Disulfide Linkage of Biotin Identifies a 106-kDa Ca\(^{2+}\) Release Channel in Sarcoplasmic Reticulum*

(Received for publication, November 23, 1988, and in revised form, October 6, 1989)

Nikhat F. Zaïdi†, Carl F. Lagenaur¶, Robert J. Hikut§, Hui Xiong, Jonathan J. Abramson†, and Guy Salama**

From Departments of Physiology and Neurobiology, Anatomy, and Cell Science, School of Medicine, University of Pittsburgh, Pennsylvania 15261; and the Physics Department, †Portland State University, Portland, Oregon 97207

*This work was supported in part by American Heart Association Grant 87-1065 and the Western Pennsylvania Affiliate of the American Heart Association (to G. S.) and by American Heart Association Grant 87-915 and the Oregon Affiliate of the AHA (to J. J. A.). 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.

†Recipient of a Muscular Dystrophy Association Postdoctoral Fellowship.

‡Established Investigator 84130 of the American Heart Association.

§Supported Investigator Training Grant 5T32-DK07458 from the National Institutes of Health.

**Recipient of Research Career Development Award 5 K04 NS09099 from the National Institutes of Health. To whom correspondence should be addressed.

Reactive disulfide reagents (RDSs) with a biotin moiety have been synthesized and found to cause Ca\(^{2+}\) release from sarcoplasmic reticulum (SR) vesicles. The RDSs oxidize SH sites on SR proteins via a thiol-disulfide exchange, with the formation of mixed disulfides between SR proteins and biotin. Biotinylated RDSs identified a 106-kDa protein which was purified by biotin-avidin chromatography. Disulfide reducing agents, like diithiothreitol, reverse the effect of RDSs and thus promoted active re-uptake of Ca\(^{2+}\) and dissociated biotin from the labeled protein indicating that biotin was covalently linked to the 106-kDa protein via a disulfide bond. Several lines of evidence indicate that this protein is not Ca\(^{2+}\),Mg\(^{2+}\)-ATPase and is not a proteolytic fragment or a subunit of the 400-kDa Ca\(^{2+}\)-ryanodine receptor complex (RRC). Monoclonal antibodies against the ATPase did not cross-react with the 106-kDa protein, and polyclonal antibodies against the 106-kDa did not cross-react with either the ATPase or the 400-kDa RRC. RDSs did not label the 400-kDa RRC with biotin. Linear sucrose gradients used to purify the RRC show that the 106-kDa protein migrated throughout 5–20% linear sucrose gradients, including the high sucrose density protein fractions containing 400-kDa RRC. Protease inhibitors disopropylfluorophosphate used to prevent proteolysis of 400-kDa proteins did not alter the migration of 106-kDa in sucrose gradients nor the patterns of biotin labeling of the 106-kDa protein. Incorporation of highly purified 106-kDa protein (free of RRC) in planar bilayers revealed cationic channels with large Na\(^{+}\) (g\(_{Na} = 375 \pm 15\) ps) and Ca\(^{2+}\) (g\(_{Ca} = 107.7 \pm 12\) ps) conductances which were activated by micromolar [Ca\(^{2+}\)]\(_{free}\) or millimolar [ATP] and blocked by micromolar ruthenium red or millimolar [Mg\(^{2+}\)]. Thus, the SR contains a sulfhydryl-activated 106-kDa Ca\(^{2+}\) channel with apparently similar characteristics to the 400-kDa "feet" proteins.

In the previous article, we have described a class of "reactive" disulfide (RDS)\(^1\) compounds (i.e. dithiopyridines) that cause Ca\(^{2+}\) release from sarcoplasmic reticulum (SR) vesicles (1). The RDSs caused release by oxidizing critical sulfhydryl groups on SR proteins through a thiol-disulfide exchange reaction and the formation of mixed disulfides between the SR protein(s) and the RDS compounds. The oxidation reaction opened a Ca\(^{2+}\) channel pathway which was reversed by reducing the mixed disulfide bond with GSH or DTT, resulting in active Ca\(^{2+}\) re-uptake by SR Ca\(^{2+}\) pumps (1). Among the dithiopyridines that were tested, SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate) is a heterobifunctional reagent for the isolation and production of intermolecular conjugates. It provides an elegant method to covalently link proteins with an easily identifiable probe, facilitating purification of proteins involved in sulfhydryl-activated Ca\(^{2+}\) release.

In the present study, two methods were used to synthesize SPDP-biotin conjugates: PDP-biocytin and PDP-biotin hydrazide. Both were effective at low concentrations (10–20 μM) in causing SR Ca\(^{2+}\) release, with characteristics similar to those described for RDSs (1). The RDS-biotin conjugates labeled an SR protein which was identified by biotin-avidin peroxidase reaction. The biotinylated protein isolated and purified by biotin-avidin chromatography had an apparent molecular mass of 106,000 daltons, did not cross-react with monoclonal antibodies to the Ca\(^{2+}\),Mg\(^{2+}\)-ATPase, and comprised about 0.3% of total SR protein. Immunological evidence indicated that the 106-kDa is neither a fragment nor a subunit of the RRC (2–6). Incorporation of purified 106-kDa protein in lipid bilayers revealed a cationic channel with a large Na\(^{+}\) conductance and exhibited three additional subconductance states. Like the RRC, the sulfhydryl-activated 106-kDa channel was activated by micromolar [Ca\(^{2+}\)] or millimolar [ATP] and inhibited by micromolar ruthenium red or millimolar [Mg\(^{2+}\)] free. Preliminary reports of these studies have been presented (7, 8).

\(^1\) The abbreviations used are: RDS, reactive disulfide; SR, sarcoplasmic reticulum; iHEPES, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; MOPS, morpholinoopropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[[(cholamidopropyl)dimethylammonio]1-propanesulfonate; EGTA, [ethylenebis(oxyethylenenitrilo)tetracetic acid; SDS, sodium dodecyl sulfate; AP III, antipyrilazo III; DIFP, diisopropylfluorophosphate; DTT, dithiothreitol; SPDP, N-succinimidyl 3-(2-pyridyl)dithiopropionate; RRC, ryanodine receptor complex; PDP, 2-pyryldithio)propionate; PAGE, polyacrylamide gel electrophoresis; IAM, iodoacetamide.

21737
**EXPERIMENTAL PROCEDURES**

**Preparation of SR Vesicles**—Heavy fraction of SR vesicles were prepared from rabbit white skeletal muscle as described previously (9). After the final centrifugation step, the vesicles were suspended at 10–20 mg/ml in 0.8 M sucrose, 10 mM Hepes, at pH 7.0, and stored in liquid nitrogen until used. Junctional SR vesicles with enriched 400-kDa RRC were prepared in the presence of DIFP (1 mM) and EGTA (2 mM) as previously described (10). Protein determinations were made by the method of Lowry et al. (11).

