Sequence similarity of calreticulin with a Ca^{2+}-binding protein that co-purifies with an Ins(1,4,5)P_3-sensitive Ca^{2+} store in HL-60 cells

Karl-Heinz KRAUSE,*†§ Heather K. B. SIMMERMAN,‡ Larry R. JONES,‡ and Kevin P. CAMPBELL†
*Division of Infectious Diseases, Geneva University Hospital, Switzerland, †Howard Hughes Medical Institute, Department of Physiology and Biophysics, The University of Iowa College of Medicine, Iowa City, IA 52242, U.S.A., and ‡Krannert Institute of Cardiology and the Department of Medicine, Indiana University School of Medicine Indianapolis, IN 46202, U.S.A.

HL-60 cells possess a 60 kDa Ca^{2+}-binding protein that is contained in a discrete subcellular compartment, referred to as calciosomes. Subcellular fractionation studies have suggested that, in HL-60 cells, this intracellular compartment is an Ins(1,4,5)P_3-sensitive Ca^{2+} store. In order to investigate the structural relationship of the 60 kDa Ca^{2+}-binding protein of HL-60 cells to other Ca^{2+}-binding proteins, we have purified the protein by ammonium sulphate extraction, acid precipitation, and DEAE-cellulose and phenyl-Sepharose column chromatography. The *N*-terminal sequence of the protein shows 93% identity with rabbit muscle calreticulin, a recently cloned sarcoplasmic reticulum Ca^{2+}-binding protein. No amino acid sequence similarity with calsequestrin was found, although the purified protein cross-reacted with anti-calsequestrin antibodies. The calreticulin-related protein of HL-60 cells might play a role as an intravesicular Ca^{2+}-binding protein of an Ins(1,4,5).P_3-sensitive Ca^{2+} store.

INTRODUCTION

There is evidence in virtually all types of non-muscle cells for the existence of intracellular Ca^{2+} storage organelles (Berridge, 1987; Carafoli, 1987). These organelles are able to sequester Ca^{2+} from the cytosol and release Ca^{2+} in response to Ins(1,4,5)P_3. The identity of these Ca^{2+} storage organelles is still a matter of debate.

Subcellular fractionation studies in the myeloid cell line HL-60 (Voipe *et al.*, 1988) have demonstrated that markers of Ins(1,4,5)P_3-sensitive Ca^{2+} pools, i.e. Ca^{2+} uptake and Ins(1,4,5)P_3-induced Ca^{2+} release, co-sediment with a Ca^{2+}-binding protein of approx. 60 kDa. Marker enzymes for other organelles, such as endoplasmic reticulum, could be separated from the Ins(1,4,5)P3-sensitive Ca^{2+} pool. As the 60 kDa Ca^{2+}-binding protein cross-reacted with anti-calsequestrin antibodies, it was tentatively referred to as 'calsequestrin-like protein'. In immunoelectron microscopy studies (Voipe *et al.*, 1988; Hashimoto *et al.*, 1988) anti-calsequestrin antibodies labelled a distinct intracellular compartment which was designated as 'calciosomes', and presumably corresponds to an Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} pool of HL-60 cells. However, in some cell types an Ins(1,4,5)P_3-sensitive Ca^{2+} pool seems to co-purify with the endoplasmic reticulum (e.g. Prentki *et al.*, 1984; Streb *et al.*, 1984). Thus the precise relationship between calciosomes, endoplasmic reticulum and Ins(1,4,5)P_3-sensitive Ca^{2+} pools is not yet known.

The 60 kDa Ca^{2+}-binding protein of HL-60 cells is of interest for several reasons: (1) it can be used as a marker for calciosomes in subcellular fractionation studies and in morphological studies, and (2) it might have a functionally important role as an intravesicular Ca^{2+}-binding protein in an Ins(1,4,5)P_3-sensitive Ca^{2+} store. In this study we have purified the 60 kDa Ca^{2+}-binding protein of HL-60 cells. We show that the *N*-terminal amino acid sequence is virtually identical with the *N*-terminal sequence of calreticulin, a Ca^{2+}-binding protein that is found in muscle sarcoplasmic reticulum as well as in a variety of non-muscle cells (Fliegel *et al.*, 1989).

