filtered solution B was added to 2 ml solution A shortly before use.

For localization of *TPPase activity*, the trimmed sections were incubated for 2 h at 37 °C in a medium containing 25 mg sodium thiamine pyrophosphate as substrate, 7 ml double-distilled water, 5 ml 0.025M $MnCl_2$, 3 ml 1% lead citrate, 1.25 g sucrose and 10 ml 0.2M Tris-maleate buffer (pH 7.2). For demonstration of *ACPase activity*, sections were incubated for 1 h at 37 °C in a medium containing 12.5 mg sodium-ß-glycerophosphate as substrate, 2.5 ml 0.2M acetate buffer (pH 5.0), 2 ml 0.2% lead nitrate, 1 ml double-distilled water and 4 ml 10% dimethyl sulphoxide. After incubation, the tissue sections were rinsed throughly in their respective buffer and then re-immersed in cacodylate buffer (pH 7.4). The tissues were then postfixed in 1% osmium tetroxide for 1 h, dehydrated in ethanol and embedded in Epon-Araldite mixture. Controls were obtained from heat-inactivated sections or from sections incubated in the substrate-free medium.

Thin sections obtained from the above were either unstained or counterstained with uranyl acetate and lead citrate and then examined in a Hitachi HU-12A electron microscope.

Results

Ultrastructural findings

The SGC cells in the rat superior cervical ganglia were characterized by their small size (10 to 15 µm in diameter) and an abundance of DCGs in their cytoplasm. A multiplicity of SGC cells was detected in rat superior cervical ganglia; however, in all SGC cells the perinuclear cytoplasm of SGC cells was occupied by endoplasmic reticulum (ER) and Golgi apparatus. The Golgi apparatus was composed of several stacks of saccules and numerous small vesicles which contained dense material of varying density. Accumulation of dense material at the blunt ends of Golgi *trans* saccules was frequently observed. The content of DCGs in the Golgi area was less osmiophilic than that in the cell periphery (Fig. 1).

ZIO impregnation

After ZIO impregnation, electron-dense precipitations were recognized in a few cisternae of rough endoplasmic reticulum (rER), in all the saccules of Golgi complex and its associated vesicles, and in the matrix and tubular cristae of some, but not all, mitochondria (Fig. 2). The *trans*-Golgi surface showed the strongest staining among all stained structure. In *trans*-Golgi structures ZIO-reactive hexagonal arrays of intertubular connection were frequently encountered (Fig. 2). Most of the DCGs in cytoplasm were not stained with the ZIO mixture.

At higher magnification, a regional inequality of staining in a ZIO-treated tissue section was seen. The DCGs on the *trans* side of the Golgi complex appeared positively-stained and those at the peripheral cytoplasm were not. The DCGs in the vicinity of the *trans*-stacks in the Golgi complex were also stained to varying degrees (Fig. 3 and inset). Frequently, the DCGs near the region of expanded rims of the Golgi cisternae were heavily-stained and those away from this region were only lightly-stained (Fig. 3 inset).

Phosphatase activities

TPPase

TPPase activity was consistently localized on the plasma membrane and in one or two inner saccules of the Golgi complex (Fig. 4). The reaction product deposited in the lumen of *trans* inner one or two Golgi saccules in a spotty pattern and was absent from the outer *cis* saccules. The most intense reaction usually occurred in the innermost saccule, with the decreasing amounts of reaction product in saccules closer to the *cis* outer face. Other organelles and the elements associated with the Golgi complex, including rER, GERL, lysosome and numerous small vesicles, did not show any reactivity. No DCGs in SGC cell showed TPPase activity (Fig. 5).

ACPase

Only weak ACPase activities were observed in the SGC cells of rat superior cervical ganglia (Fig. 6). The deposits of reaction product were immutably localized in the *trans* saccules of Golgi complex, lysosomes and multivesicular bodies in the SGC cells. GERL and DCGs were negative for ACPase activity

(Fig. 7).

