Antibodies as probes for ligand gating of single sarcoplasmic reticulum Ca^{2+} -release channels

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A large (565 kDa) junctional sarcoplasmic reticulum (SR) protein, the ryanodine receptor (RyR), may play both a structural and a functional role in the mechanism of skeletal muscle excitation–contraction coupling. Recently, the primary amino acid sequence of the RyR has been elucidated. In this paper, we introduce an immunological approach to examine the functional (electrophysiological) properties of the RyR when it is incorporated into planar lipid bilayers. The effects of two polyclonal antibodies against the SR junctional face membrane (JFM) and the RyR (anti-JFM and anti-RyR) were tested on the single-channel gating properties of the RyR SR Ca^{2+} -release channel. Junctional SR vesicles were fused into planar lipid bilayers in solutions containing caesium salts. Solutions were designed to minimize the background conductances of the SR K^+ and Cl^- channels. Three actions of the anti-JFM antibody were distinguished on the basis of single-channel gating and conductance. The anti-RyR antibody had a single action, a simultaneous decrease in single-channel open probability (P_o) and conductance. Both antibodies appear to alter single-channel gating by disrupting the Ca^{2+} -activation mechanism of the channel. Anti-RyR-antibody-induced gating abnormalities were reversed by ATP, although the ATP-re-activated channels had altered gating kinetics. Two antigenic regions, recognizing the anti-RyR antibody, in the C-terminal end of the RyR primary amino acid sequence contain or are closely associated with putative ligand (Ca^{2+} and ATP)-binding sites identified previously. Our results demonstrate (1) that the antibodies induced abnormal gating (decreased open probability and stabilization of subconducting states) of SR release channels, and (2) that abnormal gating is not associated with physical obstruction or alteration of the conduction pathway. Thus antibodies directed at specific regions of the RyR (e.g. putative ligand-binding sites) can be used as effective probes with which to study the structural and functional properties of the SR Ca^{2+} -release channel gating at the single-channel level.

INTRODUCTION

In skeletal muscle, depolarization of transverse (T)-tubular membrane triggers the sarcoplasmic reticulum (SR), an intracellular organelle, to release Ca^{2+} into the myoplasm, resulting in contraction. The mechanism which links T-tubular depolarization and SR Ca^{2+} release is termed excitation–contraction coupling (Caputo, 1983). The SR consists of two morphologically distinct regions, junctional and longitudinal SR. The junctional SR is closely associated with the T-tubular membrane, forming structures called triads. In the triad junction, the SR and T-tubular membranes are separated by a 10 nm gap which is periodically spanned by tetrameric structures called feet (Ferguson *et al.*, 1984). The principal component of the feet is the 565 kDa ryanodine receptor (RyR) protein (Campbell *et al.*, 1987; Inui *et al.*, 1987; Lai *et al.*, 1987; Takeshima *et al.*, 1989). Aside from its structural significance, the RyR may have an important functional role in the excitation–contraction coupling mechanism. It has been putatively identified as the SR Ca^{2+} -release channel (Lai *et al.*, 1988; Hymel *et al.*, 1988; Smith *et al.*, 1988).

Specific antibodies have been used to explore several functional and structural properties of ionic channels (Meiri *et al.*, 1986; Malouf *et al.*, 1987; Morton *et al.*, 1988; Fitzpatrick *et al.*, 1988). A recent study has used two polyclonal antibodies to block the Ca^{2+} -activated Ca^{2+} -release mechanism in skeletal muscle SR (Zorzato *et al.*, 1989). In the present experiments, we probe

molecular aspects of the SR Ca^{2+} -release mechanism in skeletal muscle by applying these same antibodies to single SR Ca^{2+} -release channels in planar lipid bilayers.

