Vol. 173, No. 1, 1990
November 30, 1990

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 443-448

H^+-ATPASE, A PRIMARY PUMP FOR ACCUMULATION OF NEUROTRANSMITTERS, IS A MAJOR CONSTITUENT OF BRAIN SYNAPTIC VESICLES

Yoshinori Moriyama and Masamitsu Futai

Department of Organic Chemistry and Biochemistry, Institute of Scientific and Industrial Research Osaka University, Ibaraki Osaka 567 Japan

Received October 22, 1990

SUMMARY: Upon treatment with sodium carbonate, rat brain synaptic vesicles lost ATP-dependent H^+ transport and released major polypeptide components (about 72, 57, 41, 34 and 33 kDa). These polypeptides, consisting about 15 % of the total protein, were identified as subunits of H^+-ATPase by immunoblotting with antibodies against H^+-ATPase from chromaffin granules. The same treatment also abolished the ATP-dependent, bafilomycin-sensitive uptakes of glutamate, serotonin and γ-aminobutyrate by the synaptic vesicles. These results indicated that H^+-ATPase is a major constituent of the vesicles (consisting about 20 % of their total protein) and is a primary pump for accumulation of neurotransmitters.

Synaptic vesicles, intracellular organelles in neuronal and endocrine cells, play important roles in storage of neurotransmitters and their release by exocytosis (1-7). H^+-ATPase, a multi-subunit protein, is found in the vesicles in which it establishes an electrochemical gradient of protons (8-10). This electrochemical proton gradient has been suggested to be the driving force for accumulation of neurotransmitters including glutamate, γ-amino butyric acid (GABA) and serotonin (6,10-13).

Purified synaptic vesicles have a relatively simple protein composition (5,6), but their H^+-ATPase content has not yet been

Abbreviations: GABA, γ-aminobutyric acid; SDS, sodium dodecylsulfate; SME buffer, 20 mM MOPS-NaOH (pH 7.5) containing 0.3 M sucrose and 5 mM EDTA.

0006-291X/90 $1.50

reported. In this work we found that H^+-ATPase is a major constituent of the vesicles. We also obtained evidence for energy coupling between the H^+-ATPase and transport of neurotransmitters.

MATERIALS AND METHODS

Materials. [^{125}I]-protein A (0.5 μCi/ug) was purchased from ICN Biomedicals Inc. 5-[1,2-^{3}H(N)]-Hydroxytryptamine ([^{3}H] serotonin) (30 Ci/m mol), L-(G-^{3}H) glutamic acid (46 Ci/m mol),and 4-amino-n-(2,3-^{3}H)butyric acid (99.6 Ci/m mol) ([^{3}H] GABA) were obtained from New England Nuclear or Amersham. Monoclonal antibody to synaptophysin was from PROGEN. Bafilomycin A1 was kindly donated by Dr. Altendorf (Osnabrück, FRG). Other chemicals were commercial products of analytical grade.

Preparations. Synaptic vesicles were purified from rat brain as described by Huttner et al.(5) except that 10 mM MOPS-NaOH buffer (pH 7.5) containing 0.3 M sucrose, 5 mM EDTA, 2 μg/ml leupeptin and pepstatin A (SME buffer) was used for their isolation. Synaptic vesicles were precipitated by centrifugation, suspended in SME buffer and stored at - 80°C. H^+-ATPase from chromaffin granule membranes was purified and reconstituted as described previously (14). Antibodies against individual subunits of chromaffin granule H^+-ATPase were raised by injection of H^+-ATPase or electro-eluted subunits into albino rabbits as described previously (15).

Alkaline treatment. Synaptic vesicles (about 1 mg protein/ml) in 0.3 M sucrose, 5 mM EDTA and 1 % sodium carbonate (pH 11) were incubated on ice for 1 h, collected by centrifugation at 280,000 g for 1 h, suspended in SME buffer and kept on ice bath until use.