**Measurement of Ca\(^{2+}\) Transport**—Ca\(^{2+}\) fluxes across SR vesicles were measured through the differential absorption changes of the metallochromic indicator antipyrylazo III (APIII), at 720–790 nm with a time-sharing dual-wavelength spectrophotometer. The use of APIII to measure extravesicular free [Ca\(^{2+}\)] in suspensions of SR vesicles was measured in parallel experiments through the differential absorption changes at 340–310 nm, as previously described (1).

**Synthesis of SPDP-Biotin Conjugates**—Two SPDP-biotin conjugates were synthesized by the scheme shown in Fig. 1. Equimolar concentrations (10 mM) of SPDP and biotin hydrazide or PDP-biocytin were mixed in dimethyl sulfoxide and allowed to react at room temperature for 4 h.

**Covalent Labeling of SR Proteins with Biotin**—PDP-biotin hydrazide and PDP-biocytin were used to covalently link biotin to SR proteins. SR vesicles (2 mg/ml) were incubated with an excess of an SPDP-biotin conjugate (100 pM) for 5 min in a medium consisting of (in mM) 100 NaCl, 20 Tris-HCl, 1.0 MgCl\(_2\), pH 5.0, at room temperature. The suspension was diluted by 25-fold with ice-cold medium, at pH 7.0, lacking the SPDP-biotin conjugate. The vesicles were then washed by centrifugation (20,000 rpm × 60 min, in a Sorvall SS-34 rotor) to remove unreacted sulfhydryl reagent. The pellet was resuspended in the original volume of 100 mM NaCl, 20 mM Tris-HCl, 1.0 mM MgCl\(_2\), at pH 7.0, and washed for a second time (20,000 rpm × 60 min, in an SS-34 rotor).

The labeling reaction of SR proteins with biotin was carried out in weakly acidic media, pH 5, to increase the reaction rate of thiol modification by thiol-disulfide exchange (13). However, labeling in weakly acidic media was not critical since biotinylation of SR proteins was not significantly reduced in more alkaline conditions (data not shown). The strategy for labeling SR proteins was based on the time course for SR Ca\(^{2+}\) release induced by RDS reagents followed by the interruption of the labeling reaction to minimize nonspecific biotinylation of SR proteins.

**Identification of Biotinylated SR Proteins**—Biotinylated SR vesicles (2 mg/ml) were suspended in SDS sample buffer containing 40% glycerol, 4% SDS, 0.004% bromphenol blue, 0.2 M Tris HCl at pH 6.8, and boiled for 2 min. Note that the SDS sample buffer for electrophoresis did not contain the standard sulfhydryl reducing agents. Samples (100–200 µg protein/lane for Western blots and 15 µg/lane for silver staining) were electrophoresed on 10–20% gradient polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) buffer containing 1% SDS, 0.2 M glycine, 20 mM Tris-HCl, pH 6.8, at 65 mA for 16 h (14). The gels were either stained with silver (15) or the proteins were electrothoretically transferred from polyacrylamide gel to nitrocellulose paper (45 min at 250 V in 20 mM Tris-HCl, pH 7.4, at 20 V). Nitrocellulose sheets were incubated with "blocking" buffer (10% horse serum, 1% bovine serum albumin in 50 mM Tris-HCl, pH 7.2) to prevent nonspecific binding of avidin. The sheets were then incubated with blocking buffer containing avidin linked to horseradish peroxidase diluted 1:1000 in above blocking buffer. Western blots were washed with Tris-buffered saline, and bound peroxidase was visualized by staining with 4-chloro-1-naphthol and hydrogen peroxide. An identical gel was stained with silver to detect the total SR protein. Control experiments were carried out in the presence of the reducing agent DTT (5 mM) to demonstrate that the biotin label was covalently linked to SR proteins by a disulfide bond.

**Isolation of Biotin-labeled SR Proteins by Biotin-avidin Chromatography**—Biotinylated SR vesicles were solubilized in 1 M NaCl, 25 mM NaPipes (or Tris maleate), 1.6% CHAPS, at pH 7.1, at 4 °C, for 2 h with continuous shaking. CHAPS (at 1% in 1 M NaCl) was found to efficiently solubilize the biotin-tagged SR proteins. The detergent-solubilized proteins were centrifuged (100,000 × g for 60 min) and the supernatant incubated with avidin-Affi-Gel-10 for 2 h at 4 °C. Avidin-Affi-Gel-10 beads were preincubated with avidin and Affi-Gel-10 overnight at 4 °C in 190 mM MOPS, pH 7.5. The avidin-coated bead was packed in a small column. The non-biotinylated proteins which did not bind to the column were washed with the same CHAPS-containing buffer used to solubilize SR proteins. Biotinylated proteins bound to the avidin-beads were selectively eluted with buffer containing sulfhydryl-reducing agent DTT (5 mM). The proteins eluted with DTT were no longer tagged with biotin and were identified on silver gels. Alternatively, all SR proteins bound to avidin linked to Affi-Gel-10 beads were extracted by boiling the beads in SDS sample buffer. In this case, SR proteins retained the covalently linked biotin and thus could be detected by avidin-horseradish peroxidase after Western blotting. Both procedures purified a single polysial of 106-kDa apparent molecular mass. Scheme 1 summarizes the purification protocol of SR proteins containing the critical sulfhydryl site.

**Production of Polyclonal Antibodies against the 106 kDa**—Polyclonal antibodies were raised against the biotin-avidin-purified 106-kDa protein previously described (17). Briefly, one New Zealand rabbit was immunized (intramuscular) with 20 µg (106 kDa) emulsified in Freund’s complete adjuvant. After 3 weeks, immunization was repeated with antigen in Freund’s incomplete adjuvant. Additional boosts were given intravenously in saline without adjuvant, four times at the interval of 3 days. Serum at the 3rd immunization was antiserum to 106-kDa antigen was conjugated to keyhole limpet hemocyanin using glutaraldehyde to increase its immunogenicity (17). The same immunization protocol was used as with unconjugated protein. One week after the last boost, serum was collected and tested for the presence of antibodies. For immunoprecipitations of total SR proteins were fractionated by SDS-PAGE and transferred to nitrocellulose. These Western blots were incubated either with (i) immune serum containing 106-

**Fig. 1. Scheme for the synthesis of SPDP-biotin conjugates.**
kDa antibody (1:250 dilution in blocking buffer), (ii) preimmune serum (1:250 dilution in blocking buffer), or (iii) monoclonal antibody directed against Ca\(^{2+}\),Mg\(^{2+}\)-ATPase (1:1000 dilution in blocking buffer) for 4, 4, and 1 h, respectively. Western blots were washed for 20 min with Tris-buffered saline, then incubated with goat anti-rabbit IgG-HRP for anti-106 kDa or goat anti-mouse IgG-horseradish peroxidase, for Ca\(^{2+}\),Mg\(^{2+}\)-ATPase antibody. Both horseradish peroxidase-conjugated antibodies were used at dilutions of 1:1000. After six washes with Tris-buffered saline, bound peroxidase-linked antibodies were visualized after reacting with 4-chloro-1-naphthol and hydrogen peroxide.