Discussion

Ultrastructure

The ultrastructure of SGC cells in sympathetic ganglia of various species of animals has been reported by various authors (Taxi, 1979; Case and Matthews, 1985; Lu and Chau, 1992). Features suggesting DCG formation in the Golgi region, such as: 1) dense material accumulation at the blunt ends of *trans*-most Golgi saccules; and 2) distribution of DCGs in the Golgi area, have been observed in the present and previous studies (Taxi, 1979; Hsiao and Lu, 1982; Lu and Chau, 1992). Therefore, an intimate relationship between the Golgi complex and the DCGs in SGC cells is confirmed and it is speculated that the Golgi complex may play an important role in the synthesis of DCGs.

ZIO reaction

Although the results from the ZIO staining-reduction method could be different from area to area even in the single tissue block (section), we have reported two consistent results in the SGC cells of rat superior cervical ganglia after ZIO treatment: 1) all the saccules of Golgi complex profiles and DCGs associated with Golgi complex were positively stained; and 2) the DCGs at the peripheral cytoplasm were non-reactive. These are essentially similar to the results of SGC cells in rat coeliac-superior mesenteric ganglion complex (Hsiao and Lu, 1982). In the present study, therefore, we re-confirmed the ZIO-staining pattern in SGC cells and extended the findings to the SGC cells in rat superior cervical ganglia.

It is worthy to note that the ZIO reagent is not specific for any transmitter itself, and ZIO staining products are associated with a high affinity Ca^{++} binding site (Gilloteaux and Naud, 1979) or may

