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Chromaffin granule H^+-ATPase has F_1-like structure

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Isolated H^+-ATPase from chromaffin granules was reconstituted into liposomes and the resultant proteoliposomes were further purified by Ficoll density gradient centrifugation. Studies by electron microscopy showed that proteoliposomes had particle structures (average diameter, about 10 nm) on their outer surface. These particles could be removed from the proteoliposomes by cold treatment. Immuno-electron microscopy showed that these particles were recognized by antibodies against the hydrophilic sector of the enzyme. These results indicate that the H^+-ATPase has a peripheral membrane structure similar to that of F_1-ATPase.

Chromaffin granule; Vacuolar H^+-ATPase; Extrinsic membrane sector; F_1-like particle; Catalytic sector

1. INTRODUCTION

Chromaffin granules, secretory vesicles of adrenal chromaffin cells, contain vacuolar-type H^+-translocating ATPase [1,2]. This ATPase establishes an electrochemical gradient of protons across the membranes that is the driving force for uptake of catecholamines by the granules [3,4]. The ATPase has been purified to homogeneity [5] and its structure and function have been investigated ([5–13] and reviewed in [1]).

The purified ATPase is composed of 9 different polypeptides with molecular masses of 115, 72, 57, 41, 39, 34, 33, 20 and 17 kDa [5,6,8]. The ATPase is inhibited by cold-treatment in the presence of Mg-ATP and salts [8], and in parallel with this inhibition, a set of subunits (72, 57, 41, 34 and 33 kDa polypeptides; subunits A to E) are released from the enzyme complex, suggesting the presence of peripheral and membrane sectors of the enzyme.

By analogy with F_0F_1-ATPase, which consists of a peripheral F_1 portion and a membranous F_0 portion [14], it seemed possible that the putative peripheral portion of chromaffin H^+-ATPase might be detectable as a particle on the surface of the granules. In fact, small particles (average diameter 9 nm) were detected on the surface of chromaffin granules in earlier studies [15,16], although no evidence was obtained that they corresponded to the peripheral portion of the ATPase. Moreover, preparations of chromaffin granules contain mitochondria and their fragmented membranes, and thus are contaminated with F_0F_1-ATPase [7]. Thus, further studies using purified ATPase are necessary to determine whether the particles on the membranes of chromaffin granules are in fact the peripheral portions of the H^+-ATPase complex.

In the work we reconstituted proteoliposomes with purified H^+-ATPase and obtained evidence that the ATPase has a peripheral membrane structure like F_1-ATPase.

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2. MATERIALS AND METHODS

2.1. *Materials and preparations*

Most of the chemicals used were purchased from Sigma. Protein A-gold particles were prepared as described previously [17]. Bovine adrenal glands were obtained from a local slaughterhouse.

Chromaffin granule membranes were prepared from bovine adrenal glands and stored at −70°C [5,6]. The H^+-ATPase was purified and reconstituted [5,6]: the specific activity of the reconstituted ATPase was 3.0–3.5 units/mg protein. One unit of the enzyme was defined as the amount liberating 1 μ mol of inorganic phosphate from ATP per min. Antibodies were prepared by injecting the 72 or 57 kDa subunit of H^+-ATPase electro-eluted from sodium dodecyl sulfate polyacrylamide gel into albino rabbits [18,19].

2.2. *Purification and cold treatment of proteoliposomes*

Reconstituted proteoliposomes were purified by Ficoll density gradient centrifugation. Typically, 400 μl of proteoliposome suspension (0.5 mg protein/ml) was applied to a continuous Ficoll gradient (10–30%) (4 ml) containing 20 mM MOPS-Tris (pH 7.0) and 0.5 mM dithiothreitol. After centrifugation at 237 000 × *g* for 4 h, two bands were collected by pipette for further studies.