Fractionation of SR Proteins Using Linear Sucrose Gradients—SR vesicles (6 mg/ml) were solubilized with CHAPS as described above, and the supernatant after 100,000 × g for 60 min was loaded on linear sucrose gradients in buffer containing (12 ml/tube): 25 mM NaPiPES, 1 M NaCl, 0.5% CHAPS, pH 6.8. The gradients were centrifuged in an SW 41 rotor at 30,000 rpm for 17 h at 4 °C as previously described (18). In other experiments, CHAPS solubilized junctional SR proteins were loaded on 5-20% linear sucrose gradient in buffer containing (12 ml/tube): 1 M NaCl, 40 mM Tris-maleate, 0.09% CHAPS, with or without phospholipids (PC/PE/PS = 16:3:1 at a concentration of 4 mg/ml), and centrifuged at 30,000 rpm for 17 h at 4 °C in a Beckman SW 41 rotor as previously described (18). In other experiments, CHAPS solubilized functional SR proteins were loaded on 5-20% linear sucrose gradients with buffer containing 1 M NaCl, 25 mM NaPiPES, 150 μM CaCl\(_2\), 10 μM EGTA, 1 mM DTT, 2 mM ATP, pH 7.1, and were centrifuged in an SW 41 rotor at 30,000 rpm for 17 h at 4 °C as later modified by Lai et al. (19). After centrifugation, the gradients were separated into 16-17 fractions to analyze their protein distribution.

Identification of Ca\(^{2+}\),Mg\(^{2+}\)-ATPase with a Monoclonal Antibody—Biotin-avidin chromatography identified a single protein with an apparent molecular mass ~106 kDa, as determined by SDS-PAGE. This protein represented approximately 0.5% of total SR protein, a polypeptide with an apparent molecular mass of ~106 kDa as determined by SDS-PAGE. Since the bulk of SR proteins consists of Ca\(^{2+}\)-pump proteins (~100 kDa), a low concentration of a protein with approximately the same molecular mass could be readily misconstrued as Ca\(^{2+}\),Mg\(^{2+}\)-ATPase contamination. A monoclonal antibody to the Ca\(^{2+}\) ATPase, the generous gift of Dr. Kevin Campbell (University of Iowa) was used to distinguish the two proteins. Protein samples to be tested were first run on SDS-polyacrylamide slabs gels and analyzed by Western blotting.

Reconstitution of 106-kDa Protein in Lipid Bilayer—Purified protein at 0.1-0.4 μg/ml was added to both sides of a Mueller-Rudin type bilayer (phosphatidylethanolamine/phosphatidylserine/phosphatidylcholine = 5:3:2; total lipid concentration = 50 mg/ml in decane) formed across an 80 or 150 μm hole drilled in a polystyrene cup, manufactured at the machine shop of the Department of Physiology (University of Pittsburgh). The maximum CHAPS concentration used in these measurements (64 μg/ml) was far less than the concentration that affected bilayer conductance in the absence of added protein. An Axopatch 1C (Axon Instruments) amplifier with a CV-30 headstage was used to amplify picocapere current fluctuations. Data were digitized (Instrutech Corp. model VR-10) stored on a video recorder and subsequently analyzed for channel activity. Analog data output from the Instrutech was digitized with an analog to digital converter (Labmaster TM-40, Scientific Solutions, Solon, OH) and analyzed using pCLAMP (a software package from Axon Instruments, Burlington, CA).

Materials—All reagents were of analytical grade. CHAPS was purchased from Boehringer Mannheim. PIPES, HEPES, Tris, ATP, creatine phosphate, creatine kinase DTT, GSH, and EGTA, SDS-PAGE molecular weight standards, biocytin, and avidin were purchased from Sigma. AP III was obtained from ICN Pharmaceuticals (Plainview, NY). The ionophore A 23187 was from Behring Diagnostics; SPDP and biotin hydrazide were from Pierce. Horseradish peroxidase-avidin D was purchased from Vector Laboratories (Burlingame, CA). Affi-Gel-10 was obtained from Bio-Rad; goat anti-rabbit IgG, and anti-biotin antibody were from Pierce. SPDP-Biotin hydrazide was purchased from Organon Teknika Cappal (Malvern, PA). Lipids were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). PDP-biocytin became available from Behring Diagnostics after we had initially synthesized the product. Several experiments with the commercial product (99% pure) produced qualitatively similar results. However, PDP-biotin hydrazide was the superior cross-linking agent for SH sites.

RESULTS

SPDP and SPDP-Biotin Conjugates Induce SR Ca\(^{2+}\) Release via an SH Oxidation Reaction—To test the effect of SPDP and its biotin conjugates on SR Ca\(^{2+}\) release, the vesicles were first loaded with Ca\(^{2+}\) using an ATP-regenerating system. SR vesicles (0.5 mg/ml) were suspended in 2 ml of 100 mM KCl, 20 mM Tris-HCl, 0.5 mM MgCl\(_2\), 0.1 mM AP III, pH 6.8, at room temperature. Aliquots of Ca\(^{2+}\) (25 μM) were sequentially added to calibrate the differential absorption changes of AP III, then Mg-ATP (50 μM) along with the ATP-regenerating system consisting of creatine phosphate (5 mM) and creatine kinase (5 units). As shown in Fig. 2, after the phase of Ca\(^{2+}\) uptake (not shown), lower concentrations of oxidizing reagents were added to cause Ca\(^{2+}\) release. After the phase of Ca\(^{2+}\) release, DTT was added to reduce the disulfide bond and induce active re-uptake of Ca\(^{2+}\) (Fig. 2, A and B) then the ionophore A 23187 was added to determine the maximum Ca\(^{2+}\) that could be released by the vesicles. The newly synthesized SPDP-biotin conjugates were more potent at releasing Ca\(^{2+}\) than the parent compound SPDP, and PDP-biotin hydrazide was more effective than PDP-biocytin (Fig. 2). Control experiments indicated that additions of DTT fully reversed the effects of the biotinylated sulfhydryl reagents and when added alone, the two biotin compounds (i.e. biotin hydrazide and biocytin) did not cause Ca\(^{2+}\) release nor did they alter active Ca\(^{2+}\) uptake at the concentration used in these experiments. In principle, the lower the concentration of sulfhydryl reagent that causes rapid Ca\(^{2+}\) release from the SR, the more likely it is to oxidize specifically the critical SH sites involved in this Ca\(^{2+}\) release pathway, with fewer interactions with other accessible SH groups that are not involved in Ca\(^{2+}\) release. An enhanced selectivity of reactive RDS could be demonstrated by preincubating SR vesicles with other sulfhydryl reagents such as iodoacetic acid or iodoacetamide (IAM) which were found to have no significant effect on either Ca\(^{2+}\) uptake or release, at up to 2 mM concentrations. The presence of IAM, which is known to alkylate SH sites not involved in Ca\(^{2+}\) release, significantly enhanced the potency of PDP-biocytin at causing Ca\(^{2+}\) release, from 20 μM in the absence of any other SH reagents to 5 μM in the presence.
of 1 mM IAM (Fig. 3). Similar results were obtained with PDP-biotin hydrazide. From the chemical reaction described in the previous paper (1), PDP-biotin conjugates like other RDSs (1) cause SR Ca\(^{2+}\) release through the oxidation of critical free sulfhydryls on a Ca\(^{2+}\) release channel protein. The oxidation reaction should then result in a disulfide link-}

of these reagents increased the rates and extent of SR Ca\(^{2+}\) release. Higher concentrations 2 nmol of cysteine to calibrate the optical signal.