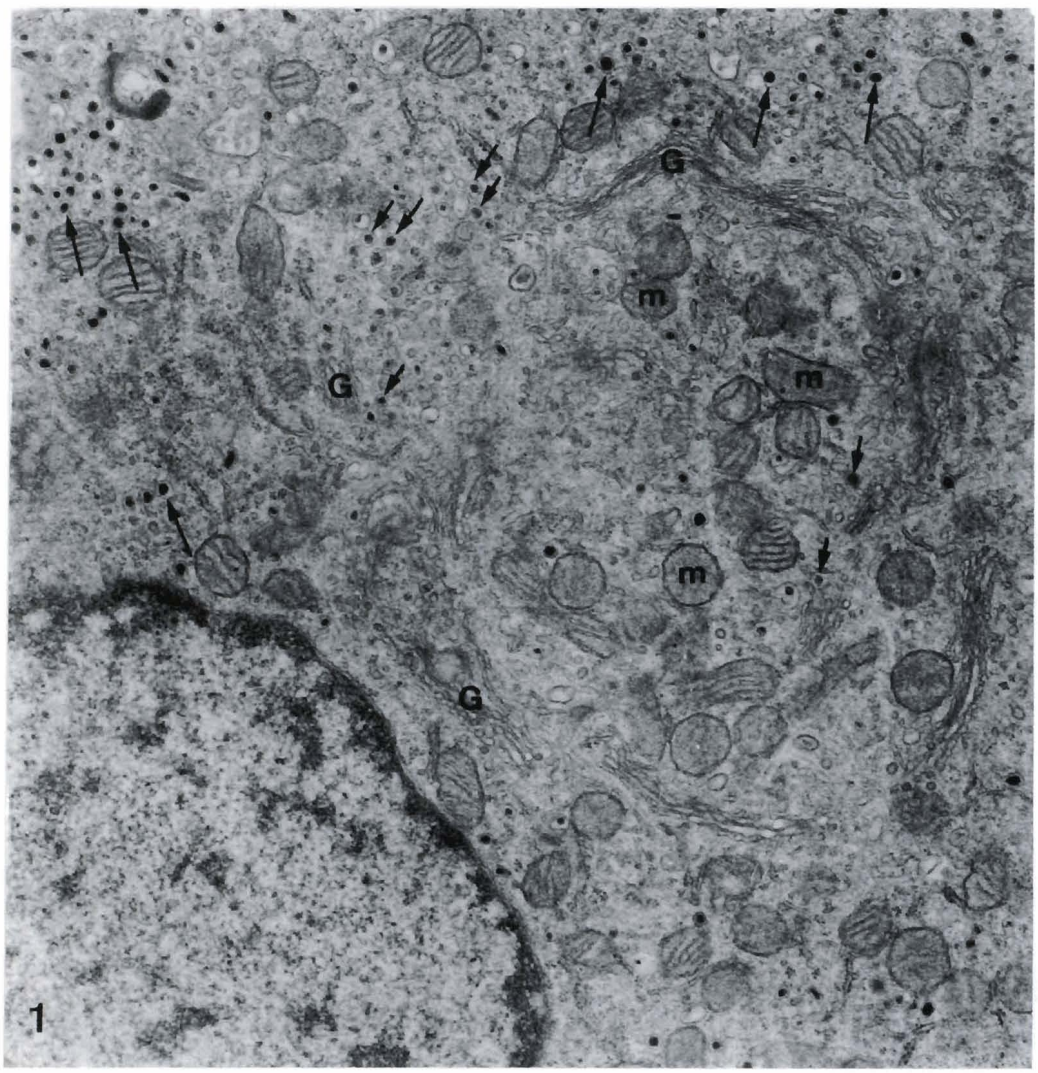

Fig. 1. Electron micrograph of Golgi area of an SGC cell in the rat superior cervical ganglion. Golgi apparatus (G) and associated vesicles are present. Note the staining gradient of the core material in the DCGs as indicated by the lenght of arrows; the longer the arrow the stronger the osmiophilia of the core material. m: mitochondria; Nu: nucleus. x 20,000

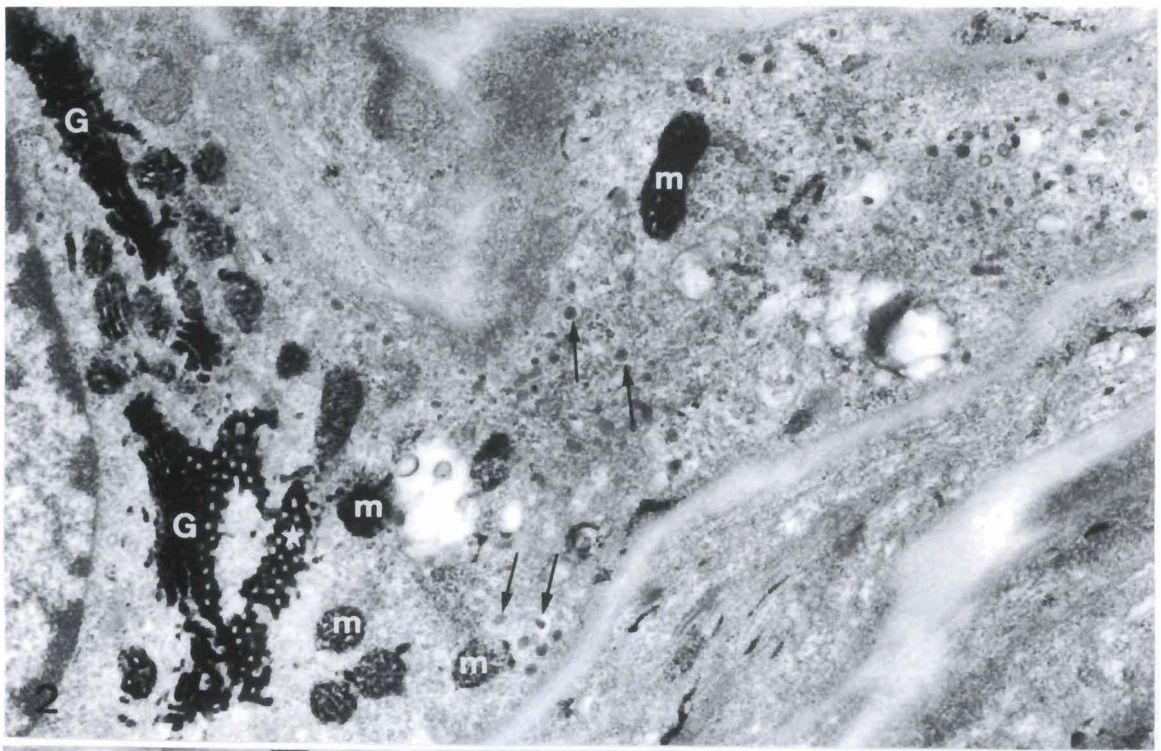

Fig. 2. Part of the cytoplasm of SGC cells in the rat superior cervical ganglion after ZIO staining. The Golgi complex (G) is heavily impregnated by the ZIO mixture. On the *trans* surface of the Golgi complex, there are hexagonal arrays of intertubular connections (star). The matrix of some, but not all, mitochondria (m) is also impregnated by the ZIO mixture. Note that the DCGs (long arrows) located at the peripheral cytoplasm are not stained. x 18,000

