Ca\(^{2+}\) Transport by Reconstituted Synaptosomal ATPase Is Associated with H\(^{+}\) Countertransport and Net Charge Displacement*  

(Received for publication, March 13, 1998, and in revised form, April 24, 1998)  

Jesús M. Salvador‡, Giuseppe Inesi§, Jean-Louis Rigaud¶, and Ana M. Mata†  
From the Departamento de Bioquímica y Biología Molecular y Genética, Facultad de Ciencias, Universidad de Extremadura, 06071 Badajoz, Spain, the "Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland 21201, and the "Institute Curie, Section de Recherche, UMR-CNRS 168 and LRC-CEA 8,11 Rue P. et Marie Curie, 75231 Paris CEDEX, France  

The synaptosomal plasma membrane Ca\(^{2+}\)-ATPase (PMCA) purified from pig brain was reconstituted with liposomes prepared by reverse phase evaporation at a lipid to protein ratio of 150/1 (w/w). ATP-dependent Ca\(^{2+}\) uptake and H\(^{+}\) ejection by the reconstituted proteoliposomes were demonstrated by following light absorption and fluorescence changes undergone by arsenazo III and 8-hydroxy-1,3,6-pyrene trisulfonate, respectively. Ca\(^{2+}\) uptake was increased up to 2–3-fold by the H\(^{+}\) ionophore carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone, consistent with relief of an inhibitory transmembrane pH gradient (i.e. lumenal alkalization) generated by H\(^{+}\) countertransport. The stoichiometric ratio of Ca\(^{2+}/H^{+}\) countertransport was 1.0/0.6, and the ATP/Ca\(^{2+}\) coupling stoichiometry was 1/1 at 25°C. The electrogenic character of the Ca\(^{2+}/H^{+}\) countertransport was demonstrated by measuring light absorption changes undergone by oxonol VI. It was shown that a 20 mV steady state potential (positive on the lumenal side) was formed as a consequence of net charge transfer associated with the 1/1 Ca\(^{2+}/H^{+}\) countertransport. Calmodulin stimulated ATPase activity, Ca\(^{2+}\) uptake, and H\(^{+}\) ejection, demonstrating that these parameters are linked by the same mechanism of PMCA regulation.

Regulation of the intracellular free Ca\(^{2+}\) concentration in nerve terminals is a key factor in the mechanism of synaptic transmission (1, 2). Reduction of cytosolic Ca\(^{2+}\) in nerve terminals is dependent on removal by the plasma membrane Ca\(^{2+}\)-ATPase (PMCA)\(^1\) and sequestration by the intracellular (SERCA) Ca\(^{2+}\)-ATPase (3). Coexistence of both ATPases, as well as the presence of various transport and permeability channels in synaptosomal preparations (4), has rendered difficult a detailed characterization of the synaptosomal PMCA with regards to Ca\(^{2+}\) and H\(^{+}\) transport and net charge trans-
Ca<sup>2+</sup>/H<sup>+</sup> Countertransport through Synaptosomal Ca<sup>2+-</sup>ATPase

**RESULTS**

**Purification and Reconstitution of Synaptosomal PMCA—**

The pig brain PMCA was purified from solubilized synaptic vesicles by calmodulin affinity chromatography. Electrophoretic analysis of the synaptic vesicles (Fig. 1, lane 1) demonstrates the presence of several protein bands in addition to the PMCA which is hardly visible because of its very low quantity. In contrast, the purified protein eluted from the calmodulin column appears as a single band of apparent 140-kDa molecular weight (Fig. 1, lane 2). We found that the purified enzyme had an ATPase activity of 1.10 ± 0.10 IU in the presence of pure EPC, as compared with 0.063 ± 0.011 IU in the solubilized synaptosomal vesicles.

**ATP-dependent Ca<sup>2+</sup> Uptake—**The pig brain purified PMCA was reconstituted into EPC/EPA liposomes at a lipid/protein ratio of 150/1 (w/w). The reconstitution procedure was performed by detergent removal from lipid-protein-detergent microsomal suspensions by Bio-Beads SM-2 as detergent removing agent. The reconstitution procedure and Ca<sup>2+</sup> transport activity were optimized by using different detergents and ionic conditions. The highest ATP-dependent Ca<sup>2+</sup> uptake was obtained when C<sub>p</sub>E<sub>p</sub> was used as a detergent in the reconstitution procedure, and Cl<sup>−</sup> (rather than SO<sub>4</sub><sup>2−</sup> or isothiocyanate) was used as the prevailing anion in the reaction medium. Similar findings were reported by Hao et al. (12) for reconstitution of the erythrocyte PMCA.