**FIG. 4. Comparison of thiopyridone production with Ca\(^{2+}\) release.** SR vesicles were loaded with Ca\(^{2+}\) as described under “Experimental Procedures” and Ca\(^{2+}\) release was initiated by adding 10 nM of PDP–biotin hydrazide (BH). At the end of the run, A 23187 was added to determine the total Ca\(^{2+}\) content of SR vesicles. Top trace, Ca\(^{2+}\) release measured by differential absorption of AP III at 720–790 nm. Bottom trace, production of thiopyridone from PDP–biotin hydrazide at 340–310 nm (under identical conditions as for the top trace), then addition of 2 nmol of cysteine to calibrate the optical signal.

**Fig. 3. Enhanced Ca\(^{2+}\) releasing activity of PDP-biotin in the presence of iodoacetamide.** SR vesicles were suspended in KCl buffer in the presence or absence of a nonspecific SH reagent, 1 mM iodoacetamide (IAM), for 5 min. The vesicles were Ca\(^{2+}\) loaded using the ATP-regenerating system, then various concentrations of PDP-biotin were added to active Ca\(^{2+}\) efflux.

**FIG. 2.** SPDP and SPDP-biotin conjugates trigger SR Ca\(^{2+}\) release. SR vesicles were suspended in a KCl buffer, Ca\(^{2+}\) was loaded with an ATP-regenerating system (not shown), and release was induced by additions of either SPDP (A), PDP-biotin (B), or PDP-biotin hydrazide (C). After the completion of Ca\(^{2+}\) efflux, DTT was added to reduce the disulfide bond linking biotin to SR proteins which resulted in active re-uptake of Ca\(^{2+}\) by the vesicles. Higher concentration of these reagents increased the rates and extent of SR Ca\(^{2+}\) release.
the SR proteins containing the reactive sulfhydryl. Fig. 6A shows a typical result of avidin affinity chromatography. TheDTT-eluted fraction (B) contained a prominent band at 106 kDa which appeared identical to the biotinylated 106-kDa band seen in Fig. 5A. Since DTT elution cleaved the disulfide bond linking biotin to protein, this protein was no longer biotinylated but was detected on silver gels (Fig. 6A). To verify that the 106-kDa protein was retained on the avidin beads because it was biotinylated rather than because of a nonspecific interaction with the beads, proteins bound to the beads were eluted with boiling SDS sample buffer which denatures avidin on the column and releases SR proteins still attached to the avidin beads. A Western blot developed to detect biotinylated proteins (0.5 pg) eluted by DTT (lane B). B, Western blot developed to detect biotinylated proteins. Lane W, whole biotinylated SR proteins, only 106-kDa protein is detected even though the lane is heavily loaded as shown on silver stained gels (see A and W). Lane B, after washing the avidin beads with buffer, they were boiled in SDS sample buffer to extract all proteins non-covalently bound to the column. Lane S, the run-through of biotinylated proteins showing that the avidin beads selectively bound the biotin-labeled proteins. To the left of lane S, the reproducibility of the biotinylation protocol is shown for two different SR preparations.

Immunochemical Analysis Indicates That the 106-kDa Protein Is Different from the Ca²⁺, Mg²⁺-ATPase and the 400-kDa Feet Proteins—The 106-kDa protein containing the reactive sulfhydryl was isolated by biotin-avidin chromatography as in Fig. 6A (lane B), then used as an antigen to immunize two rabbits and raise polyclonal antibodies. One rabbit received 106 kDa; the other received 106 kDa conjugated with keyhole limpet hemocyanin in an attempt to enhance its immunogenicity. Immune serum from both rabbits contained anti-106 kDa antibodies which cross-reacted with biotin-avidin-purified 106 kDa whereas serum from control (nonimmunized) rabbits did not. To examine specificity of the antisera, sera from both rabbits were tested by Western blot analysis of whole SR protein. As shown in Fig. 7A, two heavy SR vesicles preparations from different rabbits (lanes 1 and 2) were run on Western blots and probed with the anti-106 kDa anti-sera. Only one moon-shaped band of protein cross-reacted; no cross-reaction with high molecular weight proteins or the bulk...
A 106-kDa Ca²⁺ Release Channel Protein in Skeletal SR

Fig. 7. Immunoanalysis of 106-kDa proteins using anti-106-kDa antisera and an anti-ATPase monoclonal antibody. Total SR proteins were electrophoresed in 5-15% SDS-polyacrylamide gels and transblotted onto nitrocellulose membrane. Blots were probed with either immune serum containing 106-kDa antibody (with or without keyhole limpet hemocyanin conjugation) (A) or (B) with the monoclonal antibody directed against Ca²⁺, Mg²⁺-ATPase as described under “Experimental Procedures.” Lanes 1 and 2 represent two different SR preparations probed with either of these above antibodies to examine the cross-reactivity of anti-106-kDa and anti-ATPase antibodies. In A and B, each contained 200 μg of total SR proteins. C shows staining of Ca²⁺, Mg²⁺-ATPase by monoclonal antibody as a function of total SR protein concentrations. Lanes 1–7 represent 100, 10, 5, 2, 1, 0.5, 0.3 μg of protein, respectively.

Analysis of 106-kDa Proteins into Planar Bilayers—The sulphhydryl activated 106-kDa protein was isolated by biotin-avidin chromatography as shown in Fig. 6A and incorporated into a bilayer lipid membrane. In the presence of symmetrical 0.5 M NaCl on the cis/trans sides of the membrane, large current fluctuations were measured as a function of transmembrane potential. Channel activity was strongly activated by ATP (1 mM) and inhibited by ruthenium red (Fig. 9, A–C). Little activity was observed until ATP was added to activate the channel (in eight bilayers). The frequency distribution of current fluctuations indicated the presence of a single high conductance channel with a half-saturation constant of 200 μmol (Fig. 8, B and C). The experiments were performed using a symmetrical 0.5 M NaCl on both sides of the membrane. The current fluctuations were measured as a function of applied voltage (Fig. 10). At the KCl concentrations used in this experiment, the ratio of the activities of K+ and Cl⁻ across the membrane was equal to 3.6. The theoretical Nernst reversal potential for a channel perfectly selective for K+ was calculated to be 375 ± 15 mV.