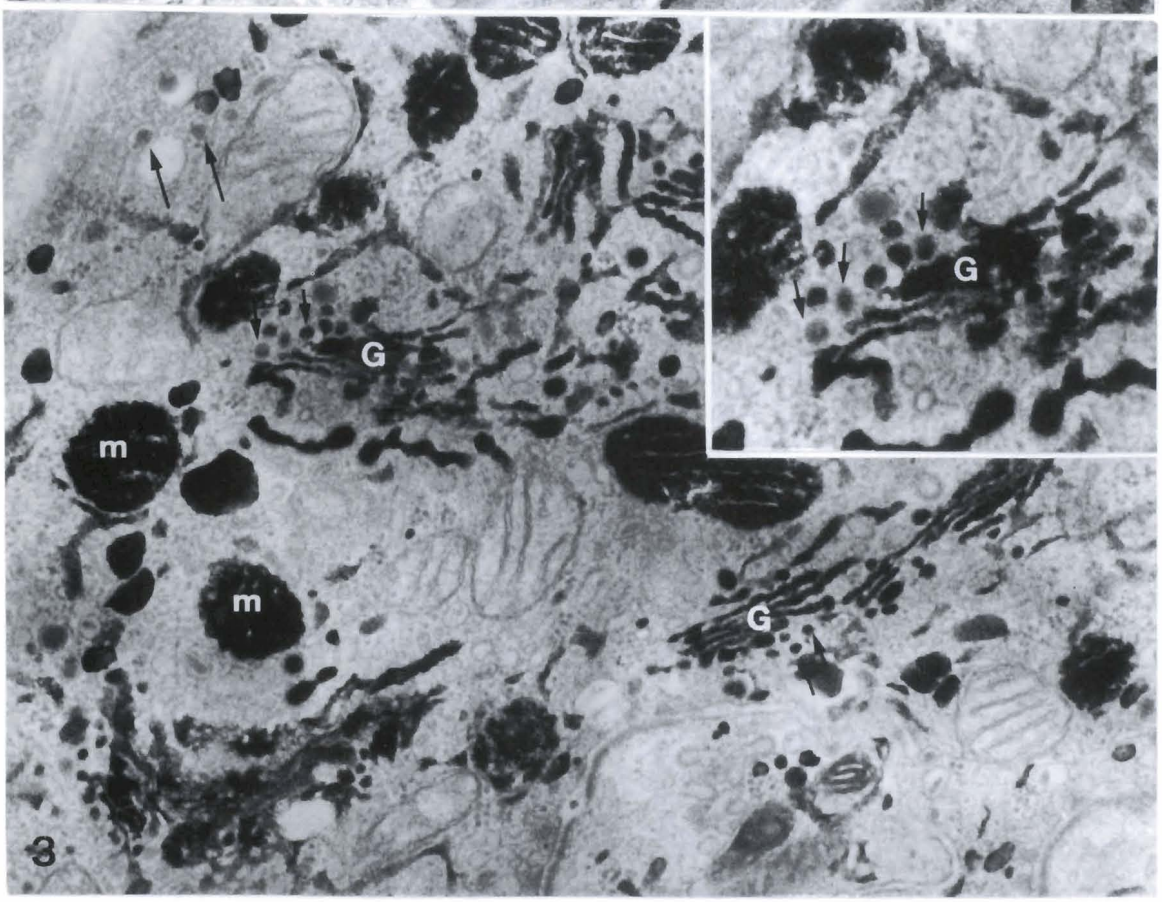

Fig. 3. High power view of cytoplasm of SGC cell in rat superior cervical ganglion treated with ZIO mixture. ZIO precipitates were recognized in some rER and all of Golgi saccules and their associated vesicles (short arrows). Note that a gradient of ZIO staining in the core-material of DCGs is roughly formed from the centre of the Golgi area toward the periphery of the cytoplasm. The DCGs (long arrows) at the peripheral cytoplasm are not stained. x 36,000. Inset: higher magnification of the Golgi area in SGC cell showing that the immature DCGs in vicinity of trans-saccule of Golgi are stained or partially stained with ZIO impregnation (short arrows). x 60,000

