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Ubiquitous presence of chromogranin A in the inner ear of guinea pig

Seung Hyun Yoo and David J. Lim

Laboratory of Cellular Biology National Institute on Deafness and other Communication Disorders National Institutes of Health, Bethesda, MD 20892, USA

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Chromogranin A (CGA), which is supposed to be responsible for the calcium storage of secretory vesicles and is also considered to be a marker protein of neurons and endocrine cells, has been found in a variety of organs and tissues. In the present study, soluble proteins from the organ of Corti, saccule, crista, utricle, tectorial membrane, stria vascularis, and the spiral ligament from the inner ear of guinea pig were extracted, and probed with both polyclonal and monoclonal CGA antibodies to determine the presence of CGA. A 75 kDa protein reactve to both antibodies was found in the organ of Corti, saccule, crista, utricle, stria vascularis, and the spiral ligament, suggesting the widespread presence of CGA in the inner ear.

Chromogranin A; Chromogranin A antibody; The organ of Corti; Inner ear

1. INTRODUCTION

Chromogranin A is the major soluble protein of adrenal medullary chromaffin cells, constituting 40% of the soluble proteins of secretory vesicles [1]. The secretory vesicles of chromaffin cells have recently been identified as a major inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store [2], and the Ca^{2+} storage function of the vesicles has been attributed to the Ca^{2+} buffering role of CGA [3,4]. Chromogranin A undergoes Ca^{2+} and pH-dependent conformational changes [5] and binds 32–55 mol of Ca^{2+}/mol of protein with dissociation constants of 2–4 mM [4]. In addition to the abundance of CGA in adrenal medulla, CGA is found in most, if not all, neurons and endocrine cells throughout the animal kingdom, ranging from human to *Paramecium* [1]. Due to its ubiquitous presence in neurons and endocrine cells [6–9], CGA is now regarded as a marker protein of neuroendocrine cells [10]. Past studies demonstrated the presence of CGA in a wide variety of organs and tissues such as brain, anterior pituitary, skin, heart, kidney, olfactory bulb, thyroid, and the central nervous system [1,6,11–13]. The widespread presence of CGA in many tissues across the species and the lack of isotypes in a given species strongly suggest that CGA is an ancient protein with potential functions of fundamental importance.

Correspondence address: S.H. Yoo, Laboratory of Cellular Biology, Bldg. 36, Room 5D-15, NIDCD, NIH, Bethesda, MD 20892, USA. Fax: (1) (301) 480 3242.

Abbreviations: CGA, chromogranin A; PBS, phosphate-buffered saline.

In spite of the extensive studies on the existence of CGA in a number of organs and tissues, the existence of CGA in the inner ear of any species has not been studied. Therefore, we examined the possible presence of CGA in several tissues of the inner ear of guinea pig, and found the presence of CGA in the organ of Corti, saccule, crista, utricle, stria vascularis, and the spiral ligament.

2. MATERIALS AND METHODS

2.1. *Animals*

A total of 13 young adult male guinea pigs (NCR 2, 200–300 g) were sacrificed by decapitation under CO_2 inhalation anesthesia and the cochleas were removed immediately for dissection under a microscope. Isolation of the organ of Corti, saccule, crista, utricle, tectorial membrane, stria vascularis, and the spiral ligament was carried out in ice-cold phosphate-buffered saline (PBS), and was completed within 1 h of the animal's death. The otoconia from the saccule and utricle were initially removed by fine tweezer during dissection and then the additional dislodged otoconia were removed by centrifugation following sonication.

2.2. *Chromogranin A purification and antibody preparation*

Chromogranin A from bovine adrenal medulla was purified as described previously [5] and was used to immunize rabbit. For antibody production, 50 μg of purified CGA in 1 ml of PBS was mixed with 1 ml of Freund's complete adjuvant and injected into a rabbit. Three weeks after the first injection 25 μg of CGA in 0.5 ml of PBS mixed with 0.5 ml of Freund's incomplete adjuvant was injected, followed by a third injection of 25 μg of CGA in 0.5 ml of PBS mixed with 0.5 ml of Freund's incomplete adjuvant two weeks thereafter. The CGA antiserum was collected one week after the third injection and the CGA antibody was affinity purified using CGA-affinity column chromatography. The bound CGA antibody was eluted with an acid buffer (0.2 M glycine-HCl, pH 2.2) and neutralized immediately with 2 M Tris-HCl, pH 8.8. The eluted antibody fractions were concentrated by ultrafiltration using Amicon centricon 10 (10,000 MW cut-

off), and dialyzed overnight against 100 volumes of PBS at 4°C. The antibody concentration was adjusted to ~0.7 mg/ml and kept in 0.02% NaN_3.