The purified proteoliposomes were suspended in 20 mM MOPS-Tris (pH 7.0) containing 0.1 M $NaNO_3$, 0.5 mM dithiothreitol and 5 mM Mg-ATP and incubated in an ice bath for 1 h (cold treatment). The mixture was then centrifuged at 22 7000 × *g* for 1 h and the precipitate (proteoliposomes after cold treatment) was suspended in 20 mM MOPS-Tris (pH 7.0), 0.1 M NaCl and 0.5 mM dithiothreitol.

2.3. *Electron microscopy of H^+-ATPase*

The reconstituted enzyme (0.5 mg protein/ml) was diluted with

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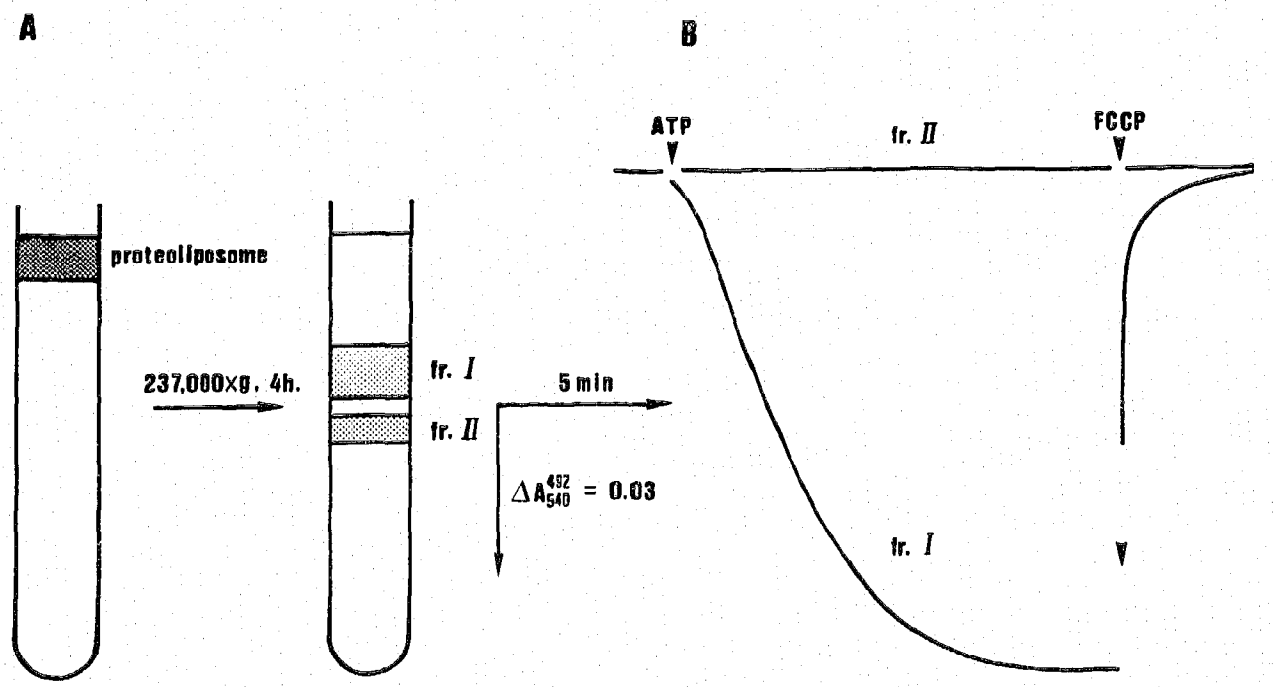

Fig. 1. Purification of reconstituted proteoliposomes. Proteoliposomes were fractionated by Ficoll density gradient centrifugation as described in section 2. Panel A illustrates the patterns of separation of samples by centrifugation. Panel B shows the ATP-dependent H^+-transport activity measured as change in absorption of acridine orange [7]. The assay mixture contained 20 mM MOPS-Tris (pH 7.0), 0.1 M KCl, 1 μg valinomycin, 15 nmol of acridine orange and 4 μg of H^+-ATPase. Mg-ATP (1 mM) and *p*-trifluoromethoxyphenylhydrazone (FCCP) (1 μM) were added at the indicated times.

