

The 30 S Lobster Skeletal Muscle Ca^{2+} Release Channel (Ryanodine Receptor) Has Functional Properties Distinct from the Mammalian Channel Proteins*

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The 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps)-solubilized ryanodine receptor (RyR) of lobster skeletal muscle has been isolated by rate density centrifugation as a 30 S protein complex. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the purified 30 S receptor revealed a single high molecular weight protein band with a mobility intermediate between those of the mammalian skeletal and cardiac *M*_r 565,000 RyR polypeptides. Immunoblot analysis showed no or only minimal cross-reactivity with the rabbit skeletal and canine cardiac RyR polypeptides. By immunofluorescence the lobster RyR was localized to the junctions of the A-I bands. Following planar lipid bilayer reconstitution of the purified 30 S lobster RyR, single channel K^+ and Ca^{2+} currents were observed which were modified by ryanodine and optimally activated by millimolar concentrations of *cis* (cytoplasmic) Ca^{2+} . Vesicle-⁴⁵ Ca^{2+} flux measurements also indicated an optimal activation of the lobster Ca^{2+} channel by millimolar Ca^{2+} , whereas ⁴⁵ Ca^{2+} efflux from mammalian skeletal and cardiac muscle sarcoplasmic reticulum (SR) vesicles is optimally activated by micromolar Ca^{2+} . Further, mammalian muscle SR Ca^{2+} release activity is modulated by Mg^{2+} and ATP, whereas neither ligand appreciably affected ⁴⁵ Ca^{2+} efflux from lobster SR vesicles. These results suggested that lobster and mammalian muscle express immunologically and functionally distinct SR Ca^{2+} release channel protein complexes.

function have been studied extensively (1, 2). The release phase is triggered by a surface membrane action potential (3, 4) that is communicated to the SR by transverse tubules via specialized areas in which the two membrane systems come into close contact and are joined by protein bridges called "feet" (5) that span the gap between the two membranes. There is now good evidence that SR Ca^{2+} release in striated mammalian and amphibian muscle is effected by a ryanodine-sensitive, high conductance Ca^{2+} release channel which is identical to the feet (6, 7). The mammalian skeletal and cardiac SR Ca^{2+} release channels have been purified as 30 S protein complexes (6, 7) and shown to be homologous channels comprised of four *M*_r 565,000 polypeptides which, however, exhibit differences in their amino acid composition (8–11) and *in vitro* regulation by the three endogenous effector molecules Ca^{2+} , Mg^{2+} , and ATP (12).

The specific mechanism of signal transduction from the transverse tubule to SR, commonly referred to as excitation-contraction coupling, remains to be defined fully. In vertebrate skeletal muscle, a voltage-sensing molecule in the transverse tubule membrane has been proposed to effect the release of Ca^{2+} through a direct physical interaction with the SR Ca^{2+} release channel (13). Recent biophysical, pharmacological, and molecular biological evidence has suggested that the dihydropyridine receptor is the voltage sensor in excitation-contraction coupling in vertebrate skeletal muscle (14, 15). In contrast to skeletal muscle, in mammalian cardiac muscle a dihydropyridine receptor isoform has been shown to function as a voltage-dependent Ca^{2+} channel which mediates Ca^{2+} influx during a surface membrane action potential (16). The rise in intracellular Ca^{2+} concentration has been suggested to induce the opening of a Ca^{2+} -activated Ca^{2+} release channel in the SR (17, 18).

Like mammalian cardiac muscle (4), crustacean skeletal muscle fails to contract when the cell's membrane is depolarized in the absence of extracellular Ca^{2+} (19, 20). Some studies have suggested that the extracellular Ca^{2+} ions which enter the muscle cell during membrane depolarization may be sufficient for tension development (19, 21, 22). Other, more recent studies have indicated that in crustacean skeletal muscle, like in vertebrate muscle, SR Ca^{2+} release is a major contributor to the intracellular Ca^{2+} required for contraction (23–25). Here, we report the purification, immunofluorescent localization, and functional characterization of a 30 S RyR- Ca^{2+} release channel complex in lobster skeletal muscle. Our studies suggest that the lobster and mammalian skeletal and cardiac Ca^{2+} release channels are related oligomeric protein complexes which, however, display major differences in their *in vitro* regulation by the three endogenous effector molecules

The sarcoplasmic reticulum (SR)¹ is an intracellular membrane compartment which plays a key role in skeletal and cardiac muscle contraction and relaxation by rapidly releasing and resequestering Ca^{2+} . The uptake phase is mediated by the SR Ca^{2+} pump or (Ca^{2+} , Mg^{2+})ATPase, whose structure and

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¹ The abbreviations used are: SR, sarcoplasmic reticulum; Pipes, 1,4-piperazinediethanesulfonic acid; RyR, ryanodine receptor; EGTA, [ethylenediamine-N,N'-bis(2-aminoethoxy)acetic acid]; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DIFP, diisopropyl fluorophosphate; SDS, sodium dodecyl sulfate; pS, picosiemens; AMP-PCP, adenosine 5' (β , γ -methylene)triphosphate.

Ca^{2+} , Mg^{2+} , and ATP. Part of this work has been presented in abstract form (26).

EXPERIMENTAL PROCEDURES

Isolation of SR Vesicles—Live Maine lobsters (*Homarus americanus*) were obtained from a local supermarket. The abdominal muscle (~70 g) was rapidly excised, minced, and homogenized in a Waring blender for 60 s (2×30 s) at a high setting in 7.5 volumes (35 g in 260 ml) of a medium containing 0.1 M NaCl, 20 mM K/Pipes, pH 6.8, 1.0 mM EDTA, 0.1 mM EGTA, and various protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 100 nM aprotinin, 1 μ M leupeptin, 1 μ M pepstatin, and 1 mM benzamide). The homogenates were centrifuged at 4,500 rpm for 30 min in a Sorvall H6000A rotor, and the resulting supernatant was passed through three layers of cheesecloth and then re-centrifuged at 17,000 rpm for 30 min in a Beckman Ti-45 rotor. Pellets were resuspended in 120 ml of medium A (0.6 M KCl, 10 mM K/Pipes, pH 7.0, 100 μ M EGTA, 75 μ M Ca^{2+} , 0.2 mM phenylmethylsulfonyl fluoride, and 1 μ M leupeptin), incubated for 30 min, and sedimented by centrifugation for 30 min at 33,000 rpm in a Beckman Ti-45 rotor. After resuspension of pellets in 16 ml of medium A, membranes were loaded onto two 20–40% linear sucrose gradients in medium A and centrifuged for 16 h at 26,000 rpm in a Beckman SW 28 rotor. Membranes sedimenting in the 25–35% sucrose region of the gradients were collected, slowly diluted with 2 volumes of water, pelleted by centrifugation for 30 min in a Beckman 42.1 rotor, then resuspended in 0.3 M sucrose, 5 mM K/Pipes, pH 7.0 (at ~10 mg of protein/ml) and stored in ~0.3-ml aliquots at $-75^\circ C$. All procedures were carried out at $4^\circ C$.

Isolation of 30 S Ca^{2+} Release Channel Complex—The Chaps-solubilized lobster 30 S RyR- Ca^{2+} release channel complex was isolated by rate density gradient centrifugation in a way similar to that described (27). Unless otherwise indicated, lobster SR vesicles (1–2 mg of protein/ml) were solubilized in medium B (1 M NaCl, 20 mM Na/Pipes, pH 7.4, 100 μ M EGTA, 200 μ M Ca^{2+} , 5 mM AMP, 5 mg/ml phosphatidylcholine, 100 μ M dithiothreitol, 1 mM diisopropyl fluorophosphate (DIFP), 1 μ M leupeptin), containing 1.6% Chaps and 2 nM [3H]ryanodine. The solubilized proteins were loaded onto a linear 5–20% sucrose gradient in medium B containing 1% Chaps and centrifuged in a Beckman SW28 or SW41 rotor for 16 h at 26,000 or 27,000 rpm, respectively. Fractions of 1–2 ml were collected and analyzed for [3H]ryanodine radioactivity. [3H]RyR peak fractions were pooled, concentrated using a Centriprep 30 concentrator (Amicon), diluted to ~5% sucrose using a 0.1 M NaCl, 0.1% Chaps, 20 mM Na/Pipes, pH 7.4, buffer, and layered at the top of a second 10–20% linear sucrose gradient. After centrifugation, gradient fractions were analyzed for [3H]ryanodine and protein content and stored at $-135^\circ C$ before use.

