THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1988 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 263, No. 1, Issue of January 5, pp. 340-343, 1988
Printed in U.S.A.

Endoplasmic Reticulum of Rat Liver Contains Two Proteins Closely Related to Skeletal Sarcoplasmic Reticulum Ca-ATPase and Calsequestrin*

(Received for publication, July 20, 1987)

Ernesto Damiani‡§, Cornelia Spamer¶, Claus Heilmann¶, Sergio Salvatori‡, and Alfredo Margreth‡

From the ‡National Research Council Unit for Muscle Biology and Physiopathology, Institute of General Pathology, Via Loredan 16, 35100 Padova, Italy and the ¶Department of Gastroenterology, Medizinische Universitatklinik, 7800 Freiburg, Federal Republic of Germany

Rat liver endoplasmic reticulum (ER) membranes were investigated for the presence of proteins having structural relationships with sarcoplasmic reticulum (SR) proteins. Western immunoblots of ER proteins probed with polyclonal antibodies raised against the 100-kDa SR Ca-ATPase of rabbit skeletal muscle identified a single reactive protein of 100 kDa. Also, the antibody inhibited up to 50% the Ca-ATPase activity of isolated ER membranes. Antisera raised against the major intraluminal calcium binding protein of rabbit skeletal muscle SR, calsequestrin (CS), cross-reacted with an ER peptide of about 63 kDa, by the blotting technique. Stains-All treatment of slab gels showed that the cross-reactive peptide stained metachromatically blue, similarly to SR CS. Two-dimensional electrophoresis (Michalak, M., Campbell, K. P., and MacLennan, D. H. (1980) *J. Biol. Chem.* 255, 1317–1326) of ER proteins showed that the CS-like component of liver ER, similarly to skeletal CS, fell off the diagonal line, as expected from the characteristic pH dependence of the rate of mobility of mammalian CS. In addition, the CS-like component of liver ER was released from the vesicles by alkaline treatment and was found to be able to bind calcium, by a ^{45}Ca overlay technique. From these findings, we conclude that a 100-kDa membrane protein of liver ER is the Ca-ATPase, and that the peripheral protein in the 63-kDa range is closely structurally and functionally related to skeletal CS.

Many physiological functions of animal cells, depending on specific tissue differentiation, are controlled by the cytosolic free calcium concentration (1), such as the contraction-relaxation cycle of skeletal muscle and secretion in glandular tissue cells (1). In skeletal muscle, the free calcium concentration is in turn regulated by the sarcoplasmic reticulum (SR),[1] through its dual control on the membrane calcium fluxes, *i.e.* on calcium release, and on the active transport and reaccumulation of calcium into the lumen of the terminal cisternae where calcium binds to the protein calsequestrin (CS) (2, 3), through the Ca-ATPase pump (4), localized in the extrajunctional SR (5).

Recent experimental evidence supports the view that some aspects in membrane composition and properties, so far assigned exclusively to muscle SR, may be shared by the endoplasmic reticulum (ER) of non-muscle cells. Electron probe analysis of intracellular calcium stores of liver cells (6, 7), and electron microscopy of rat pancreas cells following calcium uptake in the presence of oxalate (8), showed that calcium accumulates at discrete intracellular sites corresponding to ER. Furthermore, ER isolated from rat liver (9, 10) and pancreas (8) showed a Ca-ATPase activity, and an oxalate facilitated Ca-transport system sharing biochemical and kinetic properties with the Ca-transport system of skeletal muscle SR (11–13). A protein of approximately the same molecular size of skeletal SR Ca-ATPase was tentatively identified as the Ca-ATPase of ER of liver (11, 14) and of pancreas (13) cells, based on its ability to form an acid-stable, Ca-dependent phosphoprotein intermediate, as for the SR Ca-ATPase (4).

Here, we demonstrate that rat liver ER contains a protein having molecular weight properties closely similar to those of the Ca-ATPase of rabbit skeletal muscle SR, and that the two proteins share antigenic determinants. We also provide new evidence for the presence in rat liver ER of an extrinsic protein which shares extensive electrophoretic and calcium binding ability, as well as antigenic properties, with skeletal CS.