phosphate-buffered saline (pH 7.4) and negatively stained with 2% uranyl acetate. For immuno-staining, the reconstituted enzyme attached to the grid was incubated in phosphate-buffered saline containing 2% gelatin for 10 min, and then treated with antibodies diluted 100 × with phosphate-buffered saline containing 2% gelatin for 15 min. After repeated washings with the same buffer, the grid was incubated for an additional 15 min with protein A-gold particles (4 nm, OD_{525}=0.03) diluted in phosphate-buffered saline containing 2% gelatin. The grids were then washed with 0.1 M cacodylate buffer (pH 7.4) and fixed for 10 min with 2% glutaraldehyde in the same buffer. Absorption staining of the reconstituted enzyme on the grids was carried out using 0.3% uranyl acetate and 3% polyvinyl alcohol as described by Tokuyasu [20].

2.4. *Other procedures*

Published methods were used for measurement of protein concentration [21], and assay of ATPase activity [5] and ATP-dependent H^+-transport activity [5].

3. RESULTS AND DISCUSSION

3.1. *Purification of functionally active proteoliposomes*

For topological analysis of H^+-ATPase, it was important to prepare sealed proteoliposomes with active ATPase incorporated into their surface. However, preliminary examination by electron microscopy showed that the fraction reconstituted with purified H^+-ATPase consisted of a mixture of sealed proteoliposomes and membranous fragments (not shown). Thus, the reconstituted membranes were further fractionated by Ficoll density gradient centrifugation to obtain functionally active proteoliposomes.

Two populations of reconstituted membranes were separated by this centrifugation (Fig. 1). These 2 fractions contained similar amounts of ATPase, as determined by assay of ATPase activity and staining of subunits with Coomassie briliant blue after their separation by gel electrophoresis in the presence of sodium dodecyl sulfate (not shown). However, ATP-dependent H^+-transport activity was recovered only in the upper fraction (fr. I) and no activity was detectable in the lower fraction (fr. II). Addition of a detergent (0.05% polyoxyethylene 9 laurylether ($C_{12}E_9$) or Triton X-100) completely abolished the ATP-dependent H^+-transport activity without affecting the rate of hydrolysis of ATP. These results suggest that fraction I contained functionally active sealed proteoliposomes in which almost all the enzyme was located at the outer surface.

3.2. *Identification of a particle structure as the peripheral portion of the ATPase*

Figure 2 shows the electron microscopic appearance of reconstituted membranes in fraction I: essentially all membranes were sealed proteoliposomes, and particles (average diameter, about 10 nm) were detected on their outer surface (Fig. 2A). These particles could be removed by cold treatment in the presence of Mg-ATP (not shown). The supernatant obtained by centrifugation of fraction I after cold treatment contained hydrophilic subunits of the enzyme [8] and similar particles to those detected on the surface of proteoliposomes (Fig. 2B). These results suggested that these particles corresponded to the peripheral portion of the H^+-ATPase.