$^{45}Ca^{2+}$ Efflux Measurements—Unless otherwise indicated, lobster SR vesicles (2–3 mg of protein) were preincubated for 30 min at $0^\circ C$ in 10 ml of medium C (0.1 M KCl, 20 mM K/Pipes, pH 7.0, 1 mM DIFP, 1 μ M leupeptin, 100 μ M EGTA, and 100 μ M Ca^{2+}) and centrifuged for 30 min at 33,000 rpm in a Beckman 42.1 rotor. Pelleted membranes were resuspended at a protein concentration of 10 mg/ml in medium C and incubated with 1 mM $^{45}Ca^{2+}$ for 2–5 min at $22^\circ C$ following the addition of 5 μ l of 2 mM $^{45}Ca^{2+}$ in medium C to 5 μ l of the vesicle suspension. A relatively short $^{45}Ca^{2+}$ loading time of 2–5 min was chosen since it was sufficient to result in nearly complete equilibration of $^{45}Ca^{2+}$ across vesicle membranes containing the SR Ca^{2+} release channel. $^{45}Ca^{2+}$ efflux was initiated by diluting vesicles 100-fold into isoosmolar efflux media and was stopped by placing at various times a 0.4-ml aliquot on a 0.45- μ m Millipore filter. Filters were washed with a quench solution containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, and 2 mM EGTA.

A Bio-Logic rapid filtration device (28) was used to measure $^{45}Ca^{2+}$ efflux rates at time intervals of less than 5 s. After incubation for 2 min with 1 mM $^{45}Ca^{2+}$, vesicles (10 μ l) were diluted 50-fold into a solution containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, and 1 mM EGTA and placed on a 0.65 μ m Millipore filter under vacuum. Vesicles on filters were washed manually three times with 1 ml of 0.1 M KCl, 20 mM K/Pipes, pH 7.0, and 1 μ M free Ca^{2+} (100 μ M EGTA and 70 μ M Ca^{2+}) before $^{45}Ca^{2+}$ efflux rates were determined by applying to the filters for 0.1–3 s a solution containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, and varying concentrations of Ca^{2+} . Radioactivity remaining with the vesicles on the filters was determined by liquid scintillation counting.

$^{45}Ca^{2+}$ efflux measurements were carried out at least in duplicate

using two or more preparations. Standard deviations were $\pm 20\%$ or less.

[3H]Ryanodine Binding—[3H]Ryanodine binding was determined essentially as described (29). Unless otherwise indicated, membranes were incubated for 8 h at $23^\circ C$ in media containing 1 M NaCl, 20 mM Na/Pipes, pH 7.4, 1 mM Ca^{2+} , 5 mM AMP, 1 mM DIFP, 5 μ M leupeptin, and varying concentrations of [3H]ryanodine. Nonspecific binding was estimated using a 1,000-fold excess of unlabeled ryanodine.

Single Channel Recordings—Single channel measurements were performed by incorporating the lobster 30 S RyR fractions, purified in the absence of [3H]ryanodine, into Mueller-Rudin type planar lipid bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio 5:3:2 (50 mg/ml total phospholipid in *n*-decane) (27). Unless otherwise indicated, a symmetric solution of buffered KCl (0.25 M KCl, 20 mM K/Pipes, pH 7.0) was used to record single channel currents. Small aliquots (1–3 μ l) of the 30 S gradient fractions were added to the cis chamber (defined as the SR cytoplasmic side; Ref. 30) of the planar lipid bilayer apparatus. Applied voltages are defined with respect to the trans chamber held at virtual ground. Electrical signals were filtered at 600 Hz and digitized at 2 kHz for analysis, using a single threshold level set at 50% of the current amplitude and a modified version of a program kindly provided by Dr. H. Affolter (31). The open probability values (p_o) were determined from data stored in 35-s files.

Antibody Production and Purification—Polyclonal antibodies against the 30 S lobster skeletal muscle RyR were produced in rabbits by intradermal injection of an emulsion of 0.5 ml of Freund's adjuvant (complete) and 0.5 ml of a saline solution containing 50 μ g of purified lobster RyR protein. After two booster injections at 4-week intervals, rabbit sera were collected at 10-day intervals. Immunoblot analysis of lobster SR vesicle fractions using the complete sera showed immunoreactivity with the RyR band and protein bands of lower molecular weight (see Fig. 3A). Immunostaining of the latter was eliminated by absorption of the complete immune sera with lobster skeletal microsomal proteins (depleted of RyR, fractions 8–11 of Fig. 2A) coupled to cyanogen bromide-activated cross-linked agarose (32). Polyclonal antibodies were affinity purified as described by Lillie and Brown (33) using cyanogen bromide-activated cross-linked agarose (Sigma) and the purified lobster RyR. Essentially identical results were obtained using polyclonal antibodies purified by either procedure.

Production of polyclonal antibodies against the purified rat skeletal muscle RyR in rabbits has been described (32). Sera were reacted with Chaps-solubilized rabbit skeletal SR proteins (depleted of RyR) before use (32) and shown by immunoblot analysis to recognize the RyR from rat, pig, dog, and rabbit skeletal muscle.

Monoclonal antibodies against the canine cardiac RyR were prepared in BALB/c mice immunized by serial injection of canine cardiac SR vesicles and the purified cardiac RyR using standard conditions. Hybridomas were screened by enzyme-linked immunosorbent assay, and specificity of monoclonal antibodies was determined by immunoblot analysis using canine cardiac microsomal membrane fractions as described in Fig. 3.

SDS-Gel Electrophoresis and Immunoblot Staining—SDS-polyacrylamide gel electrophoresis was performed in the Laemmli buffer system using 3–12% linear polyacrylamide gradient gels and 3% stacking gels (32). After electrophoresis, gels were stained with 0.1% Coomassie Brilliant Blue R-250 according to Ref. 34. For immunoblot analysis, the separated proteins on the gels were electrophoretically transferred onto Immobilon polyvinylidene difluoride membranes (Millipore Co.) for 1 h at 400 mA and 20 h at 1 A. Transfer membranes were blocked with 5% non-fat dried milk proteins and then incubated with primary and peroxidase-conjugated secondary antibodies and developed using 3,3'-diaminobenzidine and H_2O_2 .

Immunolocalization of Lobster RyR—Immunofluorescent localization of lobster RyRs was carried out by whole mount labeling, Epon embedment, and thin sectioning as described (35). Briefly, small bundles of fibers from the dorsal portion of the main muscle mass in the lobster tail were rapidly dissected under fixative (1% paraformaldehyde in 500 mM NaCl, 20 mM sodium phosphate, pH 7.3) and pinned out under slight tension. After fixation for 20 min, fibers were permeabilized with Triton X-100, treated with NH_4Cl to block remaining aldehydes, incubated with purified lobster RyR antibodies or nonspecific rabbit IgG followed by labeling with rhodamine goat anti-rabbit IgG (Jackson ImmunoResearch, Inc, West Grove, PA), fixed with 4% paraformaldehyde, dehydrated, and wafer-embedded in Epon 812 between Aclar sheets. Longitudinal sections (0.3–0.6- μ m

thickness) were mounted on slides, treated to remove the Epon, and examined for fluorescence and phase contrast. Additional tests of immunofluorescence were carried out on cryostat sections of fixed lobster muscle using preimmune serum and the purified immune serum with and without preincubation with purified RyR. Purified immune sera (1:20 dilution) were preincubated with RyR at concentrations as low as 1 μ g of protein/ml.

Materials— $[^3H]$ Ryanodine (54.7 Ci/mmol) and $^{45}Ca^{2+}$ were obtained from DuPont-New England Nuclear and unlabeled ryanodine from AgriSystems International (Wind Gap, PA). Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL), Chaps from Boehringer Mannheim, ruthenium red from Flukka, SDS-gel molecular weight markers from Sigma, and peroxidase-conjugated secondary antibodies from Calbiochem. All other chemicals were of analytical grade.