2.3. *Protein extraction from the inner ear tissues*

For the extraction of soluble proteins, the tissues in PBS were extensively sonicated in cold room while maintaining the tissues at 4°C. This procedure released soluble proteins from each tissue, which can be separated from the remaining tissues by centrifugation at 15,000×*g* for 10 min. After centrifugation, the supernatants containing the soluble proteins were collected and used as protein samples.

2.4. *Immunoblot*

The soluble proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and either visualized by Coomassie blue or silver staining, or electrotransferred to nitrocellulose membrane for immunublot analysis. For immunoblot analysis, the affinity purified rabbit polyclonal antibody directed against bovine CGA and the mouse monoclonal CGA antibody directed against human CGA (Boehringer Mannheim) were used both at 1:1000 dilution. The CGA antibody binding was detected with a biotinylated secondary antibody and the streptavidin-biotinylated alkaline phosphatase complex system using the amplified alkaline phosphate immunoblot assay kit (Bio-Rad) as described by the manufacturer.

2.5. *2-dimensional PAGE*

The 1D isoelectric focusing was carried out using the small tube (1.5 mm × 7.5 cm) gel unit of Hoefer (San Francisco, CA, USA) in the pH range of 4 to 8, and the 2D PAGE was carried out on a 10% SDS-gel according to Laemmli [14]. The 2D gel was stained with Coomassie blue first and followed by silver staining to enhance the detection limit.

3. RESULTS

To obtain proteins from the various tissues of inner ear, the soluble proteins from the pooled organ of Corti, crista, saccule, utricle, tectorial membrane, stria vascularis, and the mixture of the stria vascularis and spiral ligament were obtained from guinea pigs as described in section 2. Among these, the crista yielded largest amount of protein. In order to examine the contents of the soluble proteins from crista, the proteins were separated by SDS-PAGE, along with purified CGA, and visualized by Coomassie blue staining (Fig. 1A). To determine the presence of immunoreactivity to the CGA antibodies, bovine CGA and the crista proteins were transferred to nitrocellulose membrane and probed with the polyclonal rabbit antibody raised against purified bovine CGA (Fig. 1B).

As shown in Fig. 1B, the antibody binding was observed in a ~75 kDa region. In particular, the polyclonal antibody reacted very strongly with the purified bovine CGA (Fig. 1B), showing a major reactivity in the ~75 kDa region (intact CGA). The minor reactivity in the lower molecular weight region appears to be due to the reactivity of antibody to the proteolyzed CGA although the fragments were not visible in the Coomassie blue stained gel. The same antibody also reacted with the