MATERIALS AND METHODS

Preparation of HL-60 cells and subcellular fractions

HL-60 cells were grown in RPMI medium enriched with 10 % fetal calf serum, incubated with the proteinase inhibitor di-isopropyi fluorophosphate (DFP; 5 μM) and disrupted by nitrogen cavitation as previously described (Krause & Lew, 1987; Voipe *et al.*, 1988). Nuclei and unbroken cells were removed from the homogenate by centrifugation at 1000 ***g*** for 5 min. After incubation with 15 μM-digitonin for 10 min, the homogenate was separated on a discontinuous Percoll density gradient consisting of two cushions of 19% and 33% Percoll. All Percoll solutions contained 100 mM-KCl and 25 mM-Hepes, pH 7.0. Gradients were centrifuged at 160000***g*** for 10 min. Three fractions were obtained: fraction 1 (below the 33% cushion), fraction 2 (between the 33 % and the 19 % cushions) and fraction 3 (above the 19% cushion). The fractions were collected, diluted 20-fold in a 100 mM-KCl/25 mM-Hepes (pH 7.0) buffer, centrifuged at 100000 ***g*** for 30 min and rediluted in the same buffer at a protein concentration of approx. 2 mg/ml. Protein concentrations were determined as described (Peterson, 1977). All steps were performed at 4 °C.

Isolation of the 60 kDa Ca^{2+}-binding protein from HL-60 cells

Whole HL-60 cells, pretreated with DFP, were homogenized in a Dounce homogenizer (10 strokes) in a buffer containing 0.1 M-KH_2PO_4, pH 7.1, 2.66 M-$(NH_4)_2SO_4$, 1 mM-EDTA and the following proteinase inhibitors: benzamidine (0.75 mM), phenylmethanesulphonyl fluoride (0.2 mM), aprotinin (0.5 μg/ml), iodoacetamide (1 mM), leupeptin (0.5 μg/ml) and pepstatin A (0.5 μg/ml). After centrifugation at 14300 ***g*** for 30 min, $(NH_4)_2SO_4$, (150 g/1), was added to the supernatant and the pH was lowered to 4.7 using phosphoric acid. The solution was stirred for 150 min and centrifuged at 14300 ***g*** for 30 min. The pellet was dissolved in 0.1 M-KH_2PO_4/1 mM-EDTA, pH 7.1, and dialysed in 50 mM-NaCl/0.1 M-KH_2PO_4/1 mM-EDTA, pH7.1,

Abbreviations used: DFP, di-isopropyi fluorophosphate; DTT, dithiothreitol.

§ To whom correspondence should be addressed, at: Division of Infectious Diseases, Hôpital Cantonal Universitaire, CH-1211 Genève 4, Switzerland.

overnight, followed by a buffer change the next morning. The dialysed sample was centrifuged at 14300 ***g*** for 10 min and applied to a DEAE-cellulose column. The column was washed with 3 vol. of a 150 mM-NaCl buffer and eluted with 3 vol. of 210 and 260 mM-NaCl buffers. All steps were performed at 4 °C and all buffers contained benzamidine (0.75 mM) and phenylmethanesulphonyl fluoride (0.2 mM).

The 260 mM-NaCl eluate of the DEAE column was further separated by phenyl-Sepharose column chromatography. This procedure was performed at room temperature. The sample was brought to 3 M-NaCl, 1 mM-dithiothreitol (DTT), 1 mM-EGTA and 20 mM-Mops by addition of solid reagents, adjusted to a pH of 7.0 and applied to the column. The column was washed with a 3 M-NaCl buffer, and proteins were eluted with 2 M-, 1 M_τ, 0.35 M- and 0 M-NaCl concentrations. All buffers contained 1 mM-DTT, 1 mM-EGTA and 20 mM-Mops, pH 7.0.