RESULTS

Purification of 30 S Lobster RyR—The neutral plant alkaloid, ryanodine, has been shown to bind with nanomolar affinity to the mammalian skeletal and cardiac SR Ca^{2+} release channels (for review see Ref. 7). High affinity binding is dependent on Ca^{2+} concentration and enhanced by increasing the ionic strength of the assay media. In preliminary studies, we found that $[^3H]$ ryanodine also bound with high affinity to lobster SR vesicles in assay media that contained 1 mM Ca^{2+} and 1 M NaCl. Scatchard analysis of binding data (Fig. 1) indicated the presence of a high affinity site with a B_{max} value of 17 pmol/mg protein and K_D of 6.5 nM.

The $[^3H]$ ryanodine-labeled lobster skeletal muscle RyR was purified by rate density gradient centrifugation, using a procedure essentially identical to that employed for the purification of the rabbit skeletal and canine cardiac RyRs (27, 36). Lobster skeletal muscle SR vesicles were solubilized with Chaps in the presence of 2 nM $[^3H]$ ryanodine and loaded onto a linear 5–20% sucrose gradient containing 1% Chaps and 5 mg/ml phosphatidylcholine. Chaps-solubilized $[^3H]$ ryanodine-labeled rabbit skeletal and canine cardiac RyRs were placed onto two parallel sucrose gradients and then centrifuged with the gradient containing the lobster RyR. Analysis of gradient fractions for 3H radioactivity indicated a single peak of bound radioactivity in the bottom fractions of the lobster (Fig. 2A) and rabbit skeletal and canine cardiac (not

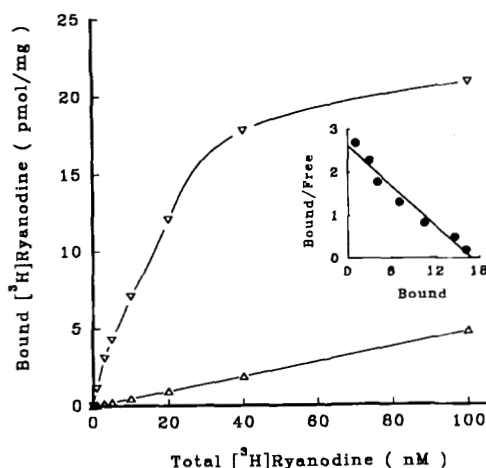


FIG. 1. $[^3H]$ Ryanodine binding to lobster SR vesicles. Lobster SR vesicles were incubated for 8 h at 23 °C in 1.0 M NaCl, 1 mM $CaCl_2$, 5 mM AMP, 1 mM DIFP, 5 μ M leupeptin, 20 mM Na/Pipes, pH 7.4, and 1–100 nM $[^3H]$ ryanodine. In parallel experiments, it was established that equilibrium of $[^3H]$ ryanodine binding was reached after incubation for 8 h at 23 °C. Total (∇) and nonspecifically (Δ) bound $[^3H]$ ryanodine were determined as described (29). Nonspecific binding was assessed using a 1,000-fold excess of unlabeled ryanodine. Inset, Scatchard plot of $[^3H]$ ryanodine binding. Data are averages of two experiments carried out in triplicate (S.D. = 10% or less).

shown) gradients corresponding to an apparent sedimentation coefficient of 30 S (27). SDS-polyacrylamide gel analysis of lobster gradient fractions revealed the presence of a > 90% pure high molecular weight protein band whose distribution on gradients was coincident with that of $[^3H]$ ryanodine binding (not shown). After further purification on a second sucrose gradient, a single high molecular weight lobster protein band was observed which migrated on the gels with a mobility intermediate between the M_r 565,000 polypeptides of the rabbit skeletal and canine cardiac RyRs (Fig. 2B and panel a of Fig. 3B).

Using 30 S receptor fractions purified on a single sucrose gradient, we obtained K_D and B_{max} values for specifically bound $[^3H]$ ryanodine of 4.5 nM and 135 pmol/mg protein, respectively (Fig. 2C). Assuming a single high affinity ryanodine binding site/tetrameric 30 S complex comprised of M_r 565,000 polypeptides, the maximum value of specific high affinity $[^3H]$ ryanodine binding would be 440 pmol/mg protein. The experimentally obtained value of 135 pmol/mg protein was appreciably lower, which was probably a result of partial inactivation of the lobster RyR during purification and/or the prolonged (8-h) incubation period required for reaching equilibrium of $[^3H]$ ryanodine binding.

Immunoblot Analysis and Immunolocalization of Lobster RyR—Immunoblot analysis of lobster microsomal proteins showed that polyclonal antibodies raised against the purified lobster skeletal muscle RyR in rabbits cross-reacted with a large number of lobster skeletal muscle proteins, in addition to the RyR (Fig. 3A, lanes 1 and 2). No immunoreactive protein bands were observed when preimmune sera were used or rabbit skeletal and canine cardiac SR proteins were probed with immune sera (not shown). A single absorption (see "Experimental Procedures") of immune sera with lobster skeletal microsomal proteins (depleted of RyR) greatly diminished immunostaining of nonRyR proteins (Fig. 3A, lane 3). After a second absorption, only one immunoreactive band with a mobility corresponding to that of RyR was observed (Fig. 3A, lane 4). Very similar results were obtained with three rabbits immunized with different purified RyR preparations. We speculate that minor contaminants in our preparations produced antibodies because of prior stimulation of the immune system of rabbits by their diet.

Immunoblot analysis of 30 S lobster RyR gradient fractions with purified antiserum against the lobster RyR revealed an immunoreactive high molecular weight protein band but no cross-reactivity with the M_r 565,000 protein bands of the rabbit skeletal and canine cardiac receptors (panel c of Fig. 3). Conversely, a polyclonal antibody to the rat skeletal RyR cross-reacted with the M_r 565,000 rabbit skeletal protein band but not with the lobster protein band (panel b of Fig. 3). A monoclonal antibody to the canine cardiac RyR (C3-33) reacted with the canine cardiac receptor protein band but did not show any noticeable immunoreactivity with the lobster and rabbit muscle high molecular weight protein bands (panel d of Fig. 3). The immunoblot data show that the lobster and mammalian 30 S RyRs are comprised of immunologically distinct polypeptides.

The lobster RyR was further characterized by immunofluorescent localization in muscle fibers dissected from the dorsal portion of the main muscle mass of the lobster tail. Binding of the purified anti-RyR polyclonal antibody was confined to small dots aligned in two roughly parallel lines in each sarcomere (Fig. 4, middle panel). The lines were essentially coincident with the junctions of the A-I bands, as judged from phase-contrast images (not shown directly; phase-contrast images of immediately adjacent regions are shown in the upper