Transport Assays. The transports of serotonin and GABA into synaptic vesicles were assayed as follows: synaptic vesicles (about 20 μg protein) were introduced into 20 mM MOPS-Tris buffer (pH 7.0) containing 0.1 M KCl, 0.2 M sucrose and 5 mM MgATP (total vol. 0.5 ml). Assays were started at room temperature by the addition of 1 μM (1 μCi) [^{3}H]serotonin or 0.1 mM (5 μCi) [^{3}H]GABA. Aliquots (150 μl) were taken at intervals, and filtered through 0.45 μm Millipore filters (type HA). The filters were washed with 5 ml of 20 mM MOPS-Tris (pH 7.0) containing 0.1 M KCl, and their radioactivity was counted in a liquid scintilation counter. Glutamate transport was also examined in the same way except that the reaction mixture consisted of 20 mM MOPS-Tris (pH 7.0) containing 0.3 M sucrose, 4 mM KCl and 0.1 mM (2.5 μCi) [^{3}H]-L-glutamic acid.

Other procedures. Published methods were used for polyacrylamide gel electrophoresis in the presence of SDS (16), immunoblotting (9), autoradiography (9), measurement of ATP-dependent H^+-transport (17), cold inactivation of vacuolar H^+-ATPase (9,15,17,18) and protein measurement (19).

RESULTS AND DISCUSSION

H^+-ATPase as a major constituent of synaptic vesicles

Purified synaptic vesicles from rat brain showed vacuolar type H^+-ATPase activity (8-10), which was inactivated by

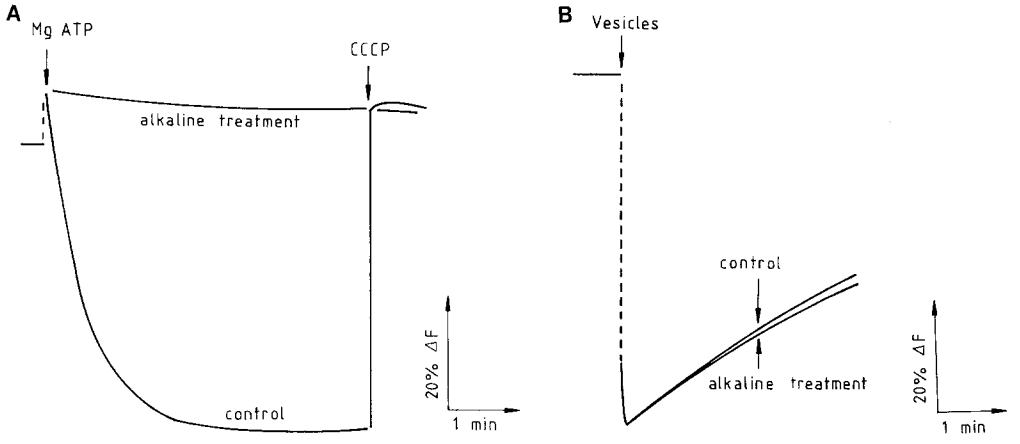

Fig. 1. Alkaline treatment inhibited ATP-dependent H^+-transport in brain synaptic vesicles without changing permeability to protons.
A. ATP-dependent H^+-transport was measured in 20 mM MOPS-Tris (pH 7.0) containing 0.1 M KCl, 0.2 M sucrose, 1 μg valinomycin, 2 μM acridine orange and synaptic vesicles (20 μg protein). MgATP (0.5 mM) and carbonylcyanide m-chlorophenylhydrazone (CCCP) (0.5 μM) were added as indicated.
B. Passive diffusion of protons. Synaptic vesicles (0.5 mg/ml) were incubated in 40 mM MES-Tris (pH 5.5) containing 0.3 M sucrose at room temperature for 1 h. Then an aliquot (20 μl) was rapidly mixed with 20 mM Bicine-Tris (pH 8.0) containing 0.3 M sucrose and 2 μM acridine orange. Passive diffusion of protons from the vesicles was measured as recovery of fluorescence.

carbonate treatment (Fig. 1-A). This treatment did not change the H^+ permeability of the vesicles, since the rates of decrease of an artificial ΔpH across the membranes before and after treatment were similar (Fig. 1-B). These results indicated that H^+-ATPase was inactivated directly by its treatment.