SDS/PAGE analysis of HL-60 proteins

The analysis of proteins by SDS/PAGE was performed using the discontinuous buffer system (Laemmli, 1970) in 1.5 mm thick gradient gels (3-12% or 5-16% acrylamide). Gels were stained with Coomassie Blue and destained with a solution of 10% acetic acid and 5 % methanol. Staining with the cationic carbocyanine dye Stains-All was carried out as described (Campbell *et al.*, 1983). Two-dimensional gel electrophoresis at two different pH values was carried out as previously described (Michalak *et al.*, 1980). Apparent molecular masses were calculated from a graph of relative mobilities versus log molecular mass for standard proteins. The following molecular mass standards (native or prestained) were used: myosin (205-206 kDa), β-galactosidase (116 kDa), phosphorylase B (100 kDa), BSA (66-68 kDa), ovalbumin (42-45 kDa), α-chymotrypsinogen (25-26 kDa), soybean trypsin inhibitor (19 kDa), β-lactoglobulin (18 kDa) and lysozyme (15 kDa).

Immunochemical analysis of HL-60 proteins

Proteins were separated by SDS/PAGE (Laemmli, 1970) and transferred electrophoretically on to nitrocellulose membranes or Immobilon (Towbin *et al.*, 1979). The transfers were blocked with 10 mM-Tris/HCl, pH 7, 500 mM-NaCI, 0.05 % Tween-20 and 0.5 % non-fat dry milk prior to incubation with the primary antibody. Polyclonal antibodies to calsequestrin were prepared as previously described (Jorgensen & Campbell, 1984). Secondary antibodies coupled to alkaline phosphatase were used and blots were developed using 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as described (Blake *et al.*, 1983).

Protein sequencing

Approx. 10μg (160pmol) of the purified 60 kDa HL-60 protein was subjected to SDS/PAGE on an 8 % Porzio gel and electroblotted on to Immobilon (Matsudaira, 1987). Following transfer, the protein was stained with Coomassie Blue and the band was cut out. The sequence was identified by automated Edman degradation using an Applied Biosystems Model 477A protein sequenator equipped with a Model 120A h.p.l.c. for on-line analysis of phenylthiohydantoin residues. A total of 40 % of the phenylthiohydantoin derivatives was analysed at each cycle, and the residues identified were present in amounts of 5-10 pmol (background approx. 1 pmol).

Materials

SDS, acrylamide, *NN′*-methylenebisacrylamide, 2-mercaptoethanol and *NNN′N′*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad. The cationic carbocyanine dye Stains-All was obtained from Eastman Organic Chemicals and prepared as a 0.1% stock solution in formamide. Alkaline phosphatase-coupled secondary antibodies, 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium were purchased from Sigma. All other chemicals were of reagent grade or the highest purity available.

RESULTS AND DISCUSSION

We have previously shown that a 60 kDa Ca^{2+}-binding protein in HL-60 cells cross-reacts with polyclonal antibodies against skeletal muscle calsequestrin (Voipe *et al.*, 1988). We have therefore tentatively referred to this protein as 'calsequestrin-like protein'. In order to analyse whether the primary structure of