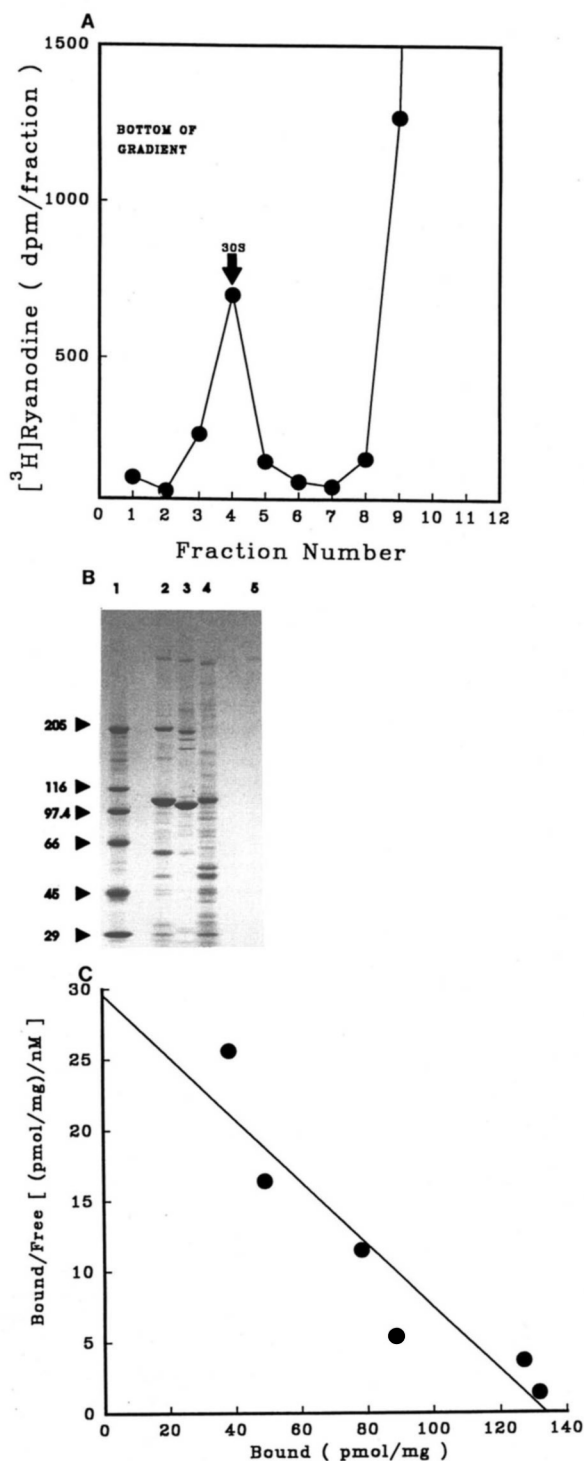


FIG. 2. Sedimentation profile, SDS-gel analysis, and $[^3H]$ ryanodine binding of Chaps-solubilized, purified lobster RyR. A, lobster SR vesicles (1.2 mg of protein/ml) were solubilized with Chaps (1.6%) in a medium containing 1.0 M NaCl, 20 mM Na/Pipes, pH 7.4, 100 μ M free Ca^{2+} , 5 mM AMP, 5 mg/ml phosphatidylcholine, 100 μ M dithiothreitol, 1 μ M leupeptin, 1 mM DIFP, and 2 nM $[^3H]$ ryanodine. The solubilized proteins were loaded onto a linear 5–20% sucrose gradient in the above medium containing 1% Chaps and centrifuged at 2 $^{\circ}$ C in a Beckman SW 41 rotor for 16 h at 27,000 rpm. Twelve fractions of 1 ml each were collected and analyzed for 3H radioactivity. The arrow indicates the position of $[^3H]$ ryanodine peak fraction of gradients containing the rabbit skeletal or canine cardiac RyRs. B, SDS-polyacrylamide gel electrophoresis of molecular weight standards (lane 1), rabbit skeletal (lane 2), lobster skeletal (lane 3) and canine cardiac (lane 4) SR vesicles (25 μ g of protein each), and purified 30 S lobster RyR (lane 5, 0.8 μ g of protein). Molecular weight values ($\times 10^{-3}$) of standard proteins are indicated on the left. C, Scatchard plot of $[^3H]$ ryanodine binding to lobster RyR, purified on a single linear 5–20% sucrose gradient containing 1 mM Ca^{2+} and 1.0–0.7% Chaps. The purified, unlabeled receptor (10 μ g of protein/ml) was incubated for 8 h at 23 $^{\circ}$ C in 1.0 M NaCl, 20 mM Na/Pipes, pH 7.4, 5 mg/ml phosphatidylcholine, \sim 0.7% Chaps, 1 mM $CaCl_2$, 5 mM AMP, 1 mM DIFP, 5 μ M leupeptin, and 3–100 nM $[^3H]$ ryanodine. Nonspecific binding was assessed using a 1,000-fold excess of unlabeled ryanodine. Specific $[^3H]$ ryanodine binding was determined as described (27).

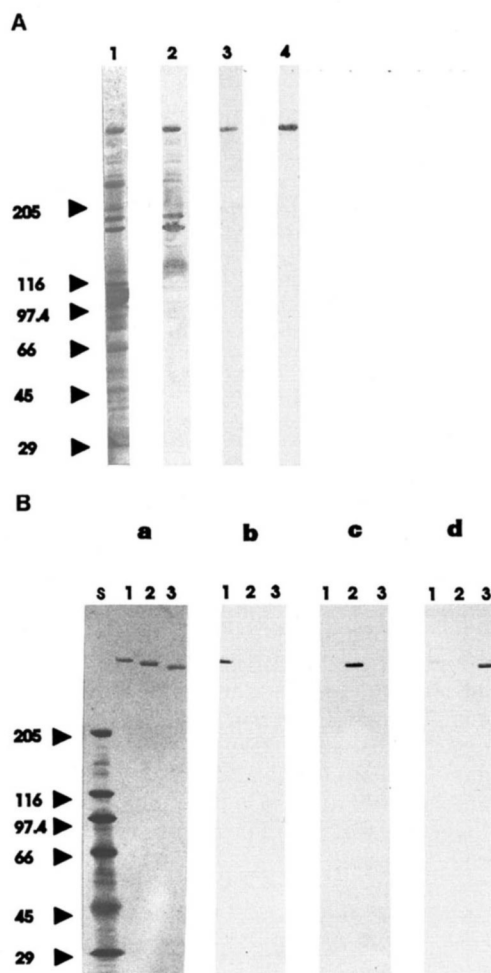


FIG. 3. Immunoblot analysis of RyRs. A, lobster skeletal microsomal proteins (30 μ g of protein each) were electrophoresed through linear 3–12% SDS-polyacrylamide gels and electrophoretically transferred onto Immobilon polyvinylidene difluoride membranes. Transfer membranes were stained with Coomassie Blue (lane 1) or probed with lobster skeletal muscle RyR antisera before (lane 2, 1:3,000 dilution) and after one (lane 3, 1:3,000 dilution) or two (lane 4, 1:600 dilution) treatments with lobster skeletal microsomal proteins depleted of RyR (see “Experimental Procedures”). B, RyR peak fractions of a second sucrose gradient (“Experimental Procedures”) containing rabbit (lane 1), lobster (lane 2), or canine (lane 3) SR proteins (1.5 μ g each) were electrophoresed through 3–12% SDS-polyacrylamide gradient gels and electrophoretically transferred onto Immobilon polyvinylidene difluoride membranes. Transfer membranes were either stained with Coomassie Blue (panel a) or probed with purified rat (panel b) and lobster (panel c) skeletal muscle RyR antisera, or canine cardiac muscle RyR monoclonal antibody C3-33 (panel d). Molecular weight values ($\times 10^{-3}$) of standard proteins (S) are indicated on the left.

and lower panels of Fig. 4). Where abrupt shifts in the positions of the lines of dots occurred (second and third sarcomeres from the left, middle panel of Fig. 4), these corresponded to shifts in the alignment of sarcomeres. No labeling could be detected when preimmune serum or nonspecific rabbit IgG

Scatchard plot of $[^3H]$ ryanodine binding to lobster RyR, purified on a single linear 5–20% sucrose gradient containing 1 mM Ca^{2+} and 1.0–0.7% Chaps. The purified, unlabeled receptor (10 μ g of protein/ml) was incubated for 8 h at 23 $^{\circ}$ C in 1.0 M NaCl, 20 mM Na/Pipes, pH 7.4, 5 mg/ml phosphatidylcholine, \sim 0.7% Chaps, 1 mM $CaCl_2$, 5 mM AMP, 1 mM DIFP, 5 μ M leupeptin, and 3–100 nM $[^3H]$ ryanodine. Nonspecific binding was assessed using a 1,000-fold excess of unlabeled ryanodine. Specific $[^3H]$ ryanodine binding was determined as described (27).