MATERIALS AND METHODS

All chemicals were obtained from Sigma, Merck A. G. (Darmastadt, Germany), or Carlo Erba (Milano, Italy). Pure nitrocellulose paper was purchased from Bio-Rad. The cationic dye Stains-All, (1-ethyl-2-[3-(1-ethylnaphthol[1,2*d*]thiazolin-2-ylidene)-2-methylpropenyl)-naphtho[1,2d]thiazolium bromide), was obtained from Sigma Chemical Co. $^{45}CaCl_2$ was from the Radiochemical Centre (Amersham, England).

Preparative Procedures—Liver endoplasmic reticulum (ER) membranes were prepared from male Wistar rats (body weight, 150–200 g), as previously described (11, 12). Sarcoplasmic reticulum (SR) membranes were isolated from rabbit fast-twitch muscle, according to Saito *et al.* (15). ER membranes were extracted by treating with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (16).

Biochemical Assay—ATPase activity was determined at 37 °C with an enzyme-coupled spectrophotometric ADP-release assay as described (17). Basal, Mg-stimulated ATPase was measured in the presence of 0.2 mM EGTA. Extra Ca-ATPase was measured after adding 0.05 mM $CaCl_2$.

* This investigation was supported by institutional funds from the Consiglio Nazionale Celle Ricerche and, in part, by grants from the Ministero della Pubblica Istruzione and Legato Dino Ferrari to A. M. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence and reprint requests should be addressed.

[1] The abbreviations used are: SR, sarcoplasmic reticulum, ER, endoplasmic reticulum; CS, calsequestrin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

This is an Open Access article under the CC BY license.

Protein concentration was determined by the method of Lowry *et al.* (18), with bovine serum albumin as a standard.

Gel Electrophoresis—One-dimensional SDS-PAGE was carried out according to Laemmli (19). Two-dimensional gel electrophoresis was carried out according to Michalak *et al.* (20). The first dimension was a disc-gel (21), and the second dimension was a regular Laemmli gel. Slab gels were stained with Stains-All, according to Campbell *et al.* (22).

Immunological Techniques—Chicken anti-(rabbit SR Ca-ATPase) IgG antibodies, previously characterized for specificity (17, 23, 24), were used. Chicken anti-(rabbit calsequestrin) antiserum was raised in adult hens, following the same immunization schedule previously described (25). One-step ELISAs were carried out as previously described (22). Western immunoblots of slab gels were carried out as described (17), except that the final color development was carried out according to Leary *et al.* (26). Before immunostaining, blots were stained with Ponceau red.

Identification of Calcium Binding Proteins—SR and ER calcium binding proteins were identified by a "^{45}Ca-overlay" technique (27). SR and ER proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose sheet. Blots were stained with Ponceau red, destained with distilled water, soaked in 5 mM EGTA, pH 7.0, for 1 h, and then, after washing with 5 mM imidazole to remove EGTA, were incubated for 20 min at room temperature with 10 μM $^{45}CaCl_2$, in a medium containing 5 mM imidazole, pH 7.0, 5 mM $MgCl_2$, and 60 mM KCl. Blots were washed four times for 5 min with 0.9% NaCl, 0.1% Tween 20, and ^{45}Ca-labeled proteins were visualized by autoradiography (Hyperfilm betamax, Amersham, England).

RESULTS

Fig. 1*A*, *lane 1*, shows the pattern of electrophoretically resolved ER proteins from rat liver, stained with Ponceau red after transfer onto nitrocellulose. Under these conditions, a narrow band of M_r - 100,000, a doublet around M_r - 80,000, and additional major bands in the M_r range between 65,000 and 50,000, approximately, are detected. When ER protein blots are probed with chicken antibody to rabbit fast muscle Ca-ATPase of known high specificity and previously shown to be highly cross-reactive with the homologous protein of human (24) and chicken (17) SR, a 100-kDa ER protein was similarly found to be selectively stained by antibody (Fig. 1*A*, *lane 2*). The low content of this peptide in liver ER membranes, as compared to the high content (80% of total protein) of Ca-ATPase in purified SR from skeletal muscle, appears to account mainly for differences in the extent of antibody binding by liver ER and skeletal SR vesicles, as tested by ELISA techniques, either by titrating the antibody (Fig. 1*B*) or the antigen (Fig. 1*C*). This finding implies that the shared antigenic determinants of the ER M_r - 100,000 protein, as in the case of the SR Ca-ATPase, are exposed on the outer surface of the vesicles.