Single SR Ca^{2+} -release channels were reconstituted and monitored in a caesium methanesulphonate gradient. Use of caesium as current carrier provided a one-step reconstitution method which significantly increases experimental productivity. Antibodies against the junctional face membrane (JFM) proteins and the RyR (anti-JFM and anti-RyR) were applied to reconstituted channels. Both antibodies decreased single-channel open probability (P_o) and conductance when applied to the myoplasmic side of the channel. The abnormal channel gating properties induced by the anti-RyR antibody were reversed by ATP. Our results indicate that bound antibody does not obstruct or alter the conduction pathway. Thus antibodies with defined sites of action (e.g. ligand-binding sites outside the pore) can be used to study the structural and functional properties of the SR Ca^{2+} -release channel at the molecular level.

METHODS

SR membrane and antibody preparation

SR of predominantly fast-twitch rabbit skeletal muscle was isolated and fractionated into longitudinal and junctional SR by the method of Saito *et al.* (1984). SR fractionation was carried out in the presence of 100 μM -phenylmethanesulphonyl fluoride.

Abbreviations used: T-tubule, transverse tubule; SR, sarcoplasmic reticulum; RyR, ryanodine receptor; JFM, junctional face membrane; P_o , open probability; PBS, 0.1 M-sodium phosphate (pH 7.2)/0.15 M-NaCl.

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Calsequestrin-containing junctional SR was isolated by either Triton X-100 or octaethylene glycol mono-n-dodecyl ether ($C_{12}E_8$) treatment of the terminal cisternae fraction as previously described (Costello *et al.*, 1986). Calsequestrin was extracted from junctional SR membranes by incubation in 10 mM-Tris/2 mM-EDTA, pH 8.0 (Duggan & Martonosi, 1970). All fractions were resuspended in 0.3 M-sucrose/5 mM-imidazole, pH 7.4 (buffer A) and stored at -70°C until used. The protein concentration was determined according to Lowry *et al.* (1951), using BSA as standard.

Anti-JFM antibodies were raised in a hen, by weekly injections into breast muscle of approx. 300 μg of calsequestrin-free junctional face SR membrane in buffer A diluted (1:1) with 1 ml of complete Freund's adjuvant. The animal was bled 35 days after beginning the immunization. The immunoglobulins were extracted from preimmune and immune sera by identical procedures. The extraction procedure was as previously described by Orlans *et al.* (1961). The anti-RYR antibodies were raised in guinea pig as described by Zorzato *et al.* (1989) [Zorzato *et al.* (1989) referred to the anti-RYR antibody as the anti-(350 kDa protein) antibody]. The anti-RYR antibodies were affinity purified according to Bisson & Schiavo (1986). Immunoglobulins were purified from guinea pig serum by Sepharose-Protein A chromatography (Goudswaard *et al.*, 1978). Sepharose-Protein A was from Pharmacia. All other chemicals were reagent grade.

Immunoblotting procedure

The junction SR proteins were electrophoretically resolved by the method of Laemmli (1970) in SDS/polyacrylamide linear gradient (5–15%) gels, and then transferred to nitrocellulose sheets (blots) by the method of Gershoni *et al.* (1985). The transfer was carried out at constant current (50 V) for 16–18 h in a buffer containing 25 mM-Tris/192 mM-glycine, pH 8.3. The blot was preincubated for 60 min at room temperature in 0.1 M-sodium phosphate (pH 7.2)/0.15 M-NaCl (PBS) plus 10% low-fat milk, and then incubated for 150 min with either anti-JFM antibodies or anti-RYR affinity purified antibodies diluted with PBS (5 $\mu\text{g}/\text{ml}$). After a 30 min wash in PBS/10% low-fat milk, the blot was incubated with either anti-(chicken immunoglobulin) or anti-(guinea pig immunoglobulin) antibodies conjugated with alkaline phosphatase. Other steps were carried out as described by Damiani *et al.* (1986).

The specificity of the anti-JFM and anti-RYR antibodies was indicated by immunoblotting. Fig. 1 shows Western blots immunochemically stained by the anti-JFM and anti-RYR antibodies. The anti-JFM antibodies stained many proteins of the junctional SR (Fig. 1, lane A), whereas the anti-RYR affinity purified antibodies recognized only a high-molecular-mass polypeptide, identified as the RYR protein (Fig. 1, lane B). Longitudinal SR membranes had minimal reactivity with anti-JFM antibodies and none with anti-RYR antibodies (results not shown; see Zorzato *et al.*, 1989). Preimmune immunoglobulins from hen or guinea pig did not stain any SR proteins (results not shown). Thus the anti-JFM antibodies bind to many junctional SR proteins, whereas the anti-RYR antibodies bind only to the RYR.