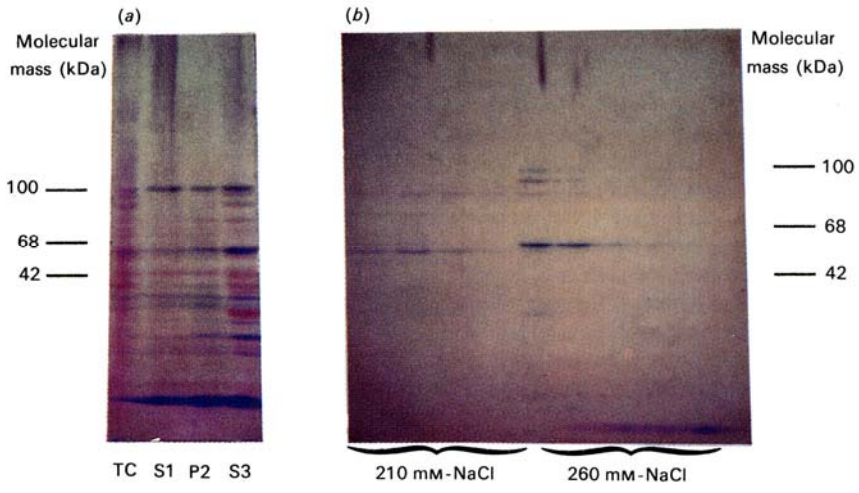

Fig. 1. Isolation of a 60 kDa Ca^{2+}-binding protein by ammonium sulphate extraction, acid precipitation and DEAE-cellulose column chromatography

HL-60 cells (*a*, TC) were homogenized and a fraction enriched in calsequestrin was obtained by ammonium sulphate extraction (S1), acid precipitation (P2), and dialysis in 100 mM-KCl and 50 mM-NaCl and removal of insoluble constituents by centrifugation (S3). The enriched fraction was loaded on a DEAE-cellulose column, washed with 150 mM-NaCl and eluted with 210 and 260 mM-NaCl buffers, (*a*) and (*b*) show Stains-All-stained SDS/PAGE gels of initial purification steps and of the 210 mM-NaCl and 260 mM-NaCl eluates from the DEAE-cellulose column respectively (100 ml/lane).

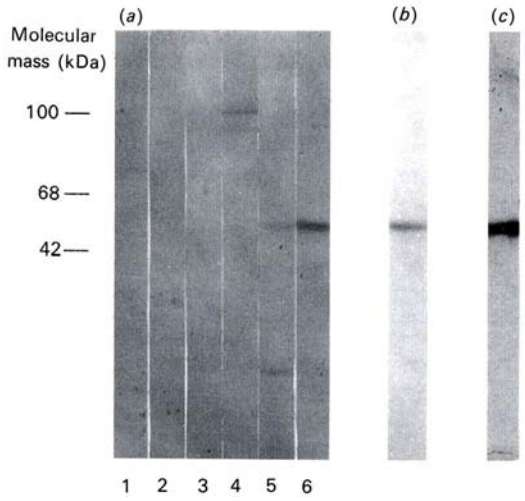

Fig. 2. Purification of a 60 kDa Ca^{2+}-binding protein by phenyl-Sepharose column chromatography

The 260 mM-NaCl eluate of the DEAE-cellulose column was made to 3 M-NaCl, 20 mM-Mops, 1 mM-EGTA and 1 mM-DTT by addition of solid reagents, adjusted to a pH of 7.0 and applied to a phenyl-Sepharose column. Proteins were eluted with 2 M-, 1 M-, 0.35 M- and 0 M-NaCl concentrations, all buffers containing 20 mM-Mops, 1 mM-EGTA, and 1 mM-DTT, pH 7.0. Void volume and various elution steps were separated by SDS/PAGE (5-16% gradient gels) and stained with Stains-All (a: void, wash, 2 M-, 1 M-, 0.35 M- and 0 M-NaCl; lanes 1, 2, 3, 4, 5 and 6 respectively; 100 µl/lane), or Coomassie Blue (*b*: 0 M-NaCl eluate, 1/µg), or transferred to nitrocellulose (*c*) and stained with polyclonal antibodies against skeletal muscle calsequestrin (0 M-NaCl eluate, 2.5 µg).

this protein is related to calsequestrin or to other known Ca^{2+}-binding proteins, we have now purified the protein and determined its *N*-terminal amino acid sequence.