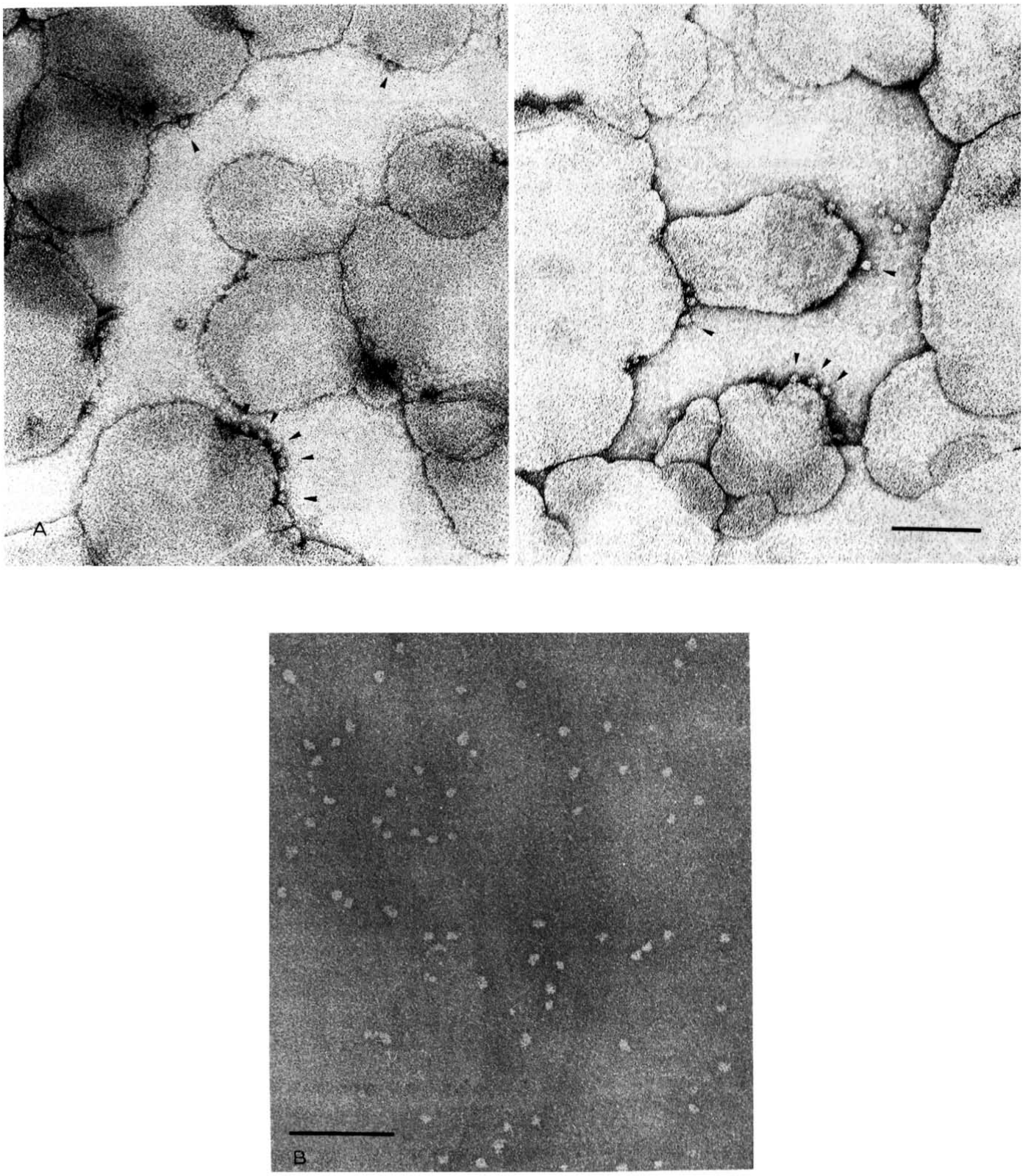

Fig. 2. Electron-microscopic appearance of reconstituted H^+-ATPase. Panel A, negatively stained reconstituted vesicles; B, fraction released by cold treatment. Particle structures are indicated by arrows. The bar represents 100 nm.

Antibodies against the 72 and 57 kDa subunits of the ATPase recognized the particles (Fig. 3), whereas the corresponding preimmune sera did not (data not shown); gold particles bound to the structures extruding