Alkaline treatment resulted in the releases of several major polypeptides from the vesicles, as shown by gel electrophoresis (Fig. 2-A). These polypeptides had similar mobilities to those of hydrophilic subunits (A to E) of chromaffin granule H^+-ATPase. Densitometric tracing of the gel indicated that the polypeptides released amounted to about 15 % of the total membrane proteins (Fig. 2-B). Antibodies against subunits A, B and D of chromaffin granule H^+-ATPase reacted with the released proteins (Fig. 3, lane 4) and their corresponding proteins in the membranes (Fig. 3, lane 2 and 3). These results indicated that the polypeptides

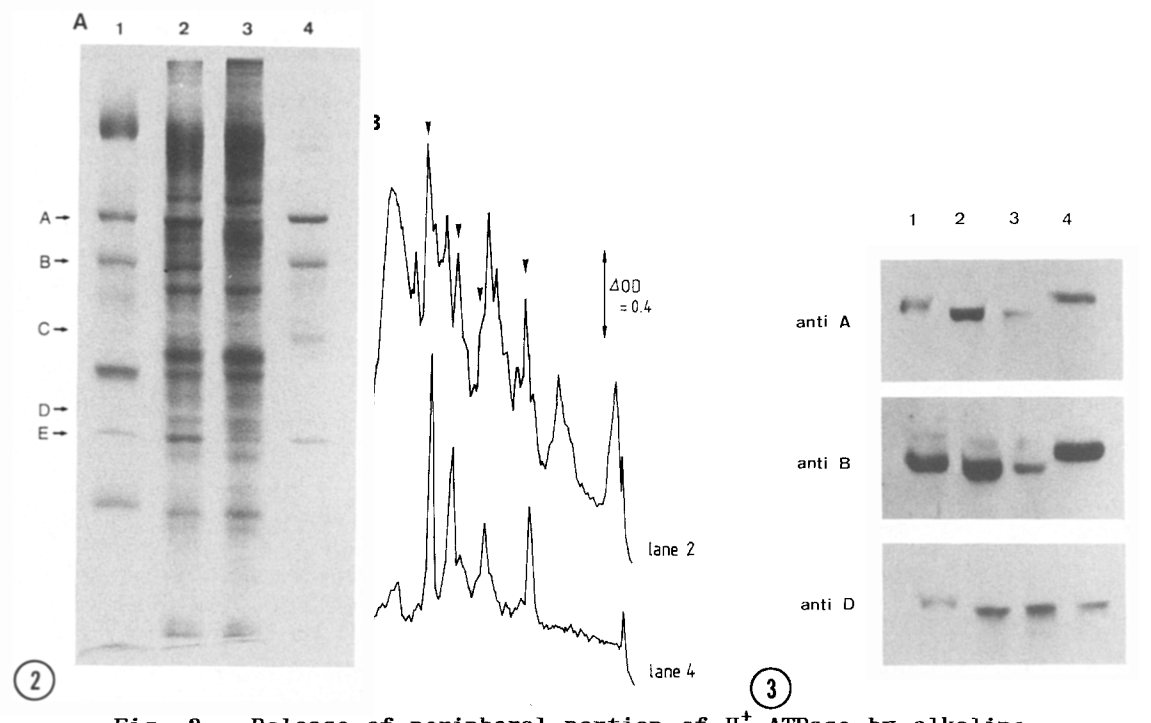

Fig. 2. Release of peripheral portion of H^+-ATPase by alkaline treatment.

Synaptic vesicles (1 mg/ml) were treated with Na_2CO_3 and hydrophilic protein released and depleted membranes were obtained as described in the text.

A. The protein composition of each fraction was analyzed by polyacrylamide gel electrophoresis in the presence of SDS and staining with Coomassie Brilliant Blue. Lane 1, vacuolar type H^+-ATPase from chromaffin granules (5 μg) as a standard; lane 2, synaptic vesicles (20 μg); lane 3, depleted membranes after alkaline treatment (20 μg); lane 4, supernatant (hydrophilic proteins) after alkaline treatment (4 μg). The polypeptides of the membranes seemed to migrate faster than the corresponding polypeptides in the released fraction, due to overloading of the gel with the membrane fraction (data not shown).

B. Densitometric tracings of lanes 2 and 4. Peaks (lane 2) corresponding to the released polypeptides are marked.

Fig. 3. Immunological identification of polypeptides from synaptic vesicles as subunits of H^+-ATPase.