In order to determine the cation versus anion selectivity of this channel a 5:1 KCl gradient was formed across the bilayer, and the single channel current fluctuations were measured as a function of applied voltage (Fig. 10). At the KCl concentrations used in this experiment, the ratio of the activities of K⁺ and Cl⁻ across the membrane was equal to 3.6. The theoretical Nernst reversal potential for a channel perfectly selective for K⁺ was calculated to be 375 ± 15 mV.
K⁺ over Cl⁻ would then = −32.5 mV. The measured reversal potential was = −20 ± 1 mV corresponding to a permeability ratio, $P_{K⁺}/P_{Cl⁻}$ of 5. Under these experimental conditions, the channel’s ionic conductance was 69 ± 1 pS. Again, addition of 10 μM ruthenium red inhibited channel activity, completely.

The Ca²⁺ conductance of the 106-kDa channel was measured in asymmetrical Ca²⁺ solutions as previously described by Lai et al. (19). The 106-kDa protein was incorporated in bilayers with asymmetrical Ca²⁺ solutions on both sides of the chamber: trans, 5 mM Ca(OH)₂/250 mM HEPES, pH 7.4; cis, 125 mM Tris, 250 mM HEPES, 20 μM CaCl₂, 20 μM EGTA, pH 7.4. As shown in Fig. 11, current fluctuations were activated by adding to the cis side either 2 mM ATP (Fig. 11, traces a and b) or ~80 μM free [Ca²⁺] (Fig. 11, traces c and d). The current to voltage relationship analyzed from three bilayer experiments indicated the presence of Ca²⁺ channels with a maximum conductance, $g_{Ca^{2+}} = 107.7 ± 12$ pS (Fig. 12). In view of reports identifying the 400 kDa feet proteins as the Ca²⁺ release channel in SR and the similarity in the channel characteristics of the 400- and 106-kDa proteins, the procedure used to purify 400-kDa feet proteins was re-examined to determine if the 106-kDa copurified with 400-kDa proteins.

Fractionation of SR Proteins by Linear Sucrose Gradients—SR vesicles were solubilized in CHAPS and fractionated on linear sucrose gradients. Fig. 13 shows an analysis of the various sucrose fractions from 3 to 15% (lanes 1-17, respectively) using SDS-PAGE and staining with silver. As previously described (6, 17), the 400-kDaryanodine receptor tends to migrate to high sucrose densities (lanes 13–15). However, in the heavily loaded silver stained gels, the same lanes contained a second high molecular mass band (~350 kDa), a band at about 150 kDa, and a dark band of protein in the range of 97–116 kDa. Lanes 16 and 17 are devoid of 400-kDa bands and contain a single protein band in the 97–116-kDa molecular mass range. A key question is whether the protein bands (~100 kDa in lanes 13–15) comigrating with the 400 kDa consist of sulphydryl-activated 106-kDa Ca²⁺ channel

**Fig. 9.** Single channel fluctuations following incorporation of the 106-kDa protein in a BLM. Isolated 106-kDa protein (as in Fig. 6A, lane B) at 0.2 μg/ml in the presence of CHAPS (84 μg/ml) was added to cis sides of bilayers (phosphatidylethanolamine/phosphatidylserine/phosphatidylcholine = 5:3:2; total lipid concentration = 50 mg/ml) containing 0.5 mM NaCl, 20 mM NaPIPES, pH 7.4, 20 μM Ca-EGTA. At an applied voltage of +40 mV: A, current trace before ATP; B, current fluctuations following an addition of 1.0 mM Tris-ATP in the cis side; C, 3 min after an addition of 10 μM ruthenium red in the cis side of the chamber; D, frequency histogram as analyzed using pCLAMP software (Axon Instruments). Left-most bar, represents the base-line current.

**Fig. 10.** $K^+$ versus $Cl^-$ selectivity of sulphydryl activated 106-kDa channel. Single channel current-voltage relationship for channels studied in 250 mM KCl, 10 mM Tris-HEPES, pH 7.4 (cis), and 50 mM KCl, 2 mM Tris-HEPES, pH 7.4, 100 mM sucrose (trans) with 2 mM ATP on both sides of the membrane. A linear regression analysis yielded a slope of 69 ± 1 pS and an $x$ intercept of −20 ± 1 mV.

**Fig. 11.** Activation of 106-kDa Ca²⁺ release channels by ATP or free-calcium. Single channel current fluctuations recorded from 106-kDa protein (0.15 μg/ml) incorporated in a planar bilayer setup. Chamber contained 55 mM Ca(OH)₂/250 mM HEPES, pH 7.4, trans side and 125 mM Tris-base, 250 mM HEPES, pH 7.4, plus the following changes on the cis side. a, −40 mV holding potential, 0 mM ATP, 50 μM CaCl₂ and 20 μM EGTA. b, −40 mV holding potential +2 mM Na₃ATP, 50 μM CaCl₂ and 20 μM EGTA. c, 0 mV holding potential with ~2 μM free Ca²⁺ (20 μM CaCl₂ and 20 μM EGTA). d, 0 mV holding potential with ~80 μM free Ca²⁺ (100 μM CaCl₂ and 20 μM EGTA). C, closed; O, open states of the channel.
potentials ranging from -40 to +50 mV. A linear regression algorithm was used to obtain the best fit through these data points. The most probable open state of the channel had a conductance, $g_{Ca^{2+}} = 107.7 \pm 12 \text{ pS}$. The current fluctuations also revealed three subconductances states. The next most probable open-state of the channel had a conductance, $g_{Ca^{2+}} = 45 \pm 10 \text{ pS}$. Reversal potential was equal to $+20 \text{ mV}$, such that $g_{Ca^{2+}}/P_{th}$ was approximately equal to 4.