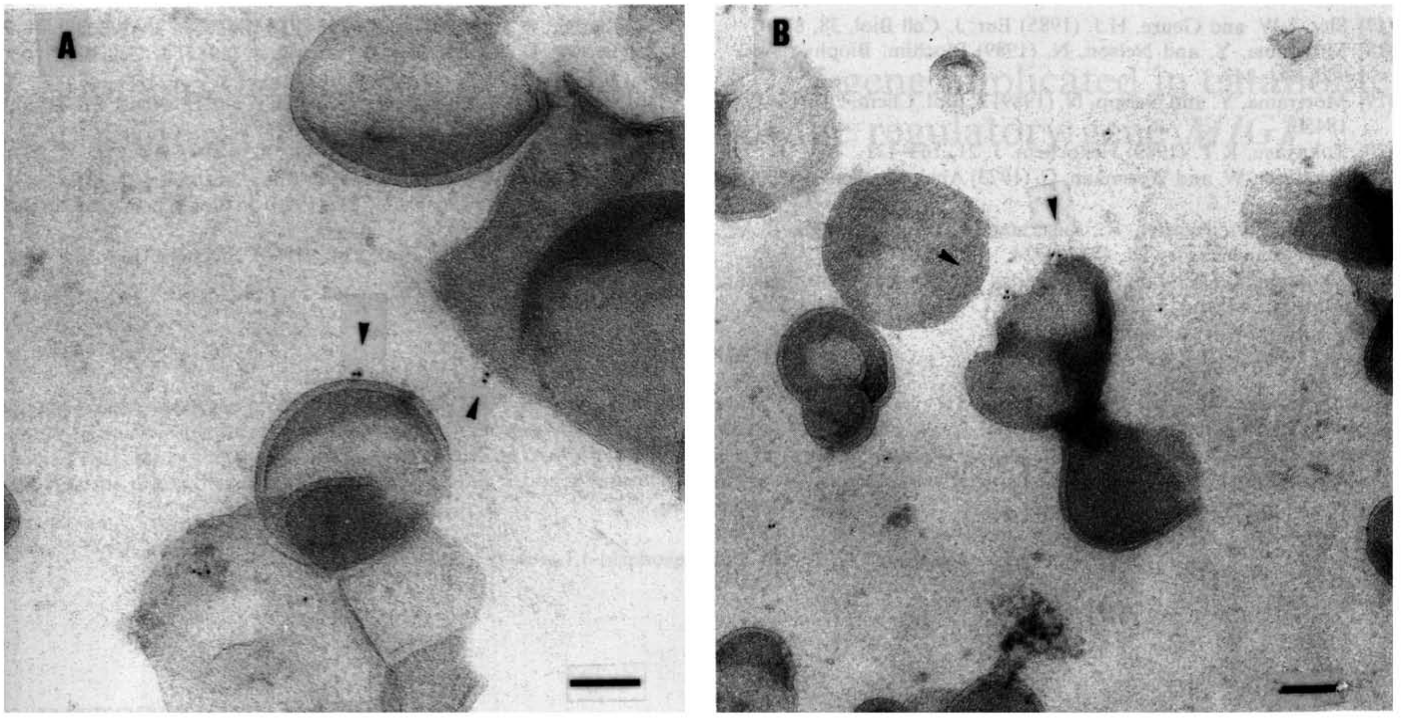

Fig. 3. Recognition of particles by antibodies against hydrophilic subunits. Immunoelectron microscopy with antibodies against the 72 kDa (panel A) or 57 kDa (panel B) subunit was carried out using protein A labeled with gold (indicated by arrows). The bar represents 100 nm.

from the membranes. Essentially no gold particles were detectable on the surface of proteoliposomes that had been subjected to cold treatment and extensive washing. These results indicated that the particles carried the 72 and 57 kDa subunits of H^+-ATPase, confirming the conclusion that they corresponded to the peripheral portion of the H^+-ATPase.

The particles of chromaffin granule H^+-ATPase were similar to F_1-ATPase in size as observed by negative staining or cryoelectron microscopy [22], probably reflecting the same subunit stoichiometry ($A_3B_3C_1D_1E_1$) as that of the peripheral portion to F_1-ATPase ($\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$) [1].

This is the first demonstration of this particle structure on proteoliposomes reconstituted with purified vacuolar type H^+-ATPases. The advantage of this system was that structural analysis could be carried out in the absence of other membrane proteins. Similar particles have been observed in various endomembrane systems containing vacuolar ATPase [23–28]. Moreover, antibodies against vacuolar H^+-ATPase recognized similar particles on kidney microsomes [25], and the particles of *Neurospora crassa* vacuoles were released by cold treatment [26]. As similar subunits could be released by cold treatment [8,18,19,29,30], these observations suggest that vacuolar H^+-ATPases in general have similar F_1-like particles as a peripheral portion of their complexes.