**Calmodulin 0.09**

**ATPase Activity—**We have studied under similar experimen-
tural conditions the effects of calmodulin, $H^+$- and $Ca^{2+}$-ionophores, and vanadate upon the $Ca^{2+}$-ATPase hydrolytic activity of the reconstituted enzyme (Fig. 3). In the presence of 0.2 mM ATP, we observed hydrolytic rates of $0.21 \pm 0.02 \mu$mol of P/mg of protein/min $^{-1}$. This activity was stimulated by addition of calmodulin, FCCP, and A23187. The initial rate was increased 2-fold by calmodulin (Table I). FCCP did not affect the initial rate, but increased the ATPase activity only after an initial period of activity, i.e. only when a pH gradient was present. The addition of vanadate totally inhibited the ATPase activity. A comparison of the initial rates of $Ca^{2+}$ transport and ATPase activity yields a coupling stoichiometry of 1/1 at 25 °C, independently of the presence of calmodulin and/or FCCP.

$ATP$-dependent $H^+$ Ejection—$H^+$ ejection from the lumen of the proteoliposomes, associated with $ATP$-dependent $Ca^{2+}$ uptake was demonstrated directly by measuring changes in luminal pH. This was accomplished by monitoring the fluorescence intensity of the pH indicator pyranine, which was trapped in the lumen of the proteoliposomes during the reconstitution procedure. Upon $ATP$ addition (Fig. 4A), the initial rate of $H^+$ extrusion calculated after 30 s was approximately $0.1 \mu$mol of $H^+$/mg of protein/min $^{-1}$. Analogous to $Ca^{2+}$ uptake, $H^+$ ejection was stimulated by calmodulin (see Table I and Fig. 4B) to a rate of approximately $0.26 \pm 0.05 \mu$mol of $H^+$/mg of protein/min $^{-1}$, reaching in 5 min a maximal amount of $0.36 \pm 0.04 \mu$mol of $H^+$ ejected/mg of protein. It is noteworthy that although $H^+$ ejection was not significantly affected by valinomycin in the presence of K$^+$, FCCP decreased the fluorescence signal to the original level. Therefore, $H^+$ ejection and formation of pH gradient are not driven by any electrical gradient, but are primary events linked to $ATP$-dependent $Ca^{2+}$ uptake in the form of a $Ca^{2+}/H^+$ countertransport. Our measurements indicate that the coupling stoichiometry of $Ca^{2+}$ uptake and $H^+$ ejection is 1.0/0.6.

$Electrical$ $Potential$—The net charge displacement associated with the observed stoichiometry of $Ca^{2+}/H^+$ countertransport suggests that a transmembrane electrical potential is generated across the proteoliposomal membrane as a consequence of this process. In fact, we observed the development of a transmembrane electrical potential by measuring light absorption undergone by oxonol VI. It is shown in Fig. 5 that activation of the $Ca^{2+}$ pump by addition of ATP under optimal conditions produces a steady-state transmembrane potential of $19 \pm 2.1$ mV. Addition of valinomycin, a $K^+$ ionophore, collapses the electrical potential by rendering the membrane permeable to $K^+$ and thereby compensating for the uneven charge displacement.

**DISCUSSION**

The aim of this study was a characterization of $Ca^{2+}$ transport by the plasma membrane $Ca^{2+}$-ATPase from pig brain in terms of countertransport and electrogenic properties. The study of ion fluxes was rendered possible by the use of PMCA reconstituted in proteoliposomes with a low electrolyte perme-
The reconstituted synaptosomal PMCA sustains a Ca\(^{2+}\) transport rate up to 0.4 μmol of Ca\(^{2+}\)/mg of protein-min\(^{-1}\), similar to those obtained by Niggli et al. (13) and Hao et al. (12) with reconstituted erythrocyte PMCA. In addition, the low lipid/protein ratio used for reconstitution has provided a large luminal volume per ATPase molecule, thereby permitting accumulation of large amounts of Ca\(^{2+}\) and relatively long experimental times before establishment of an inhibitory Ca\(^{2+}\) concentration in the lumen of the proteoliposomes. The 1/1 stoichiometric ratio of Ca\(^{2+}\) uptake and ATP hydrolysis obtained by measurements of initial rates (Table I) is in the same range as that reported with the reconstituted erythrocyte PMCA (12, 13), but lower than the ratio of 2/1 obtained with SERCA (9). This difference may be related to intrinsic characteristics of the two families of Ca\(^{2+}\) pumps (23–25).