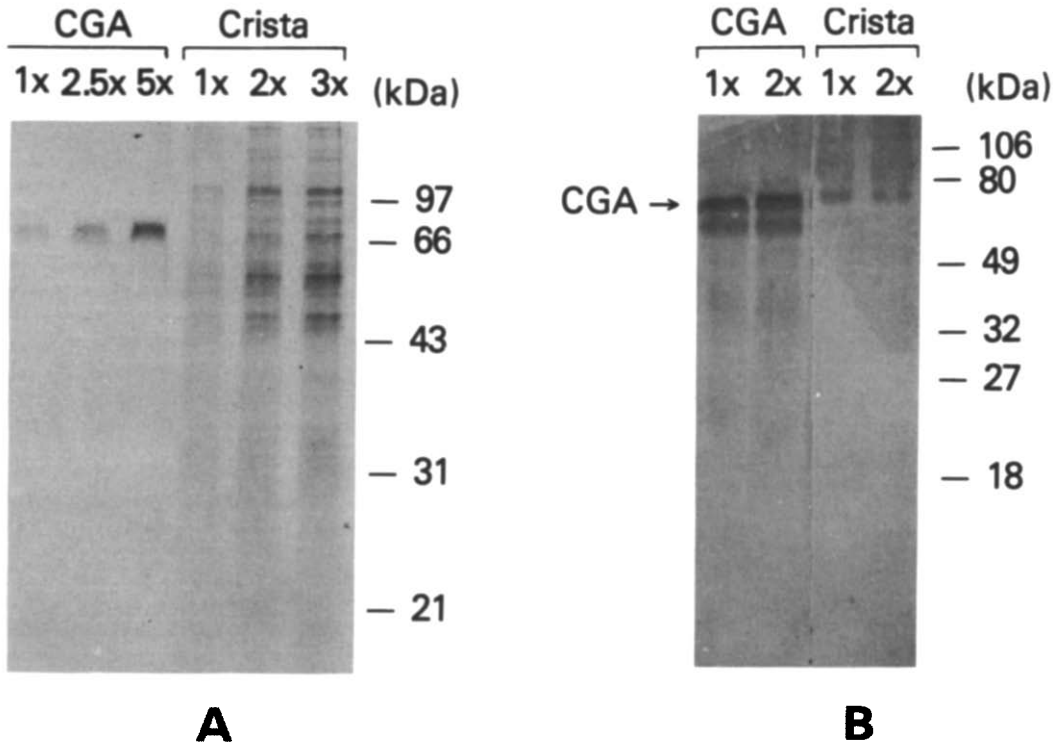

Fig. 1. SDS-PAGE and immunoblot analysis of chromogranin A antibody binding of the soluble proteins of crista. Purified bovine CGA and the soluble proteins from the crista of guinea pig inner ear were separated on a 10% SDS-gel according to Laemmli [14] and either stained with Coomassie blue (A), or subjected to immunoblot analysis using CGA antibody (B). (A) Bovine CGA in the amount of 0.1 μg (1×), 0.25 μg (2.5×), 0.5 μg (5×), and the soluble crista proteins in the amount of 5 μg (1×), 10 μg (2×), and 15 μg (3×) were stained with Coomassie blue. (B) Immunoblot analyses: bovine CGA in the amount of 0.05 μg (1×), 0.1 μg (2×), and the soluble crista proteins in the amount of 2.5 μg (1×), 5.0 μg (2×) were blotted, and probed with the polyclonal antibody (B).

crista proteins with a major reactivity in the ~75 kDa region. Since the results in Fig. 1B indicated the presence of CGA in the crista and the immunostaining method employed was sensitive enough to detect CGA from very small amounts of protein, 3–4 μg of proteins from each tissue were separated by SDS-PAGE and subjected to immunoblot analysis as in Fig. 2.

As shown in Fig. 2A, the polyclonal antibody reacted with a ~75 kDa protein from the organ of Corti, crista, stria vascularis, utricle, saccule, and the mixture of the stria vascularis and spiral ligament, but not with the tectorial membrane proteins, suggesting the presence of CGA in all of these tissues except the tectorial membrane. In addition, the same antibody also reacted with a protein of ~110 kDa in several of these tissues. However, the identity of the band is not clear at present although chromogranin B [15], another member of the chromogranin family, has a size of ~110 kDa on the SDS-gel and has previously been shown to crossreact with a chromogranin A antibody [16]. In parallel experiments, an identical protein blot was also immunoreacted with the monoclonal antibody. As shown in Fig. 2B, the antibody reacted with a ~75 kDa protein from the organ of Corti, crista, stria vascularis, utricle, saccule, and the mixture of the stria vascularis and spiral ligament, but showed no reactivity with the proteins from the tectorial membrane. Nevertheless, the CGA immunoreactivity demonstrated in all the remaining tissues agreed with the result obtained with the polyclonal antibody (Fig. 2A).

Since the proteins from the mixture of the stria vascularis and spiral ligament reacted as well as or better than those from stria vascularis alone with the antibodies, it appeared that the spiral ligament also contained CGA. Therefore, in order to determine the presence of CGA in the spiral ligament, the spiral ligament was further separated from the stria vascularis, and the soluble proteins were extracted from the spiral ligament for analysis by immunoblot (Fig. 3). As shown in Fig. 3B and C, the spiral ligament showed a major immunoreactive band in the ~75 kDa region and a minor reactive band at ~110 kDa region, indicating the presence of CGA in the spiral ligament. To further confirm the existence of CGA in the spiral ligament, the soluble proteins of spiral ligament were separated by 2D PAGE and subjected to immunoblot analysis using the monoclonal antibody (Fig. 4). As shown in Fig. 4B, the monoclonal antibody reacted with a ~75 kDa protein with an isoelectric point (pI) of 5.0, agreeing with the known pI of CGA [1].