A 106-kDa Ca$^{2+}$ Release Channel Protein in Skeletal SR

protein or Ca$^{2+}$,Mg$^{2+}$-ATPase. The issue was addressed in the following way. First, samples from lanes 3, 7, 14, and 17 were run on SDS gels, transferred by Western blot techniques to nitrocellulose sheets, and cross-reacted with monoclonal antibody against Ca$^{2+}$,Mg$^{2+}$-ATPase. As shown in Fig. 14, lanes 3 and 7, are rich in ATPase, but the low molecular mass bands ($\sim$100 kDa) in lane 14 and 17 did not significantly cross-react with anti-ATPase monoclonal antibody. The concentrations of protein layered in lanes 3, 7, 14, and 17 were 19, 8, 1.25, and 0.16 $\mu$g of protein, respectively. Moreover, the anti-ATPase monoclonal can readily detect as little as 0.1 $\mu$g of ATPase protein on Western blots which strongly implies that the low molecular mass bands in lanes 14 and 17 are primarily non-ATPase proteins. In spot tests on nitrocellulose sheets, both anti-106-kDa polyclonals were found to cross-react with proteins from lanes 14 and 17 which indicated the presence of 106-kDa channel protein comigrating with the feet proteins in lane 14. Protein in lane 17 (0.15 $\mu$g/2 ml) which did not contain 400-kDa protein was added to the cis side of a planar bilayer and upon incorporation exhibited Na$^+$ current fluctuations (Fig. 15) and a Na$^+$ conductance of about 400 pS, under the conditions described for Fig. 9. The analysis of SR proteins by linear sucrose gradients (as in Figs. 13 and 14) were reproduced with eight separate SR preparations. In each case, the vesicles were prepared with careful attention to maintaining low temperatures (4 °C) and in the presence of the protease inhibitor DIFP (1 mM), which was replenished every 4 h to ensure its potency. The latter precautions protected the 400 kDa from proteolytic breakdown and did not alter the distributions of 106-kDa protein shown in Figs. 13 and 14.

On the other hand, other studies obtained highly purified 400 kDa feet proteins using the same methods but with junctional SR vesicles that are enriched with feet proteins (16, 17). The experiments of Lai et al. (19) were reproduced and analyzed in SDS-PAGE stained with Coomassie Blue. As shown in Fig. 16, top, a single band of seemingly pure 400-kDa protein can be detected with no other proteins on the same lanes (11–14). It is important to note that this linear sucrose gradient was run with junctional SR, in the presence of ATP (2 mM), DIFP (1 mM), and free Ca$^{2+}$ (150 $\mu$M CaCl$_2$. 

FIG. 12. Ca$^{2+}$ conductance of 106-kDa Ca$^{2+}$ release channel protein incorporated in planar bilayer. The 106-kDa channel protein was incorporated in a planar bilayer in asymmetrical Ca$^{2+}$ solutions as described for Fig. 1. The current-to-voltage relationship was plotted from single channel current recorded at various holding potentials ranging from -40 to +50 mV. A linear regression algorithm was used to obtain the best fit through from -40 to +50 mV. A linear regression algorithm was used to obtain the best fit through these data points. The most probable open state of the channel had a conductance, $g_{Ca^{2+}} = 107.7 \pm 12 \text{ pS}$. The current fluctuations also revealed three subconductances states. The next most probable open-state of the channel had a conductance, $g_{Ca^{2+}} = 45 \pm 10 \text{ pS}$. Reversal potential was equal to $+20 \text{ mV}$, such that $g_{Ca^{2+}}/P_{th}$ was approximately equal to 4.

FIG. 13. Fractionation of SR proteins using linear sucrose gradients. Solubilized SR proteins were centrifuged over a 3–15% linear sucrose gradient and separated in 17 fractions of equal volume but of increasing sucrose density. Each fraction was analyzed on SDS gels followed by silver staining as shown in lanes 1–17, respectively. The RRC appears primarily on lanes 13–15 and 106-kDa protein appears in lanes 13–17. Lanes 1–6 received 7 $\mu$l from fractions 1–6 of the linear sucrose gradient and lanes 7–17 received 45 $\mu$l from fractions 7–17 of the linear gradient.

FIG. 14. Distribution of Ca$^{2+}$, Mg$^{2+}$-ATPase at various sucrose densities. Fractions 3, 7, 14, and 17 (representative of various regions of the linear sucrose gradient) were run on SDS-polyacrylamide gel and the proteins transferred to nitrocellulose sheet. Western blots were incubated with monoclonal antibody directed against Ca$^{2+}$, Mg$^{2+}$-ATPase (1:5000 dilution in blocking buffer) for 1 h. After washing off unbound antibody, blots were treated with goat anti-mouse IgG-linked horseradish peroxidase (1:1000) for 1 h. Bound peroxidase was detected using 4-chloro-1-naphthol and hydrogen peroxide. Lanes 9, 10, 14, and 17 contained 19, 8, 1.25 and 0.16 $\mu$g of protein, respectively.

FIG. 15. Single channel fluctuations following incorporation of the 106-kDa protein isolated by linear sucrose gradient (fraction 17) into a BLM. Both sides of the bilayer contain 0.5 m NaCl, 20 mM NaHEPES, 20 $\mu$M EGTA, 20 $\mu$M CaCl$_2$, 4.0 mM Tris-ATP, pH 7.4, at a holding potential of 30 mV. Solid bar denotes baseline current or closed state of the channel.
The present study shows that RDs can be used to cross-link biotin to an SR protein containing the critical sulfhydryl site involved in the opening and closing of a Ca^{2+} channel pathway. Two compounds were synthesized, PDP-biotin hydrazide and PDP-biocytin which trigger Ca^{2+} release by oxidizing a free sulfhydryl on an SR protein. Conversely, the reduction of the newly formed disulfide bond between the SR protein and the biotin resulted in active re-uptake of Ca^{2+}. Biotin-avidin chromatography made it possible to isolate a single 106-kDa protein associated with this critical and highly reactive sulfhydryl which is involved in a pathway for Ca^{2+} release. The sulfhydryl properties of the Ca^{2+} release pathway are important because they made it possible to separate and distinguish a protein which is merely 0.3% of total SR protein from the Ca^{2+}, Mg^{2+}-ATPase which has a similar molecular mass and comprises about 60% of total SR proteins. Monoclonal antibody to the Ca^{2+}, Mg^{2+}-ATPase did not label either the Ca^{2+}, Mg^{2+}-ATPase or the 400-kDa RRC. Isolation of the 400-kDa RRC by linear sucrose gradients shows that the 106-kDa migrates in even higher sucrose densities (lanes 16–17, Fig. 13) and thus provided an alternate method to separate the 106 kDa. Incorporation of purified (>95% pure) 106 kDa in planar bilayers revealed the existence of a Ca^{2+} channel with large single unit conductance (~$10^7$ ± 12 pS) which is activated by free Ca^{2+} and ATP (1 mM) on the cis side and inhibited by Mg^{2+}.

A most important issue raised by this work is the relationship between the 400-kDa ryanodine receptor complex (18, 19, 22–24) and the 106-kDa sulfhydryl-activated protein. Several possibilities come to mind: (a) the 106-kDa may be a proteolytic fragment of the 400-kDa which contains the Ca^{2+} release channel and perhaps some of the site(s) involved in the regulation of the channel; (b) it may be a subunit of the 400-kDa protein; (c) it may be an entirely different SR channel protein; or (d) the RRC is composed of high molecular mass “bridging” proteins associated with 106-kDa protein(s).