On the other hand, preincubation of ER vesicles with antibody to SR Ca-ATPase for 10 min at 37 °C before assay for ATPase activity resulted in marked inhibition of the Ca-ATPase activity, from 2.99 μmol/h/mg of protein (mean of 2) to 1.53 μmol/h/mg of protein (mean of 4). It was ascertained in control experiments that the Ca-ATPase activity of ER was not inhibited either by normal serum or by phosphate-buffered saline medium alone. Using the same batch of antibody, and under similar experimental conditions, we had previously found a 30% inhibition of the Ca-ATPase activity of SR vesicles from rabbit fast muscle (28).

Fig. 2*A* shows an immunoblot of a SDS gel of rabbit skeletal SR, following incubation with antisera against purified CS and using the same revealing system for antibody binding to protein as for the Ca-ATPase. The selective staining by antibody of CS allows its identification with the protein band of 64 kDa, in agreement with previous knowledge (22). Two additional SR proteins of 160 and 170 kDa, respectively, were found to cross-react with anti-(rabbit CS) antibody (Fig. 2*A*, *lane 1*). These peptides probably correspond to those previously described by Cala and Jones (29), that equally cross-react to anti-CS antibody (30). Under the same conditions, a single, weakly immunoreactive band appears to be present in rat liver ER, having an apparent molecular weight in the 63-kDa range, *i.e.* slightly lower than that previously reported for rat skeletal CS (65 kDa) (25) in Laemmli's system. Evidence in agreement with that is obtained by comparing the extent of cross-reactivity with the same antibody of intact SR and ER vesicles, using ELISA techniques (Fig. 2, *B* and *C*).

To further characterize the ER component cross-reactive with anti-(rabbit CS) antibody, we investigated its staining properties with the carbocyanine dye Stains-All in one-dimensional slab gels and found that, similarly to skeletal CS of mammalian species (22, 25), it stained metachromatically blue (data not shown). Furthermore, when analyzed by using the two-dimensional electrophoretic system of Michalak *et al.* (20), the 63-kDa blue-staining, cross-reactive component of rat liver ER appeared to fall off the diagonal line, as in the