4. DISCUSSION

The present results demonstrate the presence of CGA in several tissues of the inner ear, i.e. the organ of Corti,

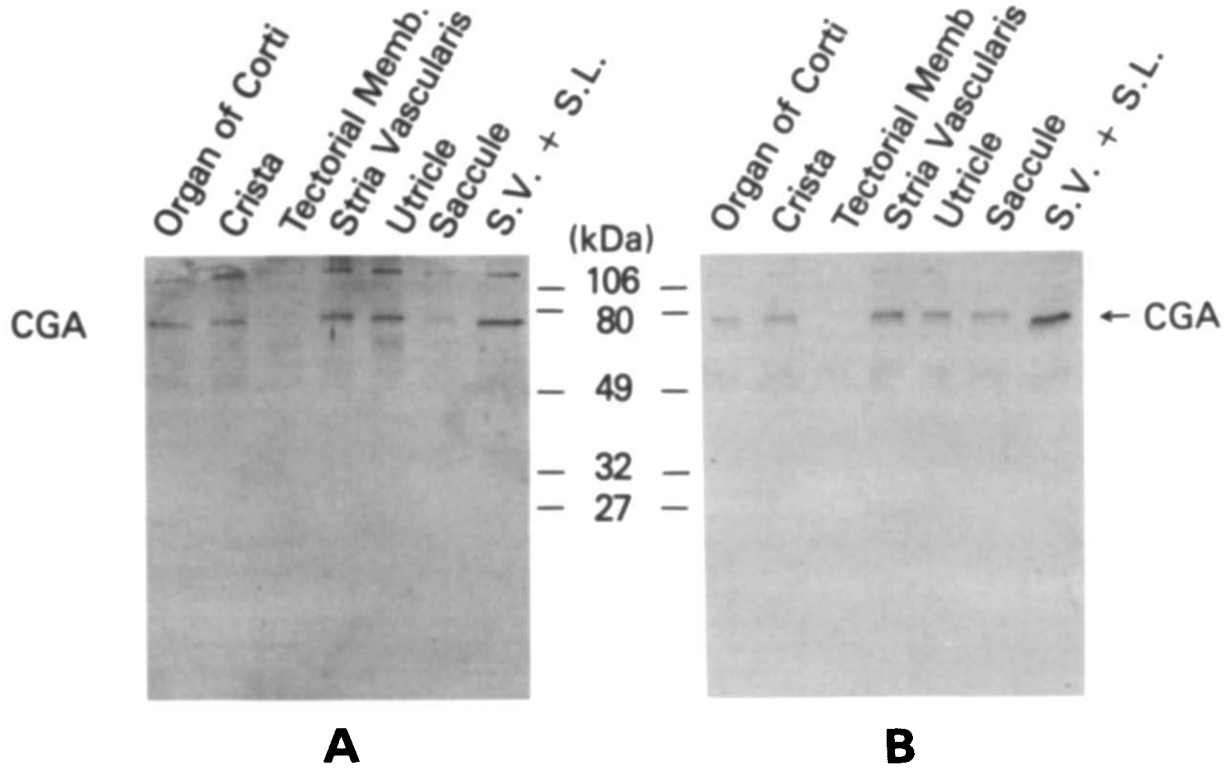

Fig. 2. Immunoblot analysis of chromogranin A antibody binding of the soluble proteins from the inner ear tissues. The soluble proteins from the several inner ear tissues of guinea pig were separated on a 10% SDS-gel, blotted, and probed with the polyclonal antibody (A) and the monoclonal antibody (B) to detect the proteins reactive to the CGA antibodies. The amount of proteins loaded in each lane is 3–4 μg except the tectrorial membrane (2 μg). S.V. + S.L. stands for the mixture of the stria vascularis and spiral ligament.