A method to isolate muscle calsequestrin based upon the physical and chemical properties of calsequestrin has been developed in our laboratory and has been used to isolate cardiac calsequestrin (Slupsky *et al.*, 1987). The 60 kDa Ca^{2+}-binding protein of HL-60 cells is increasingly enriched during the initial steps of this purification procedure (ammonium sulphate extraction, acid precipitation, and dialysis in phosphate-buffered saline and removal of non-soluble constituents; Fig. l*a*; Sl, P2 and S3 respectively). The sample obtained in this way was applied to a DEAE column and eluted with 210 mM-NaCl and subsequently with 260 mM-NaCl. The 60 kDa Ca^{2+}-binding protein eluted predominantly at 260 mM-NaCl (Fig. 2*b*).

We attempted to purify further the 60 kDa Ca^{2+}-binding protein recovered in the 260 mM-NaCl eluate. Ca^{2+}-dependent elution of calsequestrin from phenyl-Sepharose has been successfully used to purify the protein from various skeletal and cardiac preparations, attributed to a Ca^{2+}-induced decrease in hydrophobicity of the protein (Cala & Jones, 1983). We were unable to observe such a Ca^{2+}-dependent elution of the 60 kDa HL-60 Ca^{2+}-binding protein from phenyl-Sepharose, even when we increased the Ca^{2+} concentration in the elution buffer at the lowest ionic strength that would allow the 60 kDa protein to stay bound to the column (150 mM-NaCl). These results showed the first clear difference between the HL-60 protein and muscle calsequestrin. Phenyl-Sepharose column chromatography was, however, useful for the purification of the 60 kDa Ca^{2+}-binding protein. When the 260 mM-NaCl eluate of the DEAE-cellulose column was applied to a phenyl-Sepharose column in a buffer containing 3 M-NaCl, virtually all protein became bound to the

(*a*) Glu*-Pro-Ala-Val-Tyr-Phe-Lys-Glu-Gln-Phe-Leu-Asp-Gly-Asp-Gly-

(*b*) Glu-Pro-Val-Val-Tyr-Phe-Lys-Glu-Gln-Phe-Leu-Asp-Gly-Asp-Gly-

Fig. 3. *N*-Terminal sequences of the 60 kDa Ca^{2+}-binding protein of HL-60 cells and of calreticulin of muscle cells

The purified HL-60 protein (*a*) was subjected to SDS/PAGE, transferred to Immobilon, and the *N*-terminal sequence was obtained by automated Edman degradation. The *N*-terminal sequence of rabbit skeletal muscle calreticulin (*b*) is shown as published (Fliegel *et al.*, 1989). *The first amino acid of the HL-60 protein could not be unambiguously assigned.

column. In subsequent elution steps, contaminating proteins (103 kDa, 95 kDa and 20 kDa) eluted at 1 M-NaCl or 0.35 M-NaCl, but the 60 kDa protein eluted predominantly in the absence of NaCl (Fig. 2*a*). Coomassie Blue staining showed the 60 kDa protein to be apparently pure (Fig. 2*b*). The purified 60 kDa protein was recognized by polyclonal antibodies against rabbit skeletal muscle calsequestrin (Fig. 2*c*), confirming that the purified protein corresponds to the calsequestrin-like protein that we have previously described. The purified protein did not cross-react with antibodies against canine cardiac calsequestrin (results not shown).

We next investigated the *N*-terminal sequence of the purified protein. It did not display any similarities with calsequestrin, but was 93 % identical with the *N*-terminal sequence of the recently cloned rabbit muscle calreticulin (Fig. 3). Thus, from 15 amino acids identified, 14 were identical and only one substitution was found, in position 3. Calreticulin is a sarcoplasmic reticulum Ca^{2+}-binding protein. However, several non-muscle cells were found to contain proteins with *N*-terminal sequences similar to that of calreticulin (Fliegel *et al.*, 1989), and Northern blot analysis has suggested expression of the calreticulin gene in a variety of non-muscle cells (Fliegel *et al.*, 1989). To our knowledge, only one protein that shares the *N*-terminal with calreticulin has been purified from human tissue, namely from a lymphoblastoid cell line (Lieu *et al.*, 1988). This protein, also referred to as Ro/SS-A antigen, exhibits an identical sequence with all 15 TV-terminal residues identified here for the HL-60 Ca^{2+}-binding protein.