Despite the large and impressive body of evidence identifying the feet proteins as the Ca^{2+} ryanodine receptor complex and the physiological Ca^{2+} release channel, the present study questions the level of purification of 400-kDa proteins incorporated in planar bilayers. The purification of 400-kDa proteins by linear sucrose gradients and its identification as the Ca^{2+} release channel is primarily based on the correlation of radiolabeled $[^3H]$ryanodine peak with the migration of the 400 kDa (19–22). However, there are other proteins at about 100 kDa that comigrate with the 400 kDa. These “contaminating” proteins are not seen in Coomassie Blue gels (Fig. 16) or even silver gels unless heavily loaded with protein. The general consensus in the literature is that feet proteins are the high affinity ryanodine receptors which can be purified by linear sucrose density gradients. However, at least two reports suggest that the situation may be more complex. First, Pessah et al. (25) noted that proteins in the C_{12}E_{9} detergent-insoluble fractions that could be solubilized with CHAPS consisted primarily of the high molecular mass ryanodine receptor and 100-kDa proteins, possibly Ca^{2+}, Mg^{2+}-ATPase. The 100-kDa proteins could be removed from the CHAPS-soluble protein fractions (i.e., primarily ryanodine receptor) by prior treatment with increasing concentrations of C_{12}E_{9}. However, as the content of 100-kDa protein was systematically reduced, $[^3H]$ryanodine binding to the high molecular mass receptor site decreased and was abolished as the content of 100-kDa proteins (presumably Ca^{2+}, Mg^{2+}-ATPase) was removed (25). At low C_{12}E_{9} to protein ratios (0.5 mg/mg
protein), the soluble fraction contained a high density of high affinity [H]ryanodine-binding sites (18 pmol of ryanodine/ mg SR, at 10 nM free ryanodine), yet there was no detectable high molecular mass protein in the silver gels. The conclusion was that the "ATPase" and feet proteins were somehow associated, and the interaction was necessary for ryanodine binding (25). Second, Meissner et al. (26) used sucrose density purified 400-kDa proteins (by the method shown in Fig. 16, lanes 10-14) as antigens to produce polyclonal antibodies and thus follow the various fragments of 400-kDa proteins after mild tryptic digestion. The polyclonals thus produced cross-reacted with total heavy SR proteins and as anticipated were found to correctly label 400-kDa bands. But the same polyclonals also cross-reacted with a "faint" band of 100-kDa protein (26). The latter band was faint suggesting that it was a minor protein, i.e. not the bulky ATPase band. In any case, these results indicate that the 400-kDa immunogen was not free of other proteins.

The evidence gathered in this study strongly suggests that the 106-kDa protein is not a proteolytic fragment and is not a subunit of the 400 kDa. The 106-kDa was isolated by the following two different procedures: (a) fractionation of SR proteins by linear sucrose gradients, using Junctional SR prepared in the presence of EGTA (1 mM) and protease inhibitor (DIFP = 1 mM) to protect the 400-kDa RRC from proteolysis. The presence or absence of the latter agent did not cause a measurable change in the migration of 106 kDa.

(b) Biotin-avidin chromatography in the presence of DIFP resulted in labeling and isolation of the 106 kDa but did not interact with the 400-kDa RRC. The implication of the latter experiment is that the sulfhydryl site involved in cross-linking biotin to the protein is located on the 106 but not the 400-kDa protein.

Alternatively, the possibility remained that the 106 kDa was a proteolytic fragment of the 400-kDa protein and the critical SH site is part of the 400-kDa protein but is sterically protected from oxidation by PDP-biotin hydrazide or PDP-biocolitin, until proteolysis makes the site accessible. To address the latter possibility, polyclonal antibodies were obtained against 106 kDa isolated by biotin-avidin chromatography by immunizing two rabbits. The antibodies from both rabbits did not cross-react with either the 400-kDa RRC or the Ca<sup>2+</sup>,Mg<sup>2+</sup>-ATPase (Fig. 7A). These results indicate that (i) the 106 kDa is neither a fragment nor a subunit of the 400-kDa protein and that (ii) the original 106-kDa antigen used to raise these polyclonal antibodies consisted of a highly pure protein free of ATPase and 400-kDa feet proteins.

The purification and incorporation of the 106-kDa protein (isolated by biotin-avidin techniques) in planar bilayers indicate that it is a cationic channel of a large Na<sup>+</sup> conductance of 375 ± 15 pS (mean value ± S.E. from 15 bilayer measurements). The channel is activated by ATP and is inhibited by ruthenium red. Moreover, similar results have been obtained from 106-kDa proteins (Fig. 15) isolated by linear sucrose gradients (Fig. 13, lanes 16 and 17). With Ca<sup>2+</sup> solutions in the cis/trans sides of the bilayer chamber, the biotin-avidin purified 106-kDa protein had a Ca<sup>2+</sup> conductance g<sub>Ca<sup>2+</sup></sub> = 107.7 ± 12 pS; it was activated by adding on the cis side 80 μM [Ca<sup>2+</sup>]<sub>free</sub>, ATP (1 mM) (Fig. 11), Ag<sup>+</sup> (10 μM) or 2,2',3,3'-dithiodipyrindine (20 μM) (not shown). The available evidence indicates that the 106-kDa and 400-kDa channels have (within experimental error) similar conductance values for Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, and Cl<sup>-</sup> ions and respond in a similar fashion to modulators of Ca<sup>2+</sup> release (27). Further bilayer studies are needed with careful perfusion of cis and trans sides of the bilayers to fully characterize single channel properties and regulation by other agents that alter SR Ca<sup>2+</sup> release.

The molecular masses of 106-kDa Ca<sup>2+</sup> release channels and the Ca<sup>2+</sup>, Mg<sup>2+</sup>-ATPase pumps are almost identical. When biotinylated, the 106-kDa protein appears as a sharp band just above the ATPase and on gels heavily loaded with whole SR proteins, it migrates either above the ATPase or as a crescent moon just below the ATPase. Monoclonal antibody raised against Ca<sup>2+</sup>·Mg<sup>2+</sup>-ATPase did not cross-react with 106-kDa protein and polyclonal antibodies raised against 106-kDa protein did not cross-react with either the ATPase or the 400-kDa ryanodine-binding protein. The latter result was obtained with 106-kDa protein isolated by either biotin-avidin or linear sucrose gradients, as in Fig. 13 and indicates that Ca<sup>2+</sup> pump and the 106-kDa protein are two different proteins. Because of the proximity in the apparent molecular mass of these two proteins and the overwhelming concentration of ATPase in SR preparations, the 106-kDa release channel could not be previously distinguished from the ATPase without the present biotin labeling technique and the production of polyclonal antibodies against the 106 kDa. Moreover, the presence of sulfhydryl-activated 106-kDa channel protein detected in the same sucrose density as the 400-kDa RRC could be readily misconstrued as ATPase contamination. Studies that depend on highly purified fractions of Ca<sup>2+</sup>·Mg<sup>2+</sup>-ATPase may require more stringent procedures to remove non-ATPase ~100-kDa proteins from their preparations. For instance, Gould et al. (28) reconstituted presumably purified 100-kDa Ca<sup>2+</sup> pumps in liposomes and reported that Ag<sup>+</sup> ions induced Ca<sup>2+</sup> release by acting at the Ca<sup>2+</sup>·Mg<sup>2+</sup>-ATPase. From such experiments, Gould et al. (29) further argued that both processes of uptake and release from SR vesicles could be solely attributed to the properties of the Ca<sup>2+</sup>·Mg<sup>2+</sup>-activated ATPase. However, the present identification of the 106-kDa Ca<sup>2+</sup> release channel points out that such an interpretation cannot be justified without more sophisticated purification of the ATPase.