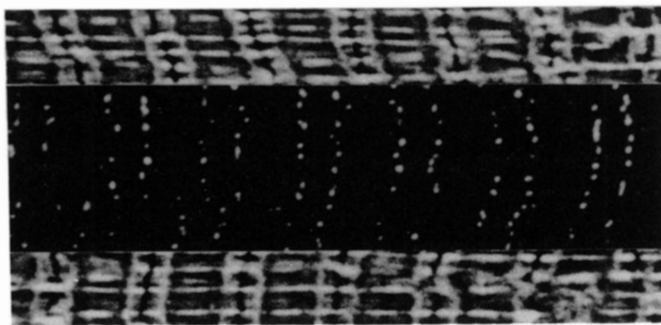


FIG. 4. Immunofluorescent localization of RyR in lobster abdominal muscle fibers. Middle panel shows the immunofluorescent image, using a purified lobster skeletal muscle RyR antiserum and rhodamine-labeled secondary antibody. Panels above and below show immediately adjacent regions of the muscle fiber in phase contrast. Magnification, $\times 1,850$. Bar, 2.5 μm .

was substituted for the purified immune serum or when the purified immune serum was preincubated with purified lobster RyR (data not shown). These results established that the RyR is localized near the junctions of the A-I bands in lobster abdominal muscle, in agreement with the presence of diads, and occasionally triads, at this position in lobster fast and slow abdominal muscle fibers (37).

Reconstitution of Purified Lobster Ca²⁺ Release Channel—The presence of a channel activity intrinsic to the 30 S RyR complex was assessed by incorporating the purified, Chaps-solubilized RyR into planar lipid bilayers. Channels were reconstituted in symmetric 0.25 M KCl medium since the reconstituted mammalian Ca²⁺ release channels have been shown to be impermeant to Cl⁻ and to conduct monovalent cations more efficiently than Ca²⁺ (27, 38). Fig. 5A shows a representative experiment in which the effects of varying Ca²⁺ concentration on channel activity were tested, following the addition of the purified lobster RyR to one chamber (defined to be the cis chamber) of the bilayer apparatus (the cis and trans chambers correspond to the cytoplasmic and luminal sides of the SR membrane, respectively; Refs. 27, 30, 38). In the upper trace, the channel was minimally activated with 6 μM free Ca²⁺ in the cis chamber. Short, often poorly resolved events were seen either separately or grouped into bursts of activity. Channel activity was greatly stimulated upon raising the free Ca²⁺ concentration in the cis chamber (traces 2–4 of Fig. 5A). Open probability of the channel was increased from 0.004 with 6 μM Ca²⁺ cis to 0.06, 0.40, and 0.97 with 0.4, 1.0, and 10 mM free Ca²⁺ cis, respectively. A nearly full activation of the lobster channel by 10 mM Ca²⁺ was observed in 8 of 12 experiments. The partially Ca²⁺-activated channel displayed a unitary conductance of (774 ± 45) pS ($n = 11$) with 260 mM K⁺ as the current carrier (Fig. 5B). Elevation of free Ca²⁺ concentration to 10 mM in the cis chamber reduced the magnitude of single channel currents at negative (bottom trace of Fig. 5A) and positive holding potentials. On perfusion of the trans chamber with 50 mM CaCl₂, a unitary Ca²⁺ conductance of 122 ± 24 pS ($n = 3$) could be measured (Fig. 5B). The reversal potential (E_{rev}) was shifted to (11 ± 1) mV ($n = 2$), from which a permeability ratio for Ca²⁺ versus K⁺ ($p_{\text{Ca}^{2+}}/p_{\text{K}^{+}}$) of 6.3 was calculated by application of constant field theory.

Fig. 6 shows the current traces of a separate channel recording with 50 μM Ca²⁺ cis (upper trace) and following the successive addition of ATP, additional Ca²⁺, and ryanodine to the same solution in the cis chamber. In contrast to the rabbit skeletal and canine cardiac channels (6, 7), the activity of the lobster channel was not appreciably changed upon the

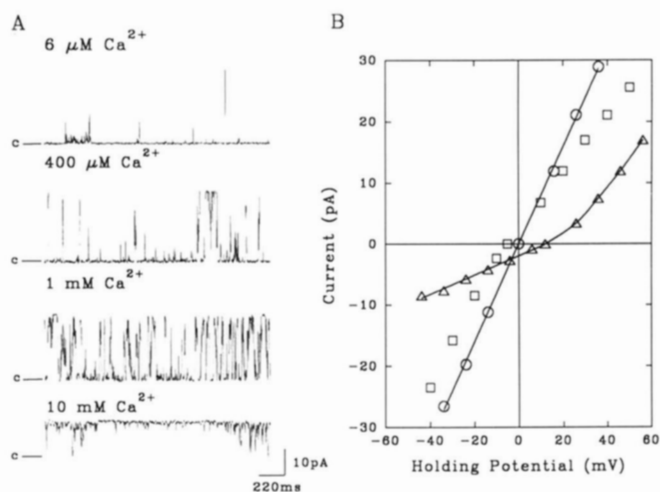


FIG. 5. Reconstitution of the purified lobster skeletal muscle 30 S RyR-Ca²⁺ release channel complex into a planar lipid bilayer. A, an aliquot (1–3 μl) of the Chaps-solubilized 30 S lobster RyR, purified on a single sucrose gradient in the absence of [³H] ryanodine, was added to the cis side of a planar bilayer. Single channel currents, shown as upward deflections, were recorded in symmetric 0.25 M KCl, 20 mM K/Pipes, pH 7.0, media containing the indicated concentrations of free Ca²⁺ in the cis chamber. Bars beside each trace indicate the level of the closed (c) state. Holding potential = -32 mV. Open probability was increased upon the addition of cis Ca²⁺ from 0.004 (upper trace) to 0.06 (second trace), 0.40 (third trace), and 0.97 (bottom trace). B, current-voltage relationships of K⁺- and Ca²⁺-conducting lobster Ca²⁺ release channel. Single channel currents were recorded in symmetric 0.25 M KCl, 20 mM K/Pipes, pH 7.0, media containing 0.4 (○, △) or 10 (□) mM free Ca²⁺ in the cis chamber, and after perfusion of the trans chamber with a 50 mM CaCl₂ solution (△). $\gamma_{\text{K}^{+}} = 775$ pS; $\gamma_{\text{Ca}^{2+}} = 140$ pS.

addition of 1.2 mM ATP cis ($p_o = 0.007$ in upper trace increased to 0.008 in the second trace of Fig. 6). In 11 of 18 experiments, the addition of millimolar concentrations of ATP cis to a medium containing micromolar concentrations of Ca²⁺ did not significantly change the p_o of the lobster channel. In the remaining experiments (7 of 18), a 2–3-fold increase in p_o of the minimally activated lobster channel was observed. In Fig. 6 (third trace), the channel was nearly fully activated, following the addition of 15 mM Ca²⁺ cis. Brief closed events and the occasional presence of a subconductance state were apparent. The two bottom traces of Fig. 6 confirm that the purified, K⁺-conducting lobster channel is a ryanodine-sensitive channel. The addition of micromolar concentrations of ryanodine cis induced the formation of an open subconductance state, whereas addition of 4 mM ryanodine cis fully closed the lobster channel. The K⁺ conductance of the reconstituted rabbit skeletal muscle Ca²⁺ release channel is modified by ryanodine in an essentially identical manner (29).

Vesicle-⁴⁵Ca²⁺ Flux Measurements—Although single channel measurements provide more direct information, an advantage of the vesicle flux technique is that it more readily yields representative data by averaging the kinetic behavior of a large number of channels. Regulation of the lobster Ca²⁺ release channel by Ca²⁺ was therefore further studied by determining the ⁴⁵Ca²⁺ efflux behavior of passively loaded SR vesicles as a function of varying concentrations of extravesicular Ca²⁺. Fig. 7 shows the time course of ⁴⁵Ca²⁺ efflux from lobster SR vesicles which were passively loaded with 1 mM ⁴⁵Ca²⁺ and then diluted into media that contained varying concentrations of free Ca²⁺. ⁴⁵Ca²⁺ efflux from the vesicles was slow in a medium containing 2 mM EGTA (free [Ca²⁺] < 10⁻⁸ M). Elevation of free Ca²⁺ concentration in efflux media