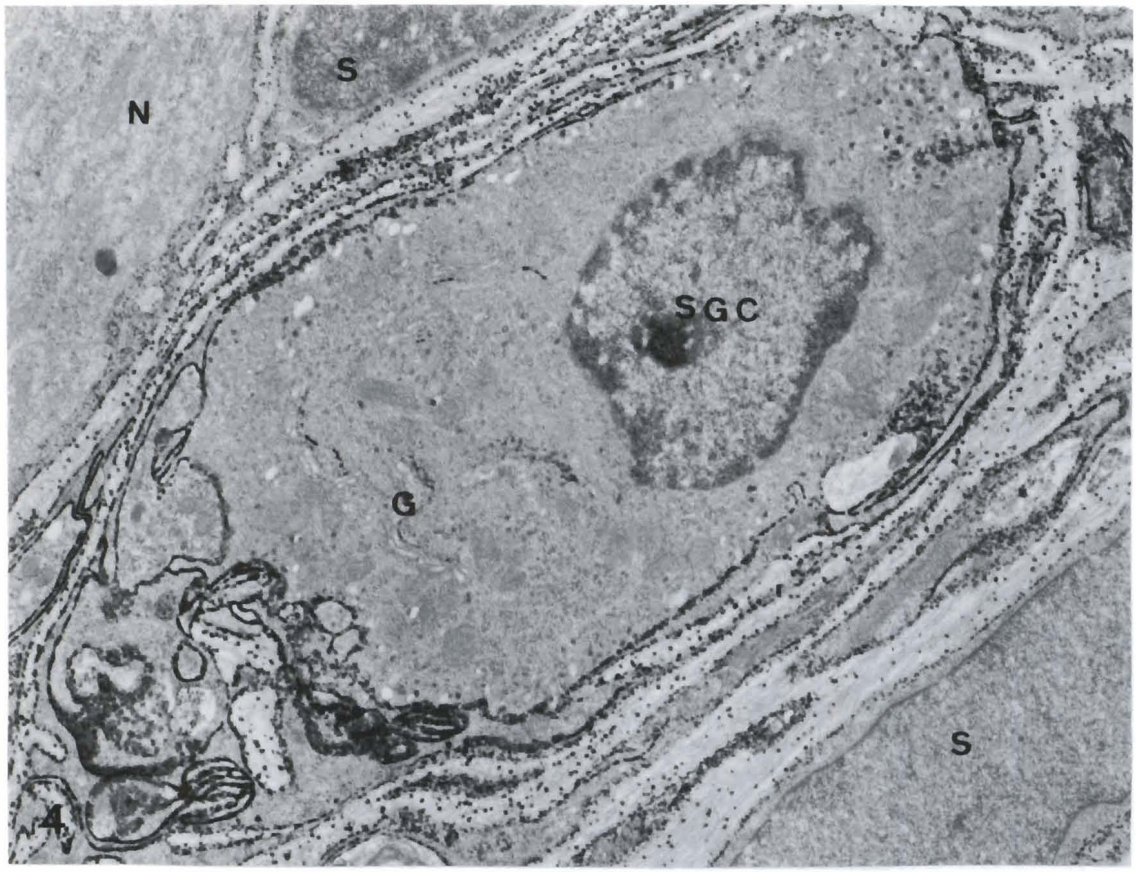

Fig. 4. Distribution of reaction product of TPPase activity in a group of SGC cells of the rat superior cervical ganglion. TPPase reaction product is deposited on the plasma membrane of the SGC cells (SGC), neurons (N), Schwann cells (S) and in the Golgi complex (G) of the SGC cells and neurons. x 6,000