Channel reconstitution in planar bilayers

Planar lipid bilayers were formed by the Mueller & Rudin (1968) technique across a 100 μm diameter aperture in the wall (thickness 50 μm) of a Delrin cup. The bilayer-forming solution contained a 5:3:2 (by vol.) mixture (50 mg/ml in decane) of palmitoyl oleoyl phosphatidylethanolamine, palmitoyl oleoyl phosphatidylserine and palmitoyl oleoyl phosphatidylcholine. Lipids were obtained in chloroform from Avanti Polar Lipids

Fig. 1. Immunoblots illustrating the specificity of anti-JFM and anti-RYR antibodies

Junctional SR membranes (20 μg) were electrophoresed and transferred to nitrocellulose blots as described in the Methods section. Blots were indirectly immunochemically stained with anti-JFM (lane A) or anti-RYR (lane B) antibodies. Arrowheads mark the RYR (565 kDa) and calsequestrin (CS, 63 kDa).

(Pelham, AL, U.S.A.). Salt-agar bridges equilibrated in the solutions present in each chamber were used to connect the chambers to Ag/AgCl electrodes immersed in 2 M-CsCl. The Ag/AgCl electrodes were connected to a voltage/current conversion amplifier.

Junctional SR was added to one side (defined as *cis*) of the bilayer to a final concentration ranging from 5 to 20 $\mu\text{g}/\text{ml}$. The other side of the bilayer was defined as *trans*. Ionic gradients were established across both the SR membrane and the planar bilayer. Fusion and recording were done (unless specified) in a standard solution containing 250 mM- CsCH_3SO_3 on the *cis* side (50 mM on the *trans* side), and 10 mM- CsHepes (pH 7.4), $\text{pCa} = 5$.

Caesium salts were used to isolate the conductance of the SR Ca^{2+} -release channel from that of other ionic channels present in the fused SR membranes (Fill *et al.*, 1990). In some experiments, once single-channel activity was observed, bilayer viability was increased by equilibrating the ionic gradient between the *cis* and *trans* chambers.

Single-channel recording and analysis

A voltage/current conversion amplifier was specifically designed to optimize single-channel recording from lipid bilayers (Hamilton *et al.*, 1989). Acquisition software (pClamp; Axon Instruments, Burlingame, CA, U.S.A.), an IBM AT-compatible 386 computer and a 12 bit A/D-D/A converter were used (Axon Instruments). Single-channel analysis was performed with commercially available (pClamp and Iproc, from Axon Instruments) software. Additional software was provided by Dr. O. Alvarez and Dr. R. Latorre (Centro de Estudios Científicos de Santiago, Santiago, Chile) and from Dr. T. VanDongen and Dr. A. Brown (Baylor College of Medicine, Houston, TX, U.S.A.). Data were filtered at 1–2 kHz by an 8-pole Bessel filter, digitized at 5–10 kHz, and collected on-line for future analysis. After SR membrane fusion, bilayers remained stable at 0 mV for up to 1.5 h. Membrane potentials are referenced to the *trans* potential. Application of a 50 mV holding potential significantly decreased bilayer lifetime (to less than 20 min). To increase bilayer viability, single-channel data were typically recorded during short (100–500 ms) pulses from 0 mV at regular intervals (0.5–0.05 Hz). Automated detection programs for single-channel events were used when possible to avoid bias due to manually set detection thresholds. When necessary, the detection threshold was set at 50% of the average channel amplitude obtained from the total

amplitude histogram (Colquhoun & Sigworth, 1983). Open-time probabilities (P_o) were calculated from total amplitude histograms fitted by a sum of Gaussian distributions using the non-linear least-squares method. Idealized open and closed events were corrected for dead time due to the sampling frequency and filter characteristics (Colquhoun & Sigworth, 1983). Logarithmically binned open and closed time histograms were fitted by the maximum likelihood method to obtain point estimates of time constants from the probability density function (Colquhoun & Sigworth, 1983; Sigworth & Sine, 1987).