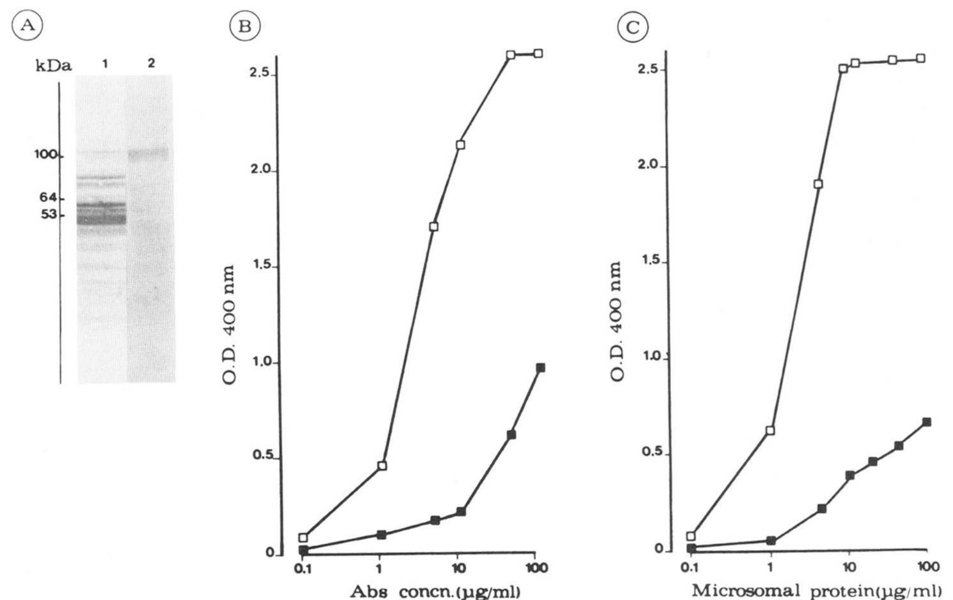

FIG. 1. **Immunological cross-reactivity of ER with anti-(rabbit Ca-ATPase) antibody.** *Panel A*, ER proteins were resolved in a 10% Laemmli gel and transferred onto nitrocellulose. Blots were stained with Ponceau red, destained with distilled water, and then incubated with anti-(rabbit Ca-ATPase) antibody (5 μg/ml). 150 μg of ER protein were loaded. *1*, ER proteins, Ponceau red-stained; *2*, immunostaining of 1. *Panel B*, titration of anti-(rabbit Ca-ATPase) antibody was carried out with microtiter wells coated with 5 μg/ml SR (□) and ER (■) membranes, at the antibody concentrations indicated on the *abscissa*. *Panel C*, titration of antigen was carried out at a fixed amount of antibody (5 μg/ml), with microtiter wells coated with SR (□) and ER (■) membranes, at the protein concentrations indicated on the *abscissa*.