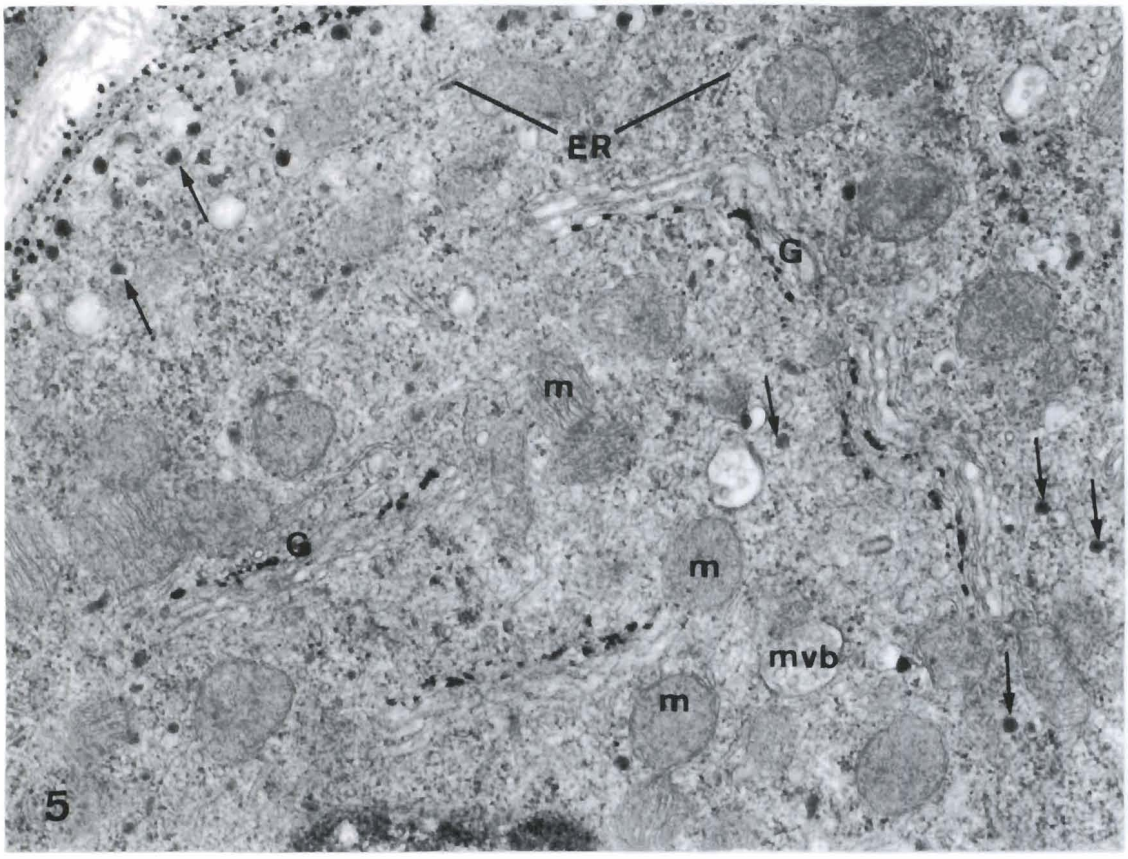

Fig. 5. Part of the cytoplasm of an SGC cell from rat superior cervical ganglion showing the TPPase activity at higher magnification. TPPase reaction product is localized within the lumen of the inner one or two saccules on the *trans* side of the Golgi complex (G). TPPase activity is not demonstrated in endoplasmic reticulum (ER), *cis* outer Golgi saccules, lysosomes, multivesicular body (mvb), mitochondria (m) or DCGs (long arrows). x 24,000

represent a range of substances probably proteinaceous in nature (Pellegrino de Iraldi, 1975, 1977). Furthermore, Reinecke and Walther (1978) and Locke and Huie (1983) suggested that the ZIO staining may be attributable to the labile disulphide bridges of polypeptides and these labile disulphide bridges in rER and Golgi cisternae may be exposed on the newly synthesized proteins that are still in modulation or glycosylation on their way to secretion. Recently, biochemical studies on the conformation of protein also supported that the protein stability can be increased by the formation of disulphide bonds (Matsumara et al., 1989). However, the chemical basis of ZIO staining is still unclear. In the present study, we believe that there is an inconspicuous ZIO staining gradient emanating from the centre of Golgi apparatus toward the peripheral cytoplasm. We also propose that during the formation and maturation of the DCGs in rat SGC cells, ZIO reactivity of these granules decreases gradually and finally, ZIO staining is lacking in the crystalline centres of secretory vesicles (Figs. 2, 3). Though the ZIO impregnation method does not give any information about the exact content of DCGs in SGC cells, it shows that ZIO staining is a powerful and selective tool to reveal the Golgi complex and its relative structures (Burgos and Gutiérrez, 1986; Uchida and Warshawsky, 1992). From this point of view, our results suggested that the DCGs of SGC cells are derived from the Golgi complex since the immature DCGs were connected to the tubular network of the Golgi complex and were ZIO-positively stained.