RESULTS

Anti-JFM antibody blockade of the SR Ca^{2+} -release channel

The goal of this study was to quantify the effects of antibodies on reconstituted SR Ca^{2+} -release channels. Several fundamental problems could arise, including: (1) random orientation of channels, (2) accessibility of antibody-binding sites on reconstituted channels, (3) non-specific actions of antibody molecules on channels, and/or (4) the need for high antibody concentrations, especially in the presence of unfused SR vesicles in the *cis* chamber. Since the anti-JFM antibody was available in greater quantities, it was used in the initial experiments to assess these possibilities.

In order to establish the sidedness of antibody action, the anti-JFM antibody was applied only to one side of the bilayer during each experiment. When added to the *cis* chamber (antibody/SR protein ratio 0.4–2.0), the antibody altered single-channel activity in 74% of the experiments ($n = 26$). The antibody/SR ratio refers to the molecular species present in solution. Since antibody was added to the *cis* chamber in the presence of SR vesicles (5–20 $\mu\text{g}/\text{ml}$), the antibody/SR ratio at the level of the bilayer was not known. At high antibody/SR ratios (1.0–2.0), antibody

effects were observed within 4 min. No antibody concentration-dependence, however, was observed after a 10 min incubation period. When the antibody was applied to the *trans* chamber (antibody/*cis* SR ratio 0.5), single-channel activity was not altered ($n = 8$). Since the *trans* side had no added vesicles, only the channels fused into the bilayer would be accessible to the antibody. Therefore the effective (*trans*) antibody concentration per exposed channel in these experiments was very high. The sidedness of our antibody data indicates that reconstituted channels are primarily oriented in one direction. Further, these results may also indicate that there are no anti-JFM antibody epitopes on the *trans* (intraluminal) side of channels.

The actions of the anti-JFM antibodies applied to the *cis* chamber are shown in Fig. 2. Figs. 2(a)–2(c) show control SR channel activity with corresponding total amplitude histograms. Figs 2(d)–2(f) show the effects of the anti-JFM antibody. The antibodies had three different effects on single-channel activity. These effects were: (1) complete blockade (Fig. 2a), (2) decreased P_o with decreased caesium conductance (Fig. 2b), and (3) decreased P_o with no effect on Cs^+ conductance (Fig. 2c). The second effect, decreased P_o accompanied by decreased Cs^+ conductance, was most frequently observed (9 of 19 experiments). The different actions of the anti-JFM antibodies may be related to the heterogeneous nature of the antibodies. The anti-JFM antibodies are a mixture of antibodies to several junctional SR proteins (see immunoblot, Fig. 1a), and possibly to several epitopes on individual proteins (see the Discussion section).

Preimmune immunoglobulins, prepared in identical fashion (see the Methods section), were applied to the channel (Fig. 3). Control channel activity is illustrated in Fig. 3(a). Preimmune immunoglobulins (antibody/SR ratio of 1.0) did not appear to alter channel activity when applied to the *cis* side of the bilayer (Fig. 3b). Several preimmune experiments are summarized in Fig. 3(c). The anti-JFM preimmune antibodies did not sig-

Fig. 2. Action of anti-JFM antibodies on single SR Ca^{2+} -release channels

Three antibody effects were distinguishable: (a) complete block ($n = 5$), (b) decrease in P_o and in channel amplitude ($n = 9$), and (c) decrease in P_o without affecting amplitude ($n = 5$). Single-channel data (opening upward, baseline marked) were recorded during 30 mV pulses from holding potential of 0 mV. Calibration bars are 15 pA and 50 ms. Solutions contained 250 (*cis*)/250(*trans*) mM- CsCH_3SO_3 and 10 mM- CsHepes (pH 7.4), pCa 5. Antibody was added to the *cis* chamber at antibody/SR protein ratios ranging from 0.4 to 2.0. The histograms of control preparations (top) and of those containing antibody (bottom) have identical total areas. The P_o values, calculated from the sum of Gaussian distributions, were as follows: (a) 0.19 (top), 0.00 (bottom); (b) 0.11 (top), 0.02 (bottom); (c) 0.10 (top), 0.02 (bottom).