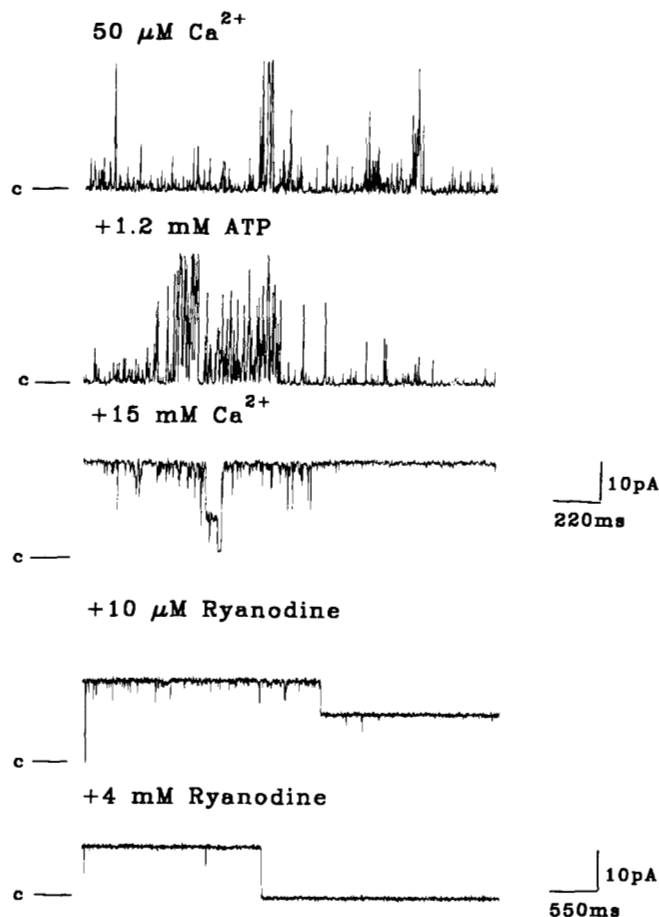


FIG. 6. Pharmacology of reconstituted K^+ -conducting lobster Ca^{2+} release channel. Single channel currents, shown as upward deflections, were recorded in symmetric 250 mM KCl, 20 mM K/Pipes, pH 7.0, media containing 50 μM free Ca^{2+} cis (upper trace, $p_o = 0.007$), following the successive addition of 1.2 mM ATP (second trace, $p_o = 0.008$), 15 mM Ca^{2+} cis (third trace, $p_o = 0.98$), 10 μM ryanodine cis (fourth trace), and 4 mM ryanodine cis (bottom trace). Appearance of subconductance (fourth trace) and fully closed (bottom trace) channel states was observed about 5 and 2 min after the addition of 10 μM and 4 mM ryanodine, respectively. Holding potential = -40 mV. Bars beside each trace indicate the level of the closed (c) state.

to 1 and 100 μM resulted in only modest increases of the initial $^{45}\text{Ca}^{2+}$ efflux rates. A more pronounced increase in the $^{45}\text{Ca}^{2+}$ efflux rates was observed in the presence of extravascular Ca^{2+} concentrations that were equal to (1 mM Ca^{2+}) or higher than (10 mM Ca^{2+}) those inside the vesicles. The Ca^{2+} dependence of $^{45}\text{Ca}^{2+}$ efflux rates from lobster SR vesicles is shown in Fig. 8, along with data from rabbit skeletal (39) and canine cardiac (12) SR vesicles. For mammalian vesicles, a bell-shaped Ca^{2+} activation curve was obtained, with $^{45}\text{Ca}^{2+}$ efflux being optimal in the micromolar Ca^{2+} concentration range. A biphasic curve like the one obtained with the mammalian vesicles has suggested that the mammalian skeletal and cardiac Ca^{2+} release channels possess high affinity activating and low affinity inhibitory Ca^{2+} binding sites. The lobster channel appeared to lack corresponding high and low affinity regulatory Ca^{2+} binding sites; over the range of Ca^{2+} concentrations tested, only low affinity activating Ca^{2+} binding sites were observed.

It was conceivable that increased $^{45}\text{Ca}^{2+}$ efflux rates at millimolar extravascular Ca^{2+} (Fig. 7) were caused by the presence of a $^{45}\text{Ca}^{2+}$ - Ca^{2+} exchanger rather than Ca^{2+} channel in lobster SR. To distinguish between these two possibilities, lobster and rabbit skeletal muscle SR vesicles were loaded

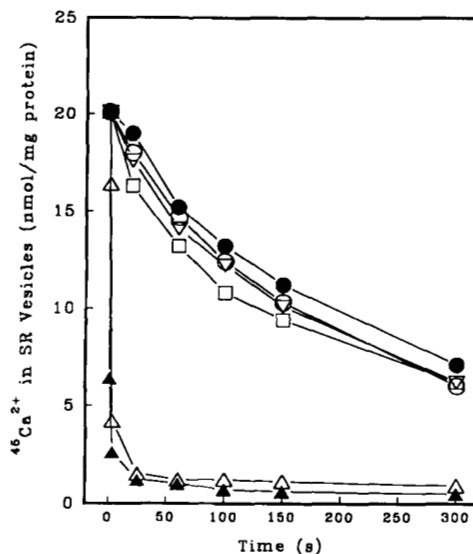


FIG. 7. Ca^{2+} -induced $^{45}\text{Ca}^{2+}$ efflux from lobster SR vesicles. Lobster SR vesicles were incubated for 2 min in 0.1 M KCl, 20 mM K/Pipes, pH 7.0, medium containing 1 mM $^{45}\text{Ca}^{2+}$ and then diluted 100-fold into isoosmolar efflux media containing 2 mM EGTA ($< 10^{-8}$ M free Ca^{2+}) (●), 10 nM (○), 1 μM (▽), 100 μM (□), 1 mM (Δ), or 10 mM (▲) free Ca^{2+} . Amounts of $^{45}\text{Ca}^{2+}$ remaining with the vesicles at the indicated times were determined as described under "Experimental Procedures."

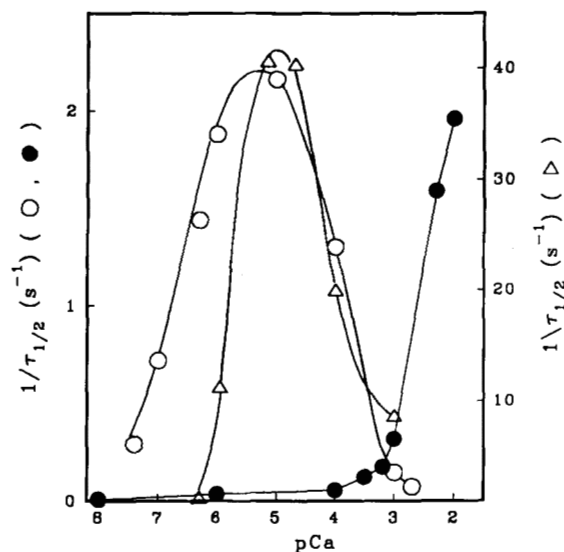


FIG. 8. Dependence of $^{45}\text{Ca}^{2+}$ efflux half-times on external Ca^{2+} concentration. Lobster (●) and rabbit (○) skeletal and canine cardiac (Δ) muscle SR vesicles were passively loaded with 1 mM $^{45}\text{Ca}^{2+}$ and then diluted into isoosmolar efflux media containing the indicated concentrations of free Ca^{2+} . The half-times ($t_{1/2}$) of $^{45}\text{Ca}^{2+}$ efflux from lobster Ca^{2+} -permeable vesicles were determined as indicated in the legend of Fig. 7 by subtracting the amount of $^{45}\text{Ca}^{2+}$ not readily released in the 10 mM Ca^{2+} efflux medium (1–2 nmol/mg of protein). Half-times of $^{45}\text{Ca}^{2+}$ efflux from Ca^{2+} -permeable rabbit skeletal and canine cardiac muscle SR vesicles were determined with a rapid mixing chemical quench apparatus and by filtration (from Meissner *et al.* (39) and Meissner and Henderson (12), respectively).

with a neutral, channel-permeating solute (^3H glucose, Ref. 40) and then diluted into media which either inhibited ($< 10^{-8}$ free Ca^{2+}) or partially activated (5 μM or 1 mM free Ca^{2+} in the presence and absence of 5 mM AMP) the lobster and rabbit Ca^{2+} release channels. ^3H Glucose efflux from lobster and rabbit SR vesicles showed a Ca^{2+} dependence comparable

to that of ⁴⁵Ca²⁺ efflux (Fig. 9, A and B; compare with Fig. 8). [³H]Glucose efflux from lobster SR vesicles was faster with 1 mM than 5 μM Ca²⁺ in the assay medium (Fig. 9A), whereas the lower Ca²⁺ concentration was more effective in causing [³H]glucose efflux from the rabbit SR vesicles (Fig. 9B). The addition of adenine nucleotide to efflux media increased [³H]glucose efflux rate from rabbit SR vesicles, in a way similar to that observed previously (40), but was without appreciable effect on [³H]glucose efflux from lobster SR vesicles. Some of the radioactivity remained with the vesicles for longer times, which suggested the presence of a subpopulation of vesicles that lacked a Ca²⁺-activated [³H]glucose efflux pathway (40). Taken together, data of Figs. 7-9 support the idea of a Ca²⁺ release channel in lobster SR which is permeable to Ca²⁺ and glucose and activated by millimolar concentrations of Ca²⁺.