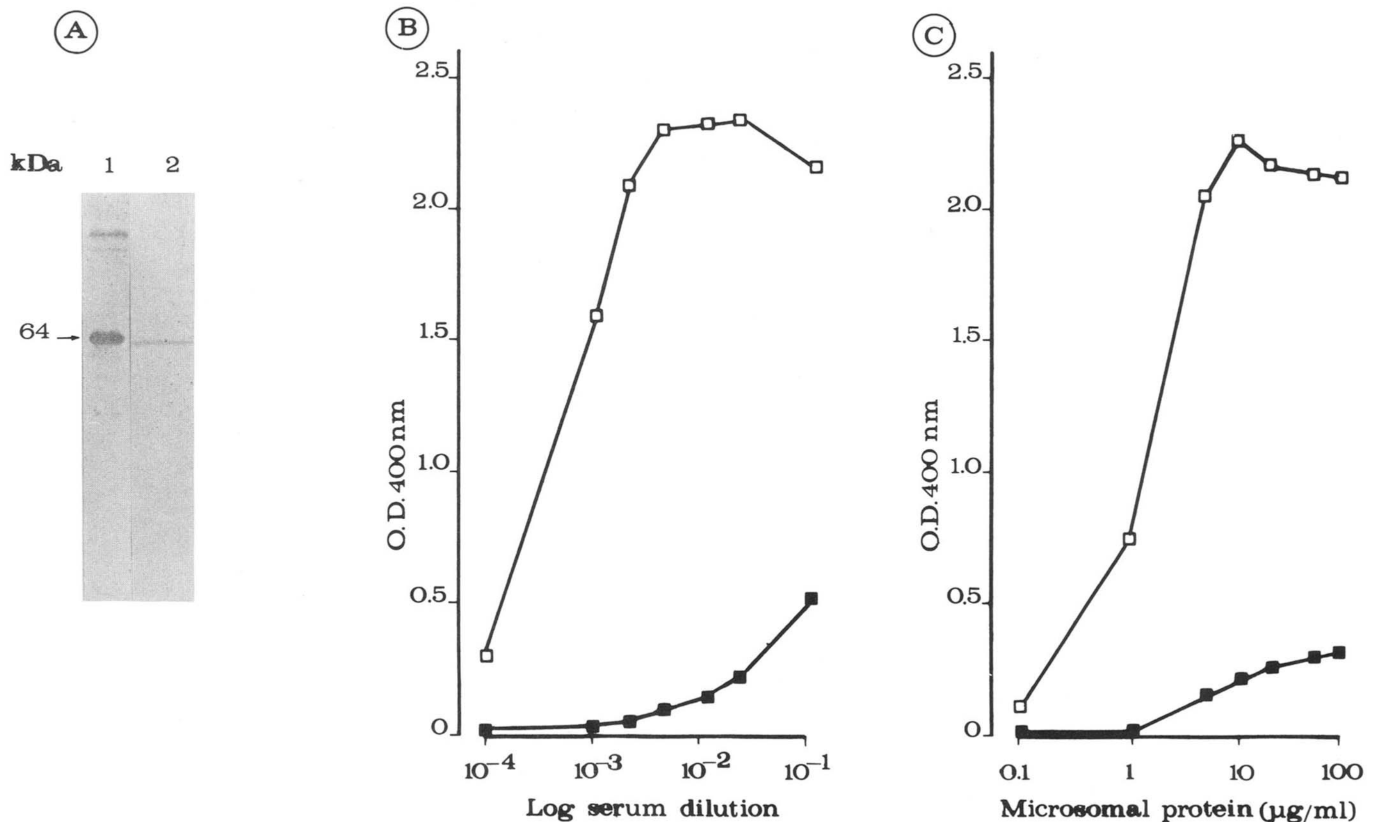

FIG. 2. **Immunological cross-reactivity with anti-(rabbit CS) antiserum.** *Panel A*, immunostaining of SR and ER proteins was carried out as in Fig. 1*A*, with anti-(rabbit CS) antiserum (1:100 dilution). 15 μg of SR and 150 μg of ER protein were loaded. *1*, SR; *2*, ER. *Panel B*, titration of anti-(rabbit CS) antiserum was carried out as in Fig. 1*B*, at the serum dilutions indicated on the *abscissa*. SR, □; ER, ■. *Panel C*, titration of antigen was carried out as in Fig. 1*C* at a fixed dilution of serum (1:100). SR, □; ER, ■.

case of all skeletal muscle CS so far investigated (25) (data not shown). Finally, the 63-kDa component of liver ER could be released from the isolated vesicles by Tris-EDTA treatment at alkaline pH, as originally described by Duggan and Martonosi (16) for rabbit skeletal CS (data not shown).

In order to investigate the calcium binding properties of the CS-like protein of rat liver ER, ^{45}Ca overlay techniques (27) were used, identical with those reported for identifying CS and other calcium binding proteins of rabbit skeletal SR (31). Rabbit SR and rat liver ER protein, following SDS-PAGE analysis, were transferred onto nitrocellulose and were incubated with ^{45}Ca, at high concentrations of MgCl and KCl, to detect specific calcium binding sites. The results are shown in Fig. 3, *lanes 3* and *4*. As expected, CS is found to be the most heavily labeled protein band in skeletal muscle SR (*lane 3*). In the case of rat liver ER, calcium binding proteins are detected as a doublet, corresponding to the 63-kDa protein, and to the faster moving component at its bottom edge. Interestingly, a protein present in the 100-kDa range was also labeled by ^{45}Ca.

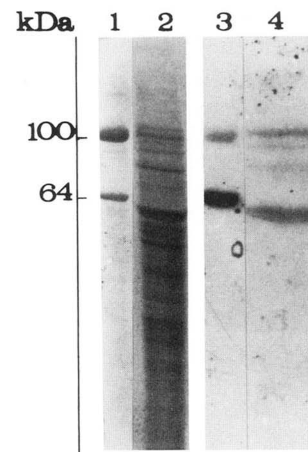

FIG. 3. **Identification of calcium binding proteins of SR and ER membranes.** Blots of SR and ER proteins were stained with Ponceau red (*lanes 1* and *2*), destained with distilled water, and then incubated with $^{45}CaCl_2$, as described under "Materials and Methods." ^{45}Ca-labeled proteins (*lanes 3* and *4*) were visualized by autoradiography, after an 8-day exposure. 30 μg of SR protein (*lanes 1* and *3*) and 150 μg of ER protein (*lanes 2* and *4*) were loaded for each lane.

DISCUSSION

The results reported here, together with previous evidence (11, 12), allow identification of the 100-kDa protein of rat liver ER, as a Ca-ATPase having several properties in common with the Ca-ATPase of skeletal muscle SR. This conclusion rests mainly on our finding that polyclonal antibodies raised against rabbit skeletal SR Ca-ATPase are cross-reactive with antigenic sites of the blotted 100-kDa protein of rat liver ER, as well as with exposed sites of this protein in membrane-bound form. We also find that the same antibody is able to inhibit the Ca-ATPase activity both of SR vesicles and of ER vesicles. That would locate the shared antigenic sites at, or near, the outer projecting catalytic domain of liver ER Ca-ATPase, as in the case of SR membrane Ca-ATPase. That, together with previous evidence (11, 12), suggests that the partial reactions of liver Ca-ATPase do not basically differ

from those of skeletal muscle SR Ca-ATPase.