The cross-reaction of the calreticulin-related protein of HL-60 cells with anti-calsequestrin antibodies is surprising, given the lack of similarity of primary structure of muscle calreticulin and calsequestrin (Fliegel *et al.*, 1989). The HL-60 protein might have, differently from muscle calreticulin, a calsequestrin-related amino acid sequence, or it might share non-linear epitopes with calsequestrin. However, we cannot exclude the possibility that our anti-calsequestrin antibodies were contaminated with anti-calreticulin antibodies.

The original studies in muscle have suggested that calreticulin has one high-affinity Ca^{2+}-binding site (K_d around 3 µM) and 25 low-affinity Ca^{2+}-binding sites (K_d around 5 mM). In liver cells, calreticulin probably corresponds to a protein previously referred to as calregulin (Waisman *et al.*, 1985). However, Ca^{2+}-binding studies of the liver protein suggested three high-affinity binding sites (K_d around 0.1 µM) and no low-affinity binding site (Waisman *et al.*, 1985).

Whereas a pH-dependence of the apparent molecular mass is a well-known feature of calsequestrin, no such phenomenon was observed with calreticulin of muscle cells (Michalak *et al.*, 1980). By contrast, the 60 kDa Ca^{2+}-binding protein of HL-60 cells clearly changes its apparent molecular mass with pH. In a two-dimensional gel system with a first separation step at a pH of 7.0 and a second separation step at a pH of 8.7 (Michalak *et al.*, 1980), the 60 kDa Ca^{2+}-binding protein falls off the diagonal formed by the majority of the proteins (Fig. 4).

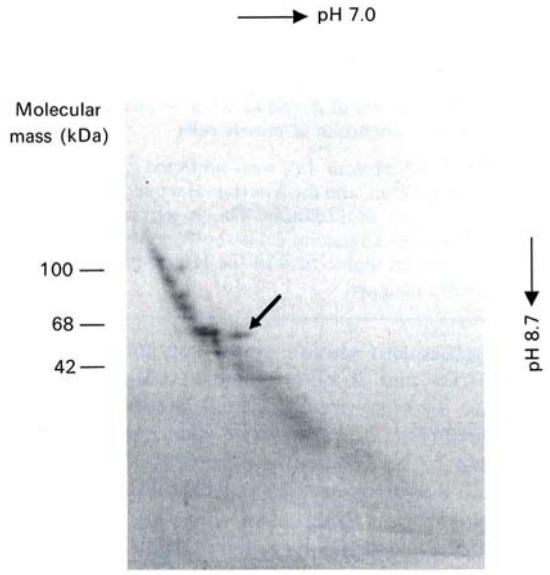

Fig. 4. Stains-All-stained two-dimensional gel electrophoresis of HL-60 membranes

A particulate fraction of HL-60 cells (200 μg) was separated by a Weber-Osborn-type gel (12% gel, pH 7.0). The lane was cut from the Weber-Osborn gel and applied to a Laemmli-type gel (5-16% gradient, pH 8.7) which was stained with Stains-All. The arrow identifies the metachromatically blue-staining 60 kDa Ca^{2+}-binding protein.

Thus studies with calreticulin-related proteins from various tissues have yielded inconsistent results with respect to Ca^{2+} binding and to pH-dependence of apparent molecular mass, despite the high degree of similarity in *N*-terminal sequences. Although technical details might explain these differing experimental results, one has to consider the possibility of different proteins that share an identical A *N*-terminus, e.g. due to alternatively spliced gene products or different post-translational processing of the protein in different tissues.