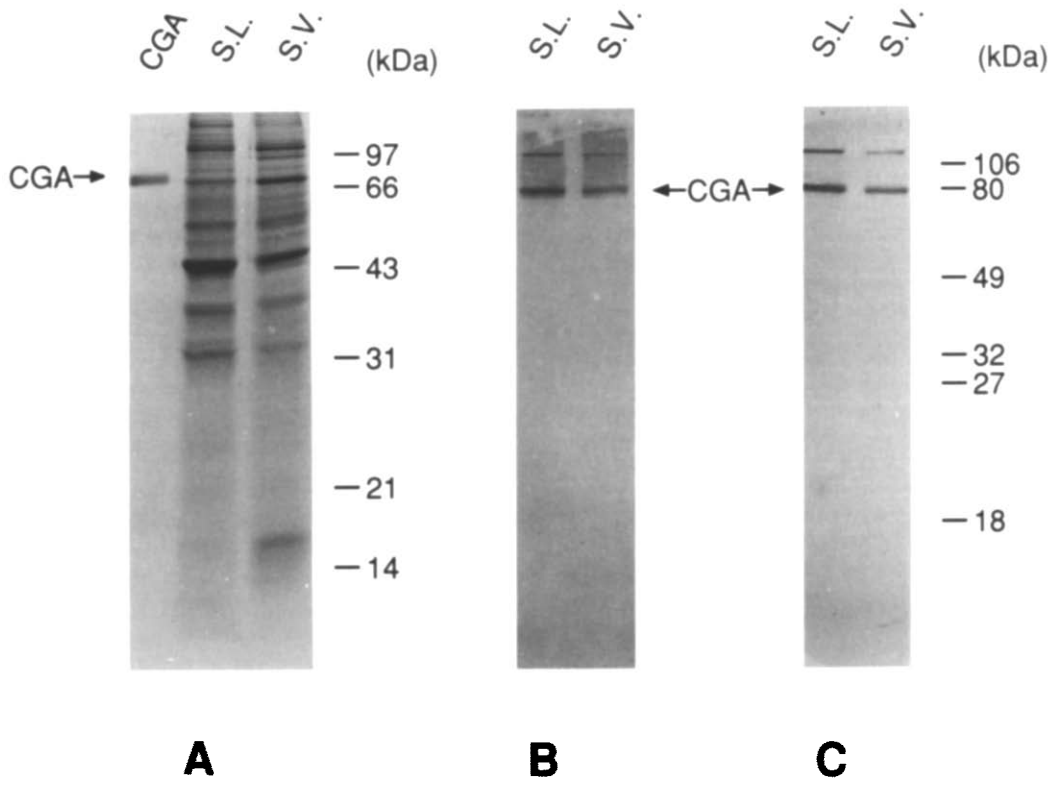

Fig. 3. SDS-PAGE and immunoblot analysis of chromogranin A antibody binding of the soluble proteins of the spiral ligament and stria vascularis. Purified bovine CGA and the soluble proteins from the stria vascularis and spiral ligament of guinea pig inner ear were separated on a 10% SDS-gel and either stained with Coomassie blue (A), or subjected to immunoblot analyses using CGA antibodies (B and C). (A) 0.5 μg of bovine CGA and 10 μg each of the soluble proteins of the spiral ligment (S.L.) and stria vascularis (S.V.) were stained with Coomassie blue. (B and C) Immunoblot analyses: 3.6 μg each of the spiral ligament and stria vascularis proteins were blotted, and probed with the polyclonal antibody (B) and the monoclonal antibody (C).

crista, saccule, utricle, stria vascularis, and the spiral ligament. Chromogranin A has been suggested to play important roles in sorting and packaging the intravesicular contents of secretory vesicles, including catecholamine, peptides, prohormones, and neurotransmitters [5,23], and in controlling Ca^{2+} storage/release of the