Fig. 3. Effects of preimmune immunoglobulins on the SR Ca^{2+} -release channel

Solutions contained 250/250 mM- CsCH_3SO_3 and 10 mM- CsHepes (pH 7.4), pCa 5. (a) Control single-channel activity (holding potential = 50 mV, baseline marked) and corresponding total amplitude histogram. (b) Single-channel activity after the addition of anti-JFM preimmune immunoglobulins (antibody/SR ratio 1) to the *cis* chamber. (c) Summary of several preimmune experiments. Results are means $\pm$ S.E.M. Paired bars represent the action of postimmune antibodies in the presence of preimmune immunoglobulins ($n = 1$ in each case).

nificantly alter control single-channel P_0 . Postimmune immunoglobulins decreased P_0 even in the presence of the preimmune antibodies. Thus the antibody effects we observe do not depend on factors present in the preimmune immunoglobulins.

Since the anti-JFM antibodies could have epitopes on other ionic channels in the junctional SR membrane, the antibodies were applied symmetrically (both sides) to the SR K^+ channel (Figs. 4a and 4b) and the SR Cl^- channel (Figs. 4c and 4d). Neither of these other channels was affected by the antibody. The lack of effect does not rule out the possibility that the anti-JFM antibodies recognize epitopes on these channel proteins. The negative effect, however, does demonstrate that the antibodies do not alter SR release-channel activity by destabilizing the bilayer or altering surface charge.

In summary, the data obtained with the anti-JFM antibody preparation indicate that antibodies can reach and alter the gating of reconstituted single SR release channels. The sidedness of antibody action reveals that reconstituted channels are primarily oriented in one direction. The anti-JFM antibodies had three actions on single-channel activity. Multiple antibody actions may reflect the heterogeneous nature of the anti-JFM antibodies. Since the anti-JFM antibody preparation recognizes several junctional SR proteins, our results do not rule out the possibility that antibodies bind to other proteins (not the RYR) which may regulate the SR Ca^{2+} -release channel.

Anti-RYR antibodies stabilize subconducting states of the SR Ca^{2+} -release channel

The anti-JFM antibody experiments established the feasibility of exploring the action of antibodies on the SR Ca^{2+} -release channel. Those experiments also demonstrated that antibody action could be observed at relatively low antibody concentrations in the *cis* chamber (antibody/SR protein ratio of 0.4).

The action of the anti-RYR antibodies (antibody/SR protein ratio of 0.4) on single-channel activity is shown in Fig. 5. Control single-channel records (Fig. 5a) were selected to illustrate the

Fig. 4. Effect of anti-JFM antibody on the SR K^+ and Cl^- channels

(a) Control SR K^+ -channel activity (opening upward, baseline marked) in 250 (*cis*)/50 (*trans*) mM-KCl at a holding potential of 10 mV. Calibration bars are 7.5 pA and 3200 ms. (b) Symmetrical addition of antibody (antibody/SR protein ratio 1) had no effect on SR K^+ channel activity. (c) Control SR Cl^- -channel activity (opening downward, baseline marked) in 250/50 mM-KCl at a holding potential of -25 mV. Calibration bars are 12.5 pA and 750 ms. (d) Symmetrical addition of antibody (antibody/SR ratio 1.4) has no effect on SR Cl^- -channel activity.