In subcellular fractionation studies in HL-60 cells, the 60 kDa Ca^{2+}-binding protein co-purifies with functional parameters of the Ins(l,4,5)P_3-sensitive Ca^{2+} stores, but not with markers of other organelles, such as endoplasmic reticulum (Voipe *et al.*, 1988). Thus the intracellular compartment of HL-60 cells that contains the 60 kDa Ca^{2+}-binding protein was designated as calciosomes and proposed to be an Ins(l,4,5)P_3-sensitive Ca^{2+} store. The last four amino acids of the *C*-terminal of rabbit muscle calreticulin are Lys-Asp-Glu-Leu (Fliegel *et al.*, 1989). This sequence has been proposed to be an endoplasmic reticulum retention signal (Munro & Pelham, 1987). Provided that the HL-60 protein has the same retention signal, the fact that it is recovered from calciosomes and not from endoplasmic reticulum might provide an important clue concerning the biosynthesis of calciosomes.

Received 5 June 1990; accepted 9 July 1990

We thank Mitch G. Gaver, Steven D. Kahl, Cynthia J. Schmitz and Elzbietha Huggler for technical assistance. Dr. William Nauseef for help with HL-60 cell culture and Dr. Robert A. dark and Dr. Daniel Lew for advice and support. K.-H.K. is supported by a grant of the Swiss National Foundation (3.829.0.87). K.P.C. is a Howard Hughes Medical Institute Investigator and a recipient of the National Institutes of Health grant HL-37187.

REFERENCES

Berridge, M. J. (1987) Annu. Rev. Biochem. **56,** 159-193
Blake, M. S., Johnston, K. H., Russel-Jones, G. J. & Gotschlich, E. C. (1983) Anal. Biochem. **136,** 175-179
Cala, S. E. & Jones, L. R. (1983) J. Biol. Chem. **258,** 11932-11936
Campbell, K. P., MacLennan, D. H. & Jorgensen, A. O. (1983) J. Biol. Chem. **258,**11267-11273
Carafoli, E. (1987) Annu. Rev. Biochem. **56,** 395-435
Fliegel, L., Burns, K., MacLennan, D. H., Reithmeier, R. A. F. & Michalak, M. (1989) J. Biol. Chem. **264,** 21522-21528
Hashimoto, S., Bruno, B., Lew, D. P., Pozzan, T., Voipe, P. & Meldolesi, J. (1988) J. Cell Biol. **107,** 2523-2531
Jorgensen, A. O. & Campbell, K. P. (1984) J. Cell Biol. **98,** 1597-1602
Krause, K. H. & Lew, P. D. (1987) J. Clin. Invest. **80,** 107-116
Laemmli, U. K. (1970) Nature (London) **227,** 680-685
Lieu, T. S., Newkirk, M. M., Capra, J. D. & Sontheimer, R. D. (1988) J. Clin. Invest. **82,** 96-101
Matsudaira, P. (1987) J. Biol. Chem. **262,** 10035-10038
Michalak, M., Campbell, K. P. & MacLennan, D. H. (1980) J. Biol. Chem. **255,** 1317-1326
Munro, S. & Pelham, H. R. B. (1987) Cell **48,** 899-907
Peterson, G. L. (1977) Anal. Biochem. **83,** 346-356
Prentki, M., Janjic, D., Biden, T. J., Blondel, B. & Wollheim, C. B. (1984) J. Biol. Chem. **259,** 10118-10123
Slupsky, J. R., Ohnishi, M., Carpenter, M. R. & Reithmeier, R. A. F. (1987) Biochemistry **26,** 6539-6544
Streb, H., Bayerdorffer, E., Haase, W., Irvine, R. F. & Schuiz, I. (1984) J. Membr. Biol. **81,** 241-253
Towbin, H., Staehlin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76,** 4350-4354
Voipe, P., Krause, K. H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J. & Dew, D. P. (1988) Proc. Natl. Acad. Sci. U.S.A. **85,** 1091-1095
Waisman, D., Salimath, B. P. & Anderson, M. J. (1985) J. Biol. Chem. **260,** 1652-1660