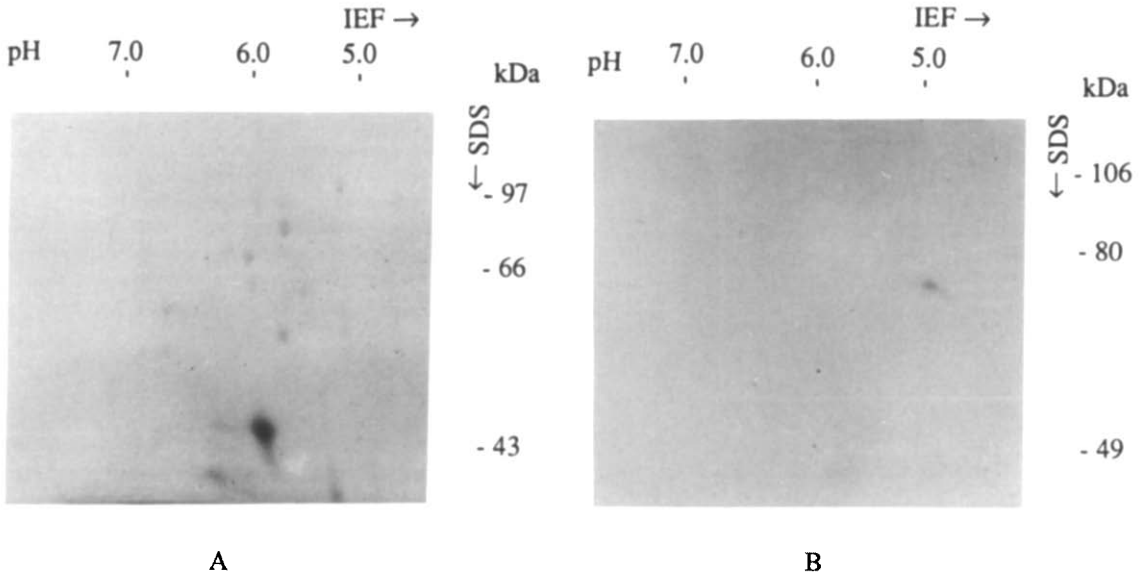

Fig. 4. Two-dimensional PAGE and immunoblot analysis of the soluble proteins of spiral ligament. The soluble proteins of spiral ligament were separated by 2D PAGE and subjected to either silver staining (A) or immunoblot analysis using the monoclonal antibody (B). The amounts of protein applied were 5 μg (A) and 3 μg (B).

inositol 1,4,5-trisphosphate-sensitive intracellular Ca^{2+} store [2,4]. Considering that CGA is a marker for neurons and endocrine cells and CGA is primarily found in the dense-core vesicles of these cells [10], the presence of CGA in several tissues of the inner ear appears to suggest the existence of neuronal or endocrine cells in these tissues and even to predict the existence of CGA-containing vesicles in the respective tissues. The immunoblot of the 2D gel of the soluble proteins of spiral ligament showed an immunoreactive protein with a size of ~75 kDa and a pI of 5.0. In light of the fact that chromogranin A is an acidic protein with acidic amino acids comprising 25–30% of the protein [17–22], a pI of 5.0 reflects the acidic property of the protein and is consistent with the known pI values of CGA, which range from 4.5 to 5.5 [1]. In addition, our preliminary immunohistochemical study of chromogranin A in the cochlea of guinea pig showed strong chromogranin A immunoreactivities in the interdental cells, spiral ligament, and in all the supporting cells of the organ of Corti (unpublished observation). Detailed immunohistochemical characterization of chromogranin A immunoreactivity in the inner ear is currently under study.

The relative amount of CGA in the soluble proteins of each tissue, quantitated by measuring the intensity of each band on the immunostained membrane using a laser densitometer, indicated that CGA constitutes 0.4–0.8% of the soluble proteins of each tissue in which CGA was found (not shown). The amount of soluble protein extracted from the spiral ligament was comparable to that obtained from the crista, both of which were among the largest amounts solubilized. Considering the intensities of the immunostaining of CGA from the crista and spiral ligament, it appeared that CGA exists in a higher concentration in the spiral ligament than in the crista. Although the significance of the abundance of CGA in the spiral ligament is not clear, it is of interest to note that the connective tissue cells near the root cells, or the cells near the Reissner's membrane attachment have been suggested to regulate the cation content of perilymph [24]. Therefore, in view of the millimolar concentration of Ca^{2+} [25] and the relative abundance of CGA in the spiral ligament, it is tempting to speculate that CGA in the spiral ligament might participate in the Ca^{2+} buffering role of the spiral ligament, thereby controlling the Ca^{2+} concentration of perilymph. Hence ultrastructural localization of CGA in each tissue would not only provide clues regarding the neuronal or endocrine origin of the cells, but also shed new light on their functions in the inner ear.