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REFERENCES

[1] Nelson, N. (1989) J. Bioenerg. Biomembr. 21, 553–571.
[2] Apps, D.K., Pryde, J.G. and Sutton, R. (1983) Neuroscience 9, 687–700.
[3] Njus, D., Kelly, P.M. and Harnadek, G.J. (1986) Biochim. Biophys. Acta 853, 237–265.
[4] Johnson, R.G. (1988) Physiol. Rev. 68, 232–307.
[5] Moriyama, Y. and Nelson, N. (1987) J. Biol. Chem. 262, 9175–9180.
[6] Moriyama, Y. and Nelson, N. (1987) J. Biol. Chem. 262, 14723–14729.
[7] Percy, J.M. and Apps, D.K. (1986) Biochem. J. 239, 77–81.
[8] Moriyama, Y. and Nelson, N. (1989) J. Biol. Chem. 264, 3577–3582.
[9] Apps, D.K., Percy, J.M. and Perez-Castineira, J.R.P. (1989) Biochem. J. 263, 81–88.
[10] Mandel, M., Moriyama, Y., Hulmes, J.D., Pan, Y.C., Nelson, H. and Nelson, N. (1988) Proc. Natl. Acad. Sci. USA 85, 5521–5524.
[11] Sutton, R. and Apps, D.K. (1981) FEBS Lett. 130, 103–106.
[12] Wang, S., Moriyama, Y., Mandel, M., Hulmes, J.-D., Pan, Y.C., Danho, W., Nelson, H. and Nelson, N. (1988) J. Biol. Chem. 263, 17638–17642.
[13] Nelson, H., Mandiyan, S., Noumi, T., Moriyama, Y., Miedel, M.C. and Nelson, N. (1990) J. Biol. Chem. 265, 20390–20393.
[14] Futai, M., Noumi, T. and Maeda, M., Annu. Rev. Biochem. 58, 111–136.
[15] Schmidt, W., Winkler, H. and Platter, H. (1982) Eur. J. Cell Biol. 27, 96–104.
[16] Eagles, P.A.M., Johnson, L.N., Van Horn, C. and Bullivant, S. (1977) Neuroscience 2, 153–158.

[17] Slot, J.W. and Geuze, H.J. (1985) Eur. J. Cell Biol. 38, 87–93.
[18] Moriyama, Y. and Nelson, N. (1989) Biochim. Biophys. Acta 980, 241–247.
[19] Moriyama, Y. and Nelson, N. (1989) J. Biol. Chem. 264, 18445–18450.
[20] Tokuyasu, K.T. (1989) Histochem. J. 21, 163–171.
[21] Schaffner, W. and Weismann, C. (1973) Anal. Biochem. 56, 502–514.
[22] Gogol, E.P., Aggeler, R., Sagermann, M. and Capaldi, R.A. (1989) Biochemistry 28, 4717–4724.
[23] Kanaseki, T. (1979) in: Neurobiology of Chemical Transmission (Ohtsuka, M. and Hall, Z.W. eds) pp. 13–24, J. Wiley, New York.
[24] Stadler, S. and Tsukita, S. (1984) EMBO J. 3, 3333–3337.
[25] Brown, D., Gluck, S. and Hartwig, J. (1987) J. Cell Biol. 105, 1637–1648.
[26] Bowman, B.S., Dshida, W.J., Harris, T. and Bowman, E.J. (1989) J. Biol. Chem. 15606–15612.
[27] Taiz, S.L. and Taiz, L. (1991) Bot. Acta 104, 117–121.
[28] Klink, R. and Luttge, U. (1991) Bot. Acta 104, 122–131.
[29] Moriyama, Y. and Futai, M. (1990) J. Biol. Chem. 265, 9165–9170.
[30] Moriyama, Y. and Futai, M. (1990) Biochem. Biophys. Res. Commun. 173, 443–448.