The ⁴⁵Ca²⁺ efflux rate from lobster SR vesicles was strongly dependent on pH. Similar to the observation of mammalian SR vesicles (12), the half-time of ⁴⁵Ca²⁺ efflux was increased from about 2 s to 300 s by decreasing the pH of assay media containing 1 mM Ca²⁺ from pH 7.4 to 6 (not shown).

Table I summarizes the results of experiments in which the effects of several compounds known to stimulate (ATP, caffeine) or inhibit (Mg²⁺, ruthenium red, tetracaine, procaine) mammalian ⁴⁵Ca²⁺ release channel activity were assessed (6, 7). At nanomolar external Ca²⁺, ⁴⁵Ca²⁺ efflux from lobster SR vesicles was slow, requiring a half-time of 130 s. The addition of 5 mM ATP (or AMP-PCP, a nonhydrolyzable ATP analog) and 10 mM caffeine to nanomolar and micromolar Ca²⁺ media resulted in a modest (~1.5-fold) stimulation of ⁴⁵Ca²⁺ efflux. The combined presence of ATP and caffeine in a nanomolar Ca²⁺ medium was somewhat more effective, decreasing the half-time of ⁴⁵Ca²⁺ efflux by a factor of about 2. The addition of 5 mM Mg·AMP-PCP, an allosteric effector of the mammalian Ca²⁺ release channels (12, 39), to 10⁻⁵ and 10⁻³ M Ca²⁺ media did not appreciably alter the ⁴⁵Ca²⁺ efflux rate from lobster SR vesicles.

The inhibitory effects of Mg²⁺, ruthenium red, tetracaine, and procaine (6, 7) were studied using conditions that resulted in a partial activation of the lobster Ca²⁺ release channel (Figs. 5 and 7). The addition of 5 and 20 mM Mg²⁺ or 10 μM ruthenium red to a 1 mM Ca²⁺ efflux medium was only moderately effective in inhibiting Ca²⁺-activated ⁴⁵Ca²⁺ efflux from lobster SR vesicles. An increase in ruthenium red concentration to 1 mM was required for a partial inhibition of

the Ca²⁺-activated efflux pathway in lobster SR. The lobster Ca²⁺ efflux pathway could be also partially inhibited by the local anesthetic tetracaine at a concentration of 1 mM. By comparison, a relatively high concentration (10 mM) of procaine increased the half-time of ⁴⁵Ca²⁺ efflux by a factor of only 2 (Table I).

Mammalian SR Ca²⁺ release channels can be partially inhibited via a direct interaction with calmodulin (41). The addition of 5 μM calmodulin or 10 μM mastoparan, a calmodulin inhibitor, to vesicle and Ca²⁺ efflux media did not appreciably change the ⁴⁵Ca²⁺ efflux rate from lobster SR vesicles (not shown).

An intracellular inositol 1,4,5-trisphosphate-sensitive Ca²⁺ release mechanism has been described in barnacle muscle (42). We found that the addition of 10 μM inositol 1,4,5-trisphosphate to 0.01, 0.5, 5, or 100 μM Ca²⁺ efflux media did not appreciably increase the ⁴⁵Ca²⁺ efflux rate from lobster SR vesicles (not shown).

DISCUSSION

The present study describes the isolation, immunofluorescent localization, and functional characteristics of a 30 S ryanodine-sensitive, Ca²⁺-, K⁺- and glucose-permeable channel complex of lobster abdominal muscle. The lobster channel shared several properties with the mammalian skeletal and cardiac RyR-Ca²⁺ release channel complexes. These included an apparent sedimentation coefficient of 30 S, isolation of a ryanodine-binding protein complex comprised of a single high molecular polypeptide band on SDS gels, the presence of a high affinity site for ryanodine, and a high conductance ion pathway which exhibited a Ca²⁺ and K⁺ conductance essentially identical to those of the mammalian skeletal and cardiac Ca²⁺ release channels. However, immunoblot analysis showed no noticeable cross-reactivity between the lobster and mammalian high molecular weight polypeptides, and the lobster and mammalian channels differed in their sensitivity to activation and inhibition by various effector molecules. These results suggest that the lobster and mammalian Ca²⁺ channels are related but immunologically and functionally distinct protein complexes.

Although many crustacean muscles are composed of "slow" (tonic) and "fast" (phasic) fibers (43) and two types of Ca²⁺ release channels have been described in avian and amphibian muscle (44), the biochemical and functional evidence obtained

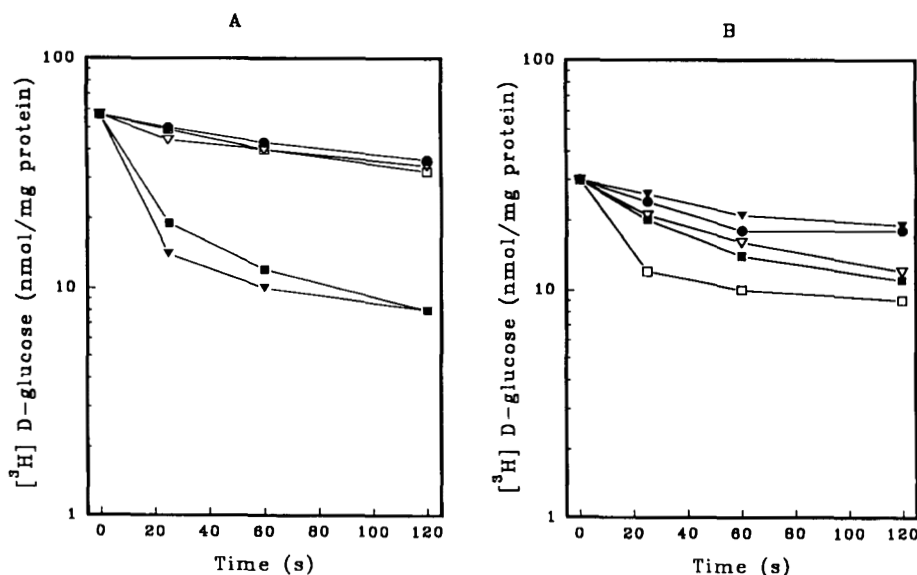


FIG. 9. Dependence of [³H]glucose efflux rates on external Ca²⁺ concentration. Lobster (A) and rabbit (B) skeletal muscle SR vesicles were passively loaded with 5 mM D-[³H]glucose for 2 and 30 min, respectively, in media containing 1 mM Ca²⁺ and then diluted into isoosmolar efflux media containing 2 mM EGTA (<10⁻⁸ M free Ca²⁺) (●), 5 μM Ca²⁺ (▽), 5 μM Ca²⁺ + 5 mM AMP (□), 1 mM Ca²⁺ (▼), or 1 mM Ca²⁺ + 5 mM AMP (■). Amounts of [³H]glucose remaining with the vesicles were determined as described under "Experimental Procedures."

TABLE I
 $^{45}\text{Ca}^{2+}$ efflux from lobster SR vesicles

The half-times ($t_{1/2}$) of $^{45}\text{Ca}^{2+}$ efflux from lobster Ca^{2+} -permeable vesicles were determined as indicated in the legend of Fig. 7 by subtracting the amounts of $^{45}\text{Ca}^{2+}$ not readily released in a 10 mM Ca^{2+} efflux medium (1–2 nmol/mg of protein). Data are average of three or more determinations (S.D. = $\pm 20\%$ or less).