Fig. 5. Action of anti-RYR antibodies on single SR Ca²⁺-release-channel gating

Channel activity (opening upward) was recorded during 320 ms pulses of 50 mV (holding potential 0 mV). The beginning and end of pulses are marked by arrowheads. (a) Control channel activity (pCa 5). Records were selected to illustrate the sub-states of the SR release channel. Calibration bars are 20 pS and 100 ms. The closed state and four open states are marked on the left. (b) Addition of antibody (*cis*, antibody/SR protein ratio 0.4) decreased P_0 and stabilized subconducting states of the channel. (c) Addition of ryanodine (*cis*, 10 μ M) increased P_0 and stabilized one subconducting state of the channel ($n = 12$). Ryanodine experiments were in the absence of antibody. (d) Current/voltage plots under all conditions. Measured open states under each condition are shown; ○, control channels; ●, plus antibody; △, plus ryanodine. The mean open time, mean amplitude and probability of each conductance level in presence and absence of antibody are summarized in Table 1.

subconducting states (opening upward, each level marked) of the SR Ca²⁺-release channel. Substates were observed in approx. 4 % of single-channel traces. Addition of antibody (Fig. 5b) dramatically decreased P_0 (0.21 to 0.05) and stabilized subconducting states of the channel. In the presence of antibody, channels opened to the middle subconducting level most frequently. Although open events were observed to all levels, open events to the highest subconducting level were rare and very fast. For comparison, the action of ryanodine on control channel activity is illustrated (Fig. 5c). Application of ryanodine increased P_0 .

Table 1. Effect of anti-RYR antibodies on single SR Ca²⁺-release-channel gating

For details, see the legend to Fig. 5. The mean open time, mean amplitude and P_0 of each conductance level are shown in the absence and presence of antibody. Values are the means from three experiments.

State	Mean dwell time (ms)		Mean amplitude (pA)		P_0	
	Control	Antibody	Control	Antibody	Control	Antibody
Open-4	3.04	0.81	42.13	41.65	0.131	0.004
Open-3	2.30	1.08	30.95	31.22	0.041	0.008
Open-2	1.30	3.79	20.64	20.43	0.022	0.031
Open-1	2.61	3.19	8.97	9.18	0.012	0.009
Closed	39.31	134.7	0.00	0.00	0.794	0.948

Fig. 6. Anti-RYR antibody blockade and ATP blockade reversal

Channel activity (opening upward, baseline marked) recorded during 320 ms/50 mV pulses from a holding potential of 0 mV. Calibration bars are 25 pA and 70 ms. Solutions contained 250 (*cis*)/50 (*trans*) mM-CsCH₃SO₃ and 10 mM-CsHepes (pH 7.4), at pCa 5. Antibody was added at an antibody/SR protein ratio of 0.4 (*cis*). (a) Control channel activity (pCa 5); (b) channel activity 5 min after antibody addition; (c) addition of Ca²⁺ (pCa 4, *cis*) to antibody-inhibited channel; (d) addition of Na₂ATP (*cis*, 2 mM, pCa 5) to antibody-inhibited channel; (e) effect of antibody and ATP on P_0 . Bars show antibody-free (□) and antibody-bound (■) conditions. The Ca²⁺-dependence of channel activation is indicated (maximal activation at approx. pCa 5). In separate experiments ($n = 5$), antibody dramatically decreased the P_0 at a pCa of 5. Addition of ATP increased the P_0 of antibody-bound channels to control levels.

(0.19 to 0.81) and stabilized the middle subconducting level. Data at each subconducting state are summarized in the current/voltage plot (Fig. 5d) and in Table 1. Since all conduction levels are present in antibody-bound channels, it appears that the conduction pathway is unaltered and, furthermore, that the action of antibody was to decrease P_0 by modifying the gating mechanism of the channel.

Action of anti-(RYR) antibody on channel gating is reversed by ATP

Under control conditions, spontaneous control channel activity is dependent on the myoplasmic (*cis*) free Ca²⁺ concentration (typically, pCa = 5). Ca²⁺-activated single-channel activity is shown in Fig. 6(a). These records (opening upward, baseline marked) were collected during 320 ms pulses to 50 mV from the holding potential (0 mV). Application of anti-RYR