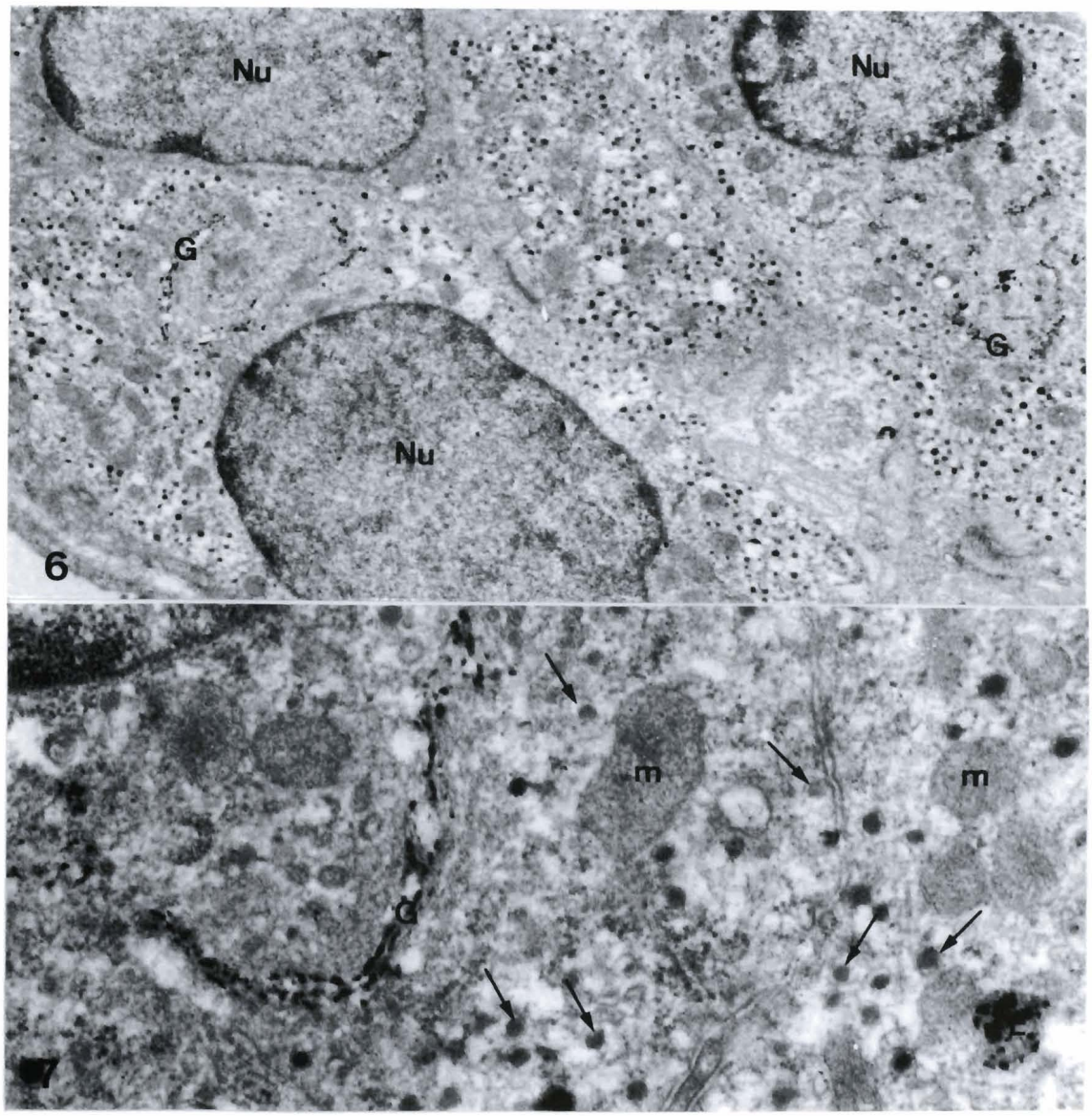

Fig. 6. A group of SGC cells after incubation for ACPase activity. Only weak ACPase activity is seen in the Golgi area (G); Nu: nucleus. x 8,000