REFERENCES

[1] Simon, J.-P. and Aunis, D. (1989) Biochem. J. 262, 1–13.
[2] Yoo, S.H. and Albanesi, J.P. (1990) J. Biol. Chem. 265, 13446–13448.
[3] Bulenda, D. and Gratzl, M. (1985) Biochemistry 24, 7760–7765.
[4] Yoo, S.H. and Albanesi, J.P. (1991) J. Biol. Chem. 266, 7740–7745.
[5] Yoo, S.H. and Albanesi, J.P. (1990) J. Biol. Chem. 265, 14414–14421.
[6] O'Connor, D.T. and Frigon, R.P. (1984) J. Biol. Chem. 259, 3237–3247.
[7] Fischer-Colbrie, R., Hagn, C. and Schober, M. (1987) Ann. N.Y. Acad. Sci. 493, 121–135.
[8] Sietzen, M., Schober, M., Fischer-Colbrie, R., Scherman, D., Sperk, G. and Winkler, H. (1987) Neuroscience 22, 131–139.
[9] Weiler, R., Meyerson, G., Fischer-Colbrie, R., Laslop, A., Pahlman, S., Floor, E. and Winkler, H. (1990) FEBS Lett. 265, 27–29.
[10] Huttner, W.B., Gerdes, H.-H. and Rosa, P., in: Markers for Neural and Endocrine Cells, Molecular and Cell Biology, Diagnostic Application (M. Gratzl and K. Langley, Eds.), VCH, Weinheim, Germany, 1991, pp. 93–131.
[11] Lahr, G., Heiss, C., Mayerhofer, A., Schilling, K., Parmer, R.J., O'Connor, D.T. and Gratzl, M. (1990) Neuroscience 39, 605–611.
[12] Hartschuh, W., Weihe, E. and Egner, U. (1989) J. Invest. Dermatol. 93, 641–648.
[13] Cohn, D.V., Zangerle, R., Fischer-Colbrie, R., Chu, L.L.H., Elting, J.J., Hamilton, J.W. and Winkler, H. (1982) Proc. Natl. Acad. Sci. USA 79, 6056–6059.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Benedum, U.M., Lamouroux, A., Konecki, D.S., Hille, A., Baeuerle, P.A., Frank, R., Lottspeich, F., Mallet, J. and Huttner, W.B. (1987) EMBO J. 6, 1203–1211.
[16] Gorr, S.-U., Shioi, J. and Cohn, D.V. (1989) Am. J. Physiol. 257, E247–E254.
[17] Benedum, U.M., Baeuerle, P.A., Konecki, D.S., Frank, R., Powell, J., Mallet, J. and Huttner, W.B. (1986) EMBO J. 5, 1495–1502.
[18] Iacangelo, A., Affolter, H.-U., Eiden, L.E., Herbert, E. and Grimes, M. (1986) Nature 323, 82–86.
[19] Konecki, D.S., Benedum, U.M., Gerdes, H.-H. and Huttner, W.B. (1987) J. Biol. Chem. 262, 17026–17030.
[20] Helman, L.J., Ahn, T., Levine, M.A., Allison, A., Cohen, P.S., Cooper, M.J., Cohn, D.V. and Israel, M.A. (1988) J. Biol. Chem. 263, 11559–11563.
[21] Iacangelo, A.L., Okayama, H. and Eiden, L.E. (1988) FEBS Lett. 227, 115–121.
[22] Wu, H.-J., Rozansky, D.J., Parmer, R.J., Gill, B.M. and O'Connor, D.T. (1991) J. Biol. Chem. 266, 13130–13134.
[23] Gerdes, H.-H., Rosa, P., Phillips, E., Baeuerle, P.A., Frank, R., Argos, P. and Huttner, W.B. (1989) J. Biol. Chem. 264, 12009–12015.
[24] Schulte, B.A. and Adams, J.C. (1989) J. Histochem. Cytochem. 37, 127–134.
[25] Ikeda, K. and Morizono, T. (1989) Hear. Res. 40, 111–116.