Fig. 7. Effect of anti-RYR antibody on channel kinetics

Representative channel records (opening upward, baseline marked) are shown. (a) Control activity; (b) ATP-activated control channel activity; (c) ATP-activated antibody-inhibited channel activity. Calibration bars are 40 pA and 50 ms. Solutions contained 250 (*cis*)/50 (*trans*) mM- CsCH_3SO_3 and 10 mM- CsHepes (pH 7.4), at pCa 5; the holding potential is 50 mV. Antibody was added at an antibody/SR protein ratio of 0.4 (*cis*). ATP (2 mM, final pCa = 5) was added to the *cis* side of the bilayer. Open (top) and closed (bottom) time histograms were logarithmically binned (Sigworth & Sine, 1987) and fitted using the maximum likelihood algorithm. Each histogram was constructed from 1000 events.

antibody (Fig. 6b) decreased P_o (0.11 ± 0.04 to 0.014 ± 0.009 , $n = 5$) and stabilized the subconducting states of the channel. No effect of the anti-RYR antibody was observed on the SR Cl^- and K^+ channels (results not shown). Application of anti-RYR preimmune immunoglobulins (antibody/SR ratio 1; $n = 5$) to the *cis* chamber did not alter channel activity (see Fig. 3). In contrast with the multiple actions of the anti-JFM antibody, only one action of the anti-RYR was observed. The single action of the anti-RYR antibody is identical to the most frequent effect of the anti-JFM antibody shown in Fig. 2(b). This similarity may indicate that both the anti-JFM and anti-RYR antisera contain antibodies which recognize the same antigenic regions of the RYR.

Myoplasmic Ca^{2+} and ATP are potent endogenous activators of the SR Ca^{2+} -release channel *in vitro* (Martonosi, 1984). To further explore the action of the anti-RYR antibody, Ca^{2+} and ATP were applied to antibody-inhibited channels. Addition of Ca^{2+} (Fig. 6c) did not significantly modify P_o or Cs^+ conductance. In contrast, addition of 2 mM-ATP (final pCa 5; Fig. 6d) reversed the antibody blockade: it increased single-channel P_o and returned Cs^+ conductance to the control level. These data are summarized and compared with control experiments in Fig. 6(e). Bars represent antibody-free ($\square$) and antibody-bound ($\blacksquare$) conditions. The Ca^{2+} -dependence of channel activation is shown on the left. All antibody experiments were performed at a pCa level of 5 (near-maximal Ca^{2+} activation). Addition of antibody ($n = 5$) dramatically decreased single-channel P_o . Application of 2 mM-ATP increased the P_o of antibody-bound channels to approximately control levels. In other experiments on anti-RYR

antibody-inhibited channels, addition of 1 mM- Mg^{2+} (to the *cis* side) further blocked channel activity (results not shown). Since ATP and Mg^{2+} can still modulate anti-RYR antibody-bound channels, these data suggest that the SR Ca^{2+} -release channel has separate binding sites for Ca^{2+} , Mg^{2+} and ATP (see the Discussion section).

Similar experiments were performed on channels inhibited by the anti-JFM antibodies. The following results were obtained; (1) Ca^{2+} addition had no effect, (2) ATP re-activated approx. 50% of the anti-JFM antibody-bound channels, and (3) Mg^{2+} further blocked the anti-JFM antibody-bound channels.

Kinetic analysis of ATP-re-activated, anti-RYR antibody-bound, SR Ca^{2+} -release channels

The degree of channel activation is dependent on the *cis* (myoplasmic) free Ca^{2+} concentration. Thus, in the absence of other channel-activating ligands, antibody inhibition of channel activity (Figs. 5b and 6b) indicates that the anti-RYR antibody interferes with the Ca^{2+} -activation mechanism. Since anti-RYR antibody-bound channels remain sensitive to Mg^{2+} and ATP, it is possible that the anti-RYR antibodies interfere with the Ca^{2+} -activation mechanism independent of Mg^{2+} and/or ATP. To explore this possibility, we further examined the single-channel gating properties of ATP-activated channels in the antibody-free and antibody-bound conditions. This analysis reveals that bound anti-RYR antibody alters the gating kinetics of ATP-re-activated channels.