Fig. 7. ACPase reaction product is localized in the *trans*-most saccules of the Golgi apparatus (G). Spotty reaction product deposits in the lumen of the saccule. Some lysosomes (L) are also reactive. Note that the DCGs (long arrows) are free from ACPase activity. m: mitochondria. x 24,000

Phosphatase activities

For the cytochemical studies on the SGC cells, TPPase and ACPase activities were consistently localized in the inner saccules of the Golgi complex and no reaction products were observed in the DCGs. TPPase and ACPase have been proposed as the «marker enzymes» of *trans* saccules and GERL system, respectively. TPPase activity is generally localized in several *trans* saccules of Golgi complex and ACPase activity is often localized in GERL, lysosome and immature secretory granules (Novikoff et al., 1971; Novikoff and Novikoff, 1977). Based on the concept of paraneuron (Fujita, 1977), it is reasonable to compare the SGC cells in sympathetic ganglia with carotid glomus type I cells. In a study on the localization of lysosomal enzymes in chromaffin cells of the rat carotid body, Böck (1987) demonstrated that ACPase staining yields reaction product in *all* cisternae of the Golgi stack in carotid body type I cells and that reactivity for ACPase is lower in the *cis* constituent of the stack as compared to its *trans* constituents, the penultimate cisternae regularly being the most reactive one. Unlike their results, we demonstrated that only the innermost, but not *all*, saccules of the Golgi stack showed the ACPase activity. This is not surprising because it has been shown that the distribution of enzymatic activities in the Golgi complex varied significantly in different cell types and that the modulation of the enzymatic activities might occur under various circumstances, such as developmental, functional or pathological alterations (Broadwell and Oliver, 1981; Sawano and Fujita, 1981; Hand and Oliver, 1984).

According to recent studies, a co-distribution of TPPase and galacto-transferase or N-acetyl-galactosaminyl-transferase has been demonstrated in the trans sacculus of the Golgi complex (Goldfischer, 1982; Malchiodi et al., 1986; Geuze and Morre, 1991) and therefore, TPPase appears to play an important role in converting UDP to UMP which is then specially transported across the Golgi saccule membrane in exchange for UDP galactose or other UDP sugar in glycosylation (Fleischer, 1983; Hirschberg and Snider, 1987). On the other hand, autoradiographic and cytochemical studies revealed that the sialation in protein synthesis is only within the *trans*-Golgi compartment and that sialic acid is transferred to glycoproteins, from CMP-sialic acid (Roth et al., 1985; Bennet and Wild, 1991). The function of ACPase in trans-Golgi saccule is therefore suggested to be involved in degradation of nucleoside phosphates after transfer of the sialic acid rather than acting as lysosomal enzyme (Farquhar and Palade, 1981; Böck, 1987; Oliver and Yuasa, 1987). The fact that both ACPase and TPPase activity was limited in Golgi trans-saccule in the present study might imply that the glycosylation and proteolytic cleavage of protein contents in the DCGs were achieved in the Golgi complex.

In the present study it is suggested, on the grounds of cytochemical analysis that (1) the DCGs of SGC cells are synthesized and released from the Golgi complex, and that (2) the proteolytic cleavage of protein contents in the DCGs is accomplished in the *trans*-saccules of Golgi complex.

Acknowledgements. This work was supported in part by grants Nos. NSC-80-0412-B002-159 and NSC-81-0412-B002-558 from the National Science Council, Republic of China. We thank Ms. M.-H. Kao, H.-L. Liou, H.-C. Chang and Mr. B.-N. Huang for their excellent technical assistance and photographic work.

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Accepted May 12, 1994