After gel electrophoresis as described in the legend of Fig. 2, protein bands were tranferred onto nitrocellulose sheets and treated with antibodies (diluted 1000-fold) against subunit A, B or D of chromaffin granule H^+-ATPase: lane 1, chromaffin granule H^+-ATPase (5 μg) as a standard; lane 2, synaptic vesicles (20 μg); lane 3, depleted membranes after alkaline treatment (20 μg); lane 4, supernatant after alkaline treatment (4 μg). Subunits bound with antibodies were detected with iodinated protein A. Only portions of gels are shown.

released by alkaline treatment were hydrophilic subunits of H^+-ATPase. As the H^+-ATPase also has four hydrophobic subunits (9), it must be the major constituent of the synaptic vesicles, comprising around 20 % of the total proteins.

Table I. Inhibition of ATP-dependent uptake of neurotransmitters by synaptic vesicles

treatment	glutamate	serotonin	GABA
control	450 (100)	76 (100)	60 (100)
bafilomycin (75 nM)	30 (7)	0 (0)	0 (0)
alkaline	15 (3)	3 (4)	5 (8)
cold	5 (1)	8 (11)	10 (17)

Uptakes of neurotransmitters are expressed as initial velocities (p mole/min/mg protein). Relative activities are shown in parentheses.

Energy coupling between H^+-ATPase and uptake of neurotransmitters

Vacuolar H^+-ATPase is suggested to be a primary pump for the uptakes of neurotransmitters (6,9,10). In fact, bafilomycin, cold or alkaline treatment, which inactivated the vacuolar H^+-ATPase, inhibited ATP-dependent glutamate uptake (Table I)(20). These treatments also caused almost complete inhibitions of the ATP-dependent uptakes of GABA and serotonin (Table I), indicating that the transport systems for glutamate, GABA and serotonin were energetically coupled with the H^+-ATPase.

In this work, several proteins in the synaptic vesicles were identified as subunits of vacuolar H^+-ATPase. It would be interesting to know which proteins are responsible for the uptakes of neurotransmitters. Identification and purification of these transport carriers seems the next step in understanding the structure and function of synaptic vesicles.

Acknowledgments. This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan, a grant for a Research Program on "Creation of New Materials through Intelligent Design" of ISIR, Osaka University, and a Human Frontier Science Program Research Grant.

REFERENCES

1 Boarder, M. R. (1989) J. Neurochem. 53, 1-11.
2 Walker, J. H., and Agoston, D. V. (1987) Biochem. J. 247, 249-258.

3 De Camilli, P., and Novone, F. (1987) Ann. NY. Acad. Sci. 493, 461-479.
4 Trimble, W. S., and Schelle, R. H. (1988) Trends. Neurosci. 11, 241-242.
5 Huttner, W. B., Schiebler, W., Greengard, P, G., De Camilli, P. (1983) J. Cell Biol. 96, 1374-1386.
6 Hell, J. W., Maycox, P. R., Stadler, H., and Jahn, R. (1988) EMBO. J. 7, 3023-3029.
7 Wiedenmann, B., and Franke, W. W. (1985) Cell 41, 1017-1028.
8 Moriyama, Y., and Nelson, N. (1989) Molecular Structure, Function, and Assembly of The ATP Synthase. Marzuki, S., ed. pp 127-137. Plenum Press.
9 Moriyama, Y., and Nelson, N. (1989) Biochim. Biophys. Acta 980, 241-247.
10 Cidon, S., and Sihra, T. S. (1989) J. Biol. Chem. 264, 8281-8288.
11 Johnson, R. G. (1988) Physiol. Rev. 68, 232-307.
12 Njus, D., Kelly, P. M., and Harnadek, G. J. (1986) Biochim. Biophys. Acta 853, 237-265.
13 Ueda, T. (1986) in Excitatory Amino Acids: Robert, P. J., Storm-Mathisen, J., and Branford, H. F. eds, pp. 173-195. MacMillian Press
14 Moriyama, Y., and Nelson, N. (1987) J. Biol. Chem. 262, 9175-9180.
15 Moriyama, Y., and Nelson, N. (1989) J. Biol. Chem. 264, 3577-3582.
16 Douglas, M. G., and Butow, R. A. (1976) Proc. Natl. Acad. Sci. USA 73, 1083-1086.
17 Moriyama, Y., and Futai, M. (1990) J. Biol. Chem. 265, 9165-9170.
18 Moriyama, Y., and Nelson, N. (1989) J. Biol. Chem. 264, 18445-18450.
19 Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.
20 Moriyama, Y., Maeda, M., and Futai, M. (1990) J. Biochem. 108, 689-693.