Kinetic analysis was performed on channels under three experimental conditions: (1) control (Fig. 7a), (2) antibody-free

plus ATP (Fig. 7b), and (3) anti-RYR-inhibited plus ATP (Fig. 7c). Single-channel records were selected to demonstrate the observed kinetic changes. Each histogram was constructed from 1000 events and represents several experiments (three antibody-free, four antibody-bound). The open (top) and closed (bottom) time histograms are plotted on a log-log scale (Sigworth & Sine, 1987), with peaks representing the mean times of each distribution. The histograms, except that of the control closed time (bottom, Fig. 7a), were best fitted by the sum of two exponentials. The control closed time histogram was best fitted by the sum of three exponentials. Comparison of control (Fig. 7a) and antibody-free/ATP (Fig. 7b) open time histograms reveals that the action of ATP is to increase the frequency (46 to 68% of openings) and mean duration (1.9 to 5.6 ms) of long openings. Comparison of control (Fig. 7a) and antibody-bound/ATP (Fig. 7c) open time histograms reveals that in the presence of antibody the action of ATP is altered. In the antibody-bound condition, ATP does increase P_o (see Fig. 6d) but by an abnormal alteration of the open or closed time kinetics (Fig. 7c). These results indicate that ATP can bind in the presence of the anti-RYR antibodies and increase channel activity, but that the ATP activation mechanism is modified by the anti-RYR antibodies. Thus the Ca^{2+} and ATP activation mechanisms are not independent and may interact with each other.

DISCUSSION

Sidedness of reconstituted SR Ca^{2+} -release channels

Determination of the sidedness of ion channels has always been a fundamental problem in channel reconstitution studies. Typically, specific membrane-impermeable ligands are used to test channel sidedness (e.g. Na^+ channel, tetrodotoxin; Ca^{2+} -activated K^+ channel, charibdotoxin; dihydropyridine-sensitive Ca^{2+} channel; D890, a membrane-impermeable derivative of verapamil). The SR Ca^{2+} -release channel, however, poses a significant problem in this respect. The channel is not very ion-selective ($\text{PCa}^{2+}/\text{PK}^+ = 5.8$, $\text{PK}^+/\text{PNa}^+ = 1$, $\text{PCa}^{2+}/\text{PMg}^{2+} = 2.3$, where P is permeability) and shows no marked rectification (Smith *et al.*, 1988). The only possible ligand with which to test SR release-channel sidedness is ATP. However, since molecules as large as glucose pass through the channel (Meissner, 1986) and the magnitude of the ATP flux (if any) through the channel is unknown, ATP sidedness may not be a reliable method. Large membrane-impermeable antibodies provide an opportunity to determine the sidedness of the SR release channel when SR vesicles are incorporated into planar bilayers. In our study, the antibodies had an effect in 74% of the experiments ($n = 26$) when applied to the *cis* chamber. If applied to the *trans* chamber, no effect was observed in any of the experiments ($n = 8$). Thus the majority of reconstituted SR release channels are oriented in one direction. According to the interpretations of Takeshima *et al.* (1989) and Zorzato *et al.* (1990), 95% of the RYR protein and all putative ligand-binding sites are myoplasmic. If this interpretation of the RYR primary structure is correct, our data indicate that reconstituted SR release channels are oriented such that the *cis* chamber corresponds to the myoplasmic space.

Specificity of antibody action

In this study we have demonstrated that two polyclonal antibodies have actions on the gating of the SR Ca^{2+} -release channel. Several factors, however, could non-specifically alter channel gating. Some conceivable examples are: (1) the immunoglobulin fraction may contain endogenous antibodies or contaminants which affect channel function; (2) binding of large immunoglobulins could sterically alter channel structure; (3) bound antibodies could physically block the conductive pathway;

and (4) immunoglobulins or contaminants could alter the physical properties of the lipid bilayer.

Premature immunoglobulins were prepared using the same procedure used to extract the anti-JFM and anti-RYR antibodies. Application of premature IgG to the myoplasmic (*cis*) side of the SR release channel had no effect on channel activity. Therefore it