The use of the proteoliposomal reconstituted system turned out to be very advantageous in demonstrating that the synaptosomal PMCA pump operates Ca\(^{2+}\)/H\(^{+}\) countertransport. Collapse of the related H\(^{+}\) gradient by the H\(^{+}\) ionophore FCCP, and not by the K\(^{+}\) ionophore valinomycin, is consistent with direct Ca\(^{2+}\)/H\(^{+}\) countertransport through the pump, rather than H\(^{+}\) extrusion secondary to electrical potential. The Ca\(^{2+}\)/H\(^{+}\) stoichiometric ratio of 0.6 found in the synaptosomal plasma membrane Ca\(^{2+}\)-ATPase, is similar to the ratios reported for the other Ca\(^{2+}\)-ATPases. For example, Hao et al. (12) obtained a stoichiometric Ca\(^{2+}\)/H\(^{+}\) ratio of 1 with the reconstituted erythrocyte PMCA at 12 °C temperature but a lower ratio at 30 °C. Although the Ca\(^{2+}\)/H\(^{+}\) ratio may be primarily determined by the stoichiometry and pKs of acidic residues operating the Ca\(^{2+}\)/H\(^{+}\) exchange (18), it is possible that higher temperatures and/or other experimental variable may produce H\(^{+}\) slippage (26). The uneven charge exchange produced by the observed Ca\(^{2+}\)/H\(^{+}\) ratios is consistent with the development of transmembrane electrical potential following addition of ATP (Fig. 5).

The Ca\(^{2+}\)/H\(^{+}\) countertransport and the net charge transfer demonstrated with purified and reconstituted brain PMCA will be useful in understanding Ca\(^{2+}\) regulation in synaptosomal function. Furthermore, demonstration of Ca\(^{2+}\)/H\(^{+}\) countertransport in several Ca\(^{2+}\) transport ATPases indicates that a shift in the affinity of protein carboxylic functions for H\(^{+}\) and Ca\(^{2+}\) occurs upon formation and cleavage of phosphorylated enzyme intermediate and that this is a common mechanistic feature in the coupling of ATP utilization and Ca\(^{2+}\) transport.

REFERENCES
1. Gill, D. L., Grollman, E. F., and Khon, L. D. (1981) J. Biol. Chem. 256, 184–192
2. Sorensen, R. G., and Mahler, H. R. (1981) J. Neurochem. 37, 1407–1418
3. Michaelis, M. L. (1994) Annu. N. Y. Acad. Sci. 747, 407–418
4. REJA, A. F. (1986) in The Ca\(^{2+}\) Pump of Plasma Membranes (Rega, A. F., and Garrahan, P. J., eds) pp. 45–48, CRC Press, Boca Raton, FL
5. Salvador, J. M., and Mata, A. M. (1996) Biochem. J. 315, 183–187
6. Salvador, J. M., and Mata, A. M. (1996) Biochem. Biophys. Acta 351, 272–278
7. Chiesi, M., and Inesi, G. (1988) Biochemistry 19, 2912–2918
8. Levy, D., Seigneur, A., Bluzat, A., and Rigaud, J. L. (1990) J. Biol. Chem. 265, 19524–19534
9. Yu, X., Carrel, S., Rigaud, J. L., and Inesi, G. (1993) Biophys. J. 64, 1232–1242
10. Waisman, D. M., Gimble, J. M., Goodman, D. B. P., and Rasmussen, H. (1981) J. Biol. Chem. 256, 415–419
11. Gassner, B., Luterbacher, S., Schatzman, H. J., and Wuthrich, A. (1988) Cell Calcium 2, 95–103
12. Hao, L., Rigaud, J. L., and Inesi, G. (1994) J. Biol. Chem. 269, 14268–14275
13. Niggli, V., Sigel, E., and Carafoli, E. (1982) J. Biol. Chem. 257, 2356–2356
14. Rigaud, J. L., Peltier, B., and Levy, D. (1990) Biochim. Biophys. Acta 1231, 223–246
15. Rigaud, J. L., Paternostre, M. T., and Bluzat, A. (1988) Biochemistry 27, 2677–2688
Ca\textsuperscript{2+}/H\textsuperscript{+} Countertransport through Synaptosomal Ca\textsuperscript{2+}-ATPase

16. Pitard, B., Richard, P., Dunach, M., Girault, G., and Rigaud, J. L. (1996) Eur. J. Biochem. 235, 769–778
17. Levy, D., Golik, A., Bluzat, A., and Rigaud, J. L. (1992) Biochim. Biophys. Acta 1107, 283–298
18. Yu, X., Hao, L., and Inesi, G. (1994) J. Biol. Chem. 269, 16656–16661
19. Rigaud, J. L. Bluzat, A., and Bluschlen, S. (1983) Biochem. Biophys. Res. Commun. 111, 373–382
20. Scarpa, A. (1979) Methods Enzymol. 56, 301–338
21. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
22. Laemmli, U. K. (1970) Nature 227, 680–685
23. Inesi, G., and Kirtley, M. R. (1992) J. Bioenerg. Biomembr. 24, 271–283
24. Raeymaekers, L., and Wuytack, F. (1993) J. Muscle Res. Cell. Motil. 14, 141–157
25. Carafoli, E., and Guerini (1997) in Calcium Cell Metabolism, Transport and Regulation (Sotelo, J. R., and Benech, J. C., eds) pp. 73–84, Plenum Press, NY
26. Yu, X., and Inesi, G. (1993) FEBS Lett. 328, 301–304