Previous studies (32) had shown the presence of a 63-kDa calcium binding protein in the high speed supernatant of bovine liver homogenates, which was described as a new protein species named calregulin. Immunocytochemical studies localized this protein to ER membranes (32). Therefore, our present findings suggest a possible relationship between this calcium binding protein (32) and the CS-like peptide of liver ER, as also reported here. On the other hand, the fact that our anti-(CS) antibody appears to be cross-reactive with the CS-like protein of ER (see Fig. 2*A*) would seem to argue against the possible identification of this protein with calregulin, since Khanna and Waisman (33), using antibody specific to calregulin found that skeletal CS and calregulin are immunologically unrelated proteins. Skeletal CS and the putative CS of liver ER share a number of significant properties. Both are intraluminal proteins readily released from the membranes by EDTA treatment at alkaline pH and both are calcium binding proteins, as shown by ^{45}Ca overlay technique. Furthermore, both proteins stain metachromatically blue with the carbocyanine dye Stains-All and show a similar pH dependence of the rate of electrophoretic mobility. Putative CS of rat liver ER is slightly heterogeneous in molecular weight from its skeletal SR counterparts, both of the rat (65 kDa) and of the rabbit (64 kDa) which, in turn, are closely immunologically related proteins (25). We do find, however, that the liver 63-kDa protein is only weakly cross-reactive with antibody to rabbit skeletal CS. A possible explanation for these negative findings is that skeletal and liver calsequestrins, even within the same mammalian species, are antigenically more heterogeneous than are skeletal calsequestrins of different species of mammals, due to tissue-specific differences in primary structure, as earlier suggested by Campbell *et al.* (34) for skeletal and cardiac CS.

Recent experimental evidence supports the view that some properties and components, previously considered to be specific to SR membranes, are instead shared by the ER of non-muscle cells. Electron probe x-ray analysis of rat liver parenchymal cells (6, 7), and of other non-muscle cells (35), and electron microscopy of exocrine pancreas cells (8) showed that the ER is the major storage site for calcium. Furthermore, it was reported that isolated ER vesicles from rat liver share with SR vesicles the ability not only to actively transport calcium (11), but also to release calcium on addition of inositol-(1,4,5)-triphosphate (36), similarly to what has been described for rabbit skeletal SR (37). Therefore, our present results, together with previous findings, encourage the interpretation that liver ER is able to carry out the three basic functions peculiar of SR, *i.e.* calcium uptake, calcium storage, and calcium release. This view is not necessarily in contrast with that according to which a new organelle, called calciosome, would exist, as suggested by Krause *et al.* (38) in human phagocytes, based on a double immunogold labeling technique using antibodies to the SR Ca-ATPase and to CS of rabbit skeletal muscle. In fact, membrane protein specificity in relation to calcium regulation would demand the existence of discrete areas of membrane specialization within the ER system of liver and, possibly, of other non-muscle cells.

Acknowledgment—We appreciate the help of Sandra Furlan, who contributed an invaluable technical assistance to this work.