Free Ca^{2+}	Addition to efflux medium	$^{45}\text{Ca}^{2+}$ efflux ($t_{1/2}$)
M	M	s
10^{-8}		130
	5×10^{-3} ATP	90
	1×10^{-2} Caffeine	90
	5×10^{-3} ATP + 1×10^{-2} caffeine	60
10^{-5}		110
	5×10^{-3} AMP-PCP	70
	5×10^{-3} Mg·AMP-PCP	90
	1×10^{-2} Caffeine	70
10^{-3}		2.5
	5×10^{-3} Mg·AMP-PCP	2.3
	5×10^{-3} Mg^{2+}	3.0
	2×10^{-2} Mg^{2+}	4.0
	1×10^{-5} Ruthenium red	5.0
	1×10^{-3} Ruthenium red	30
	1×10^{-3} Tetracaine	27
	1×10^{-2} Procaine	5

in this study is compatible with the presence in our biochemical preparations of a single species of Ca^{2+} release channel. Jahromi and Atwood (37) have described the structural and physiological characteristics of slow and fast extensor muscle fibers in the lobster abdomen. The slow muscle fibers had a greater sarcomere length ($> 6 \mu\text{m}$ versus $< 4 \mu\text{m}$ in fast fibers), contracted and relaxed more slowly, and developed greater tension than the fast fibers in high extracellular K^+ , in 30 mM caffeine, or in high K^+ and caffeine. A preliminary histological examination confirmed that the muscle masses used for our biochemical preparations contain at least two fiber types, based on A-band lengths.² However, the relative amounts of SR membranes derived from fast and slow muscle fibers in our preparations are not known and cannot even be estimated without much additional histological work.

Ultrastructural analysis of lobster abdominal slow and fast extensor (37) and fast flexor (45) muscle fibers revealed numerous diad-like, and occasionally triad-like, structures at the junctions of the A-I bands. In agreement with these earlier studies, immunofluorescent data of this study indicated that the lobster Ca^{2+} release channel is located at the junctions of the A-I bands. An apparent sarcomere length of $\sim 6 \mu\text{m}$ in immediately adjacent regions suggested that the sample in Fig. 4 was from a slow muscle fiber.

Previous studies with isolated SR vesicle fractions have shown that lobster abdominal muscle possesses a highly active ATP-dependent Ca^{2+} pump (46, 47). Two preliminary studies describing the presence of a ryanodine-sensitive Ca^{2+} release channel in lobster skeletal muscle microsomal membrane fractions have been presented (48, 49). Formelova *et al.* (50) have described the isolation of a large RyR complex from crayfish skeletal muscle. Electron microscopic examination of negative-stained receptor fractions revealed square-like protein structures with an overall dimension comparable to those of the 30 S mammalian skeletal and cardiac receptor complexes (6, 7). Upon reconstitution into planar lipid bilayers, two conductances of 50 and 100 pS were observed in 100 mM NaCl; however, the pharmacological characteristics of the two

conductance states were not described by these investigators.

The lobster and mammalian skeletal and cardiac Ca^{2+} channels displayed major differences in their sensitivity to activation or inhibition by various endogenous and exogenous effector molecules. In the two mammalian preparations, the addition of 10 mM caffeine and 5 mM ATP to a nanomolar Ca^{2+} medium or the addition of 5 mM AMP-PCP to a micromolar Ca^{2+} medium resulted in a nearly complete activation (12, 51). Intermediate $^{45}\text{Ca}^{2+}$ efflux rates were observed when the mammalian channels were activated at nanomolar Ca^{2+} by 10 mM caffeine or 5 mM ATP or by 10^{-5} M Ca^{2+} alone. In contrast, neither the addition of 5 mM adenine nucleotide and 10 mM caffeine nor an increase in free Ca^{2+} from 10^{-8} to 10^{-5} M in the absence or presence of 5 mM AMP-PCP was effective in giving more than slight activation of the lobster channel in vesicle or planar lipid bilayer experiments. Another significant difference was that Ca^{2+} -activated $^{45}\text{Ca}^{2+}$ efflux from lobster SR vesicles could not be readily inhibited by millimolar Mg^{2+} and micromolar ruthenium red concentrations. One property shared by lobster (Table I) and mammalian SR channels (6) was that Ca^{2+} -activated $^{45}\text{Ca}^{2+}$ efflux was inhibited by the local anesthetic tetracaine at a concentration of 1 mM.

The apparent lack of a high affinity Ca^{2+} -activating channel site raised the possibility that during membrane isolation the Ca^{2+} sensitivity of activation of the lobster channel was altered because of the loss or binding of another endogenous effector molecule. We tested the effects of compounds which are known or suspected to modulate mammalian ryanodine-sensitive Ca^{2+} release channel activity, such as Mg^{2+} , ATP, and calmodulin (6, 7) or inositol 1,4,5-trisphosphate (52). The presence of an endogenous inhibitor molecule in lobster muscle was tested by incubating rabbit skeletal SR vesicles with lobster skeletal muscle cytosol fractions.³ However, none of these interventions significantly affected the $^{45}\text{Ca}^{2+}$ efflux behavior of lobster or rabbit SR vesicles, respectively.

The apparent lack of ATP and Mg^{2+} modulatory as well as high affinity Ca^{2+} activating sites raises the question of the mechanism of SR Ca^{2+} release channel regulation in lobster muscle. During membrane depolarization of mammalian cardiac muscle, a dihydropyridine-sensitive surface membrane Ca^{2+} channel permits the entrance of external Ca^{2+} ions which then serve as a trigger for intracellular Ca^{2+} release by activating a high conductance SR Ca^{2+} release channel (17, 18). Although studies with isolated cardiac SR vesicles have shown that in the absence of ATP and Mg^{2+} the release of Ca^{2+} ions from the SR can be stimulated by micromolar concentrations of Ca^{2+} (Fig. 8), millimolar concentrations of Ca^{2+} were required for optimal activation when SR Ca^{2+} release was studied under more physiological conditions, *i.e.* in the presence of millimolar concentrations of Mg^{2+} and adenine nucleotide (12). The results of the present study suggest that the lobster Ca^{2+} release channel requires millimolar Ca^{2+} for activation in the absence or presence of Mg^{2+} and ATP. Attainment of such high concentrations in the regions of the feet during excitation-contraction coupling is likely in crustacean muscle because of the high extracellular Ca^{2+} concentration (16 mM, Ref. 43) and the large influx of Ca^{2+} known to occur (21, 22, 43).

In crustacean muscle, the role of the SR in regulating muscle contraction has been challenged (19, 21, 22, but see also Refs. 23–25). Bezanilla *et al.* (21) have suggested that during membrane depolarization of barnacle muscle few, if any, Ca^{2+} ions are released from intracellular Ca^{2+} stores. Rather, a primary function of the sarcoplasmic reticulum,

² N. R. Kramarcy and R. Sealock, unpublished studies.

³ J.-H. Seok and G. Meissner, unpublished studies.

together with a surface membrane Na⁺-Ca²⁺ exchanger, would be to facilitate muscle relaxation by sequestering the Ca²⁺ ions that enter the cell during membrane depolarization. The sequestered Ca²⁺ were thought to be then unloaded by a mechanism which was not further specified. ⁴⁵Ca²⁺ efflux and single channel data of this study do not support the model proposed by Bezanilla *et al.* (21). We observed a decreased, rather than increased, Ca²⁺ permeability at Ca²⁺ concentrations resembling those in relaxed muscle.

In conclusion, the studies reported here suggest that the RyR-Ca²⁺ release channel complex of lobster exhibits a less complex pattern of regulation by Ca²⁺ and other effector molecules than the mammalian skeletal and cardiac muscle channel proteins. Further comparison of the structure and function of the crustacean and mammalian channel complexes should help to define better the process of excitation-contraction coupling in muscle, and specifically the mechanism of Ca²⁺ release from sarcoplasmic reticulum.

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