It is important to note that feet proteins have been purified and reconstituted in planar bilayers by numerous methods: (a) sucrose density gradients (18, 19), (b) sequential column chromatography on heparin and hydroxylapatite columns (30, 31), and (c) immunoaffinity chromatography (23). In addition, the recent cloning of the cDNA coding for the 5037 amino acids comprising the ryanodine receptor complex and its expression resulting in ryanodine binding activity (32), all comprise overwhelming evidence in favor of the feet proteins as the physiological site for SR Ca<sup>2+</sup> release.

Nevertheless, the present data on 106-kDa Ca<sup>2+</sup> release channel proteins indicate that SR Ca<sup>2+</sup> release involves more than one pathway and suggests that linear sucrose gradients may not always separate feet proteins from the sulfhydryl-activated 106-kDa channels. It does not address the relationship between the 106- and 400-kDa proteins when feet proteins are purified by the other methods mentioned above. Certainly the understanding of the relationship between these two channels is far from complete and important questions remain regarding the role of the 106-kDa Ca<sup>2+</sup> release channel protein, such as: (a) could the 106-kDa protein still be a fragment of the feet proteins (i.e. the Ca<sup>2+</sup> channel region of the feet protein cloned by Takeshiba et al. (32)) despite the lack of cross-reactivity between anti-106 antibodies and feet proteins? (b) Could there be more than one Ca<sup>2+</sup> release channel in SR? (c) Since reactive disulfides can cause some release of Ca<sup>2+</sup> from light SR vesicles, are 106-kDa proteins distributed in the longitudinal as well as terminal cisternae of the SR network?

*I. N. Fessah, personal communication.*
A 106-kDa Ca\(^{2+}\) Release Channel Protein in Skeletal SR

The present study does not address the physiological role of sulfhydryl oxidation-reduction reactions but does demonstrate the importance of sulfhydryl chemistry to elucidate SR Ca\(^{2+}\) release pathways.

Acknowledgments—We wish to thank Drs. Christopher Miller, Jeffrey Smith, and Barbara Erlich for helpful advice on the lipid bilayer experiments, Dr. Isaac Pessah for helpful discussions, and Thomas Brown, Richard Tress, Donna Memon, and Scott Milne for their technical support.

REFERENCES

1. Zaidi N. F., Lagenaur C. F., Abramson J. J., Pessah I. N., and Salama G. (1989) J. Biol. Chem. 264, 21725–21736
2. Pessah I. N., Waterhouse A. L., and Casida J. E. (1985) Biochem. Biophys. Res. Commun. 128, 449–456
3. Pessah I. N., Francini A. O., Scales D. J., Waterhouse A. L., and Casida J. E. (1986) J. Biol. Chem. 261, 8643–8646
4. Fleischer S., Ogunbunmi E. M., Dixon M. C., and Fleer E. A. M. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7256–7259
5. Lattanzio F. A., Jr., Schlatterer R. G., Nier M., Campbell K. P., and Surko J. L. (1987) J. Biol. Chem. 262, 2711–2718
6. Inui M., Saito A., and Fleischer S. (1987) J. Biol. Chem. 262, 1740–1747
7. Salama G., Zaidi N. F., Abramson J. J., and Lagenaur C. (1988) Biochem. J. 253(2), 420 (abstr.)
8. Zaidi N. F., Abramson J. J., Lagenaur C., and Salama G. (1988) Biochem. J. 253(2), 456 (abstr.)
9. Salama G., and Scarpa A. (1985) Biochem. Pharmacol. 33(22), 3465–3477
10. Meissner, G. (1984) J. Biol. Chem. 259, 2365–2374
11. Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1951) J. Biol. Chem. 193, 265–275
12. Scarpa A., Brinley F. J., Jr., and Dubyak G. (1978) Biochemistry 17, 1378
13. Torchinsky Y. M. (1981) Sulfur in Protein, p. 27, Pergamon Press, Inc. Elmsford, NY
14. Laemmli U. K. (1970) Nature 227, 680–685
15. Merrill C. R., Switzer R. C., and Vankeuran M. L. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4355–4359
16. Towbin H., Staehelin T., and Gordon J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
17. Avrameas S. (1969) Immunochemistry 6, 43–52
18. Lai F. A., Erickson H. P., Block B. A., and Meissner G. (1987) Biochem. Biophys. Res. Commun. 134, 704–709
19. Lai F. A., Erickson H. P., Rousseau E., Liu Q.-Y., and Meissner G. (1988) Nature 331, 315–319
20. Hennessey J. P., and Scarrborough G. A. (1989) Anal. Biochem. 176, 264–296
21. Hawkes R., Niday E., and Gordon J. (1982) Anal. Biochem. 119, 142–147
22. Pessah I. N., Stambuk R., and Casida J. E. (1986) Mol. Pharmacol. 31, 232–238
23. Imagawa T., Smith J. S., Coronado R., and Campbell K. P. (1987) J. Biol. Chem. 262, 16636–16643
24. Campbell K. P., Knudson C. M., Imagawa T., Leung A. T., Surko J. L., Kahl S. D., Raab C. R., and Madison L. (1987) J. Biol. Chem. 262, 6460–6463
25. Pessah I. N., Anderson K. W., and Casida J. E. (1986) Biochem. Biophys. Res. Commun. 139, 235–243
26. Meissner G., Rousseau E., and Lai F. A. (1989) J. Biol. Chem. 264, 1715–1722
27. Smith J. S., Imagawa T., Ma J., Fill M., Campbell K. P., and Coronado R. (1988) J. Gen. Physiol. 92, 1–26
28. Gould, G. W., Coyler J., East J. M., and Lee A. G. (1987) J. Biol. Chem. 262, 7676–7679
29. Gould, G., McWhiter J. M., East J. M., and Lee A. G. (1987) Biochem. J. 245, 739–749
30. Inui M., Saito A., and Fleischer S. (1987) J. Biol. Chem. 262, 1740–1747
31. Inui M., Saito A., and Fleischer S. (1987) J. Biol. Chem. 262, 15637–15642
32. Takeshima H., Nishimura S., Matsumoto T., Ishida H., Kangawa K., Minamino N., Matsuo H., Ueda M., Hanaoka M., Hirose T., and Numa S. (1988) Nature 339, 439–445