REFERENCES

1. Carafoli, E. (1982) in *Pathophysiology of Shock, Anoxia and Ischemia* (Cowley, R. A., and Trump, B. F., eds) pp. 95-112, Williams, Wilkins, Baltimore
2. Endo, M. (1977) *Physiol. Rev.* **57,** 71–108
3. Somlyo, A. V., Gonzales-Serratos, H., Shuman, H., McClellan, G., and Somlyo, A. P. (1981) *J. Cell Biol.* **90,** 577–594
4. Tada, M., Yamamoto, T., and Tonomura, Y. (1979) *Physiol. Rev.* **58,** 1–79
5. Jorgensen, A. O., Shen, A. C.-Y., Campbell, K. P., and MacLennan, D. H. (1983) *J. Cell Biol.* **97,** 1573–1581
6. Somlyo, A. P. (1984) *Nature* **309,** 516–517
7. Somlyo, A. P., Meredith, B., and Somlyo, A. V. (1985) *Nature* **314,** 622–625
8. Bayerdorffer, E., Streb, H., Eckhardt, L., Haase, W., and Schulz, I. (1984) *J. Membr. Biol.* **81,** 69–82
9. Kraus-Friedmann, N., Carafoli, E., Biber, J., and Murer, H. (1982) *Ann. N. Y. Acad Sci.* **402,** 440–442
10. Famulski, K., and Carafoli, E. (1982) *Cell Calcium* **3,** 263–281
11. Heilmann, C., Spamer, C., and Gerok, W. (1984) *J. Biol. Chem.* **259,** 11139–11144
12. Heilmann, C., Spamer, C., and Gerok, W. (1985) *J. Biol. Chem.* **260,** 788–794
13. Imamura, K., and Schulz, I. (1985) *J. Biol. Chem.* **260,** 11339–11347
14. Fleschner, C. R., and Kraus-Friedmann, N. (1986) *Eur. J. Biochem.* **154,** 313–320
15. Saito, A., Seiler, S., Chu, A., and Fleischer, S. (1984) *J. Cell Biol.* **99,** 875–885
16. Duggan, P. F., and Martonosi, A. (1970) *J. Gen. Physiol.* **56,** 147–157
17. Damiani, E., Margreth, A., Furlan, A., Dahms, A. S., Arnn, J., and Sabbadini, R. A. (1987) *J. Cell Biol.* **104,** 461–472
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
19. Laemmli, U. K. (1970) *Nature* **227,** 680–685
20. Michalak, M., Campbell, K., and MacLennan, D. H. (1980) *J. Biol. Chem.* **255,** 1317–1326
21. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4410
22. Campbell, K. P., MacLennan, D. H., and Jorgensen, A. (1983) *J. Biol. Chem.* **258,** 11267–11273
23. Betto, R., Damiani, E., Biral, D., and Mussini, I. (1981) *J. Immunol. Methods* **46,** 289–298
24. Salviati, G., Pierobon, S., Betto, R., Damiani, E., Angelini, C., Ringel, S. P., Salvatori, S., and Margreth, A. (1985) *Muscle & Nerve* **1985,** 299–306
25. Damiani, E., Salvatori, S., Zorzato, F., and Margreth, A. (1986) *J. Muscle Res. Cell Motil.* **7,** 435–445
26. Leary, J. J., Brigati, D. J., and Ward, D. C. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 4045–4049
27. Maruyama, K., Mikawa, T., and Ebashi, S. (1984) *J. Biochem. (Tokyo)* **95,** 511–519
28. Margreth, A., Salvatori, S., Damiani, E., and Salviati, G. (1986) in *Calcium Ion: Membrane Transport and Cellular Regulation* (Carpenédo, F., Debetto, P., Floreani, M., and Luciani, S., eds) Proceedings of International Symposium in Honor of Luigi Sabbatani, pp. 75–78, Dept. of Pharmacology, Padova, Italy
29. Cala, S. E., and Jones, L. R. (1983) *J. Biol. Chem.* **258,** 11931–11936
30. Mahony, L., and Jones, L. R. (1986) *J. Biol. Chem.* **261,** 15257–15265
31. Zorzato, F., and Volpe, P. (1987) *Biophys. J.* **51,** 353a
32. Waisman, D. M., Salimah, B. P., and Anderson, M. J. (1985) *J. Biol. Chem.* **260,** 1652–1660
33. Khanna, N. C., and Waisman, D. M. (1986) *Biochemistry* **25,** 1078–1082
34. Campbell, K. P., MacLennan, D. H., Jorgensen, A. O., and Mintzer, M. C. (1983) *J. Biol. Chem.* **258,** 1197–1204
35. Somlyo, A. P., and Somlyo, A. V. (1987) *J. Cardiovasc. Pharmacol.* **8,** (suppl.), S42–S47
36. Dawson, A. P., and Irvine, R. F. (1984) *Biochem. Biophys. Res. Commun.* **120,** 858–864
37. Volpe, P., Salviati, G., Di Virgilio, F., and Pozzan, T. (1985) *Nature* **316,** 347–349
38. Krause, K.-H., Volpe, P., Zorzato, F., Hashimoto, S., Pozzan, T., Meldolesi, J., and P. D. Lew (1987) in *Cell Calcium Metabolism '87* (Fiskum, G., ed) Seventh International Washington Spring Symposium, Abstr. Volume p. 64, Washington University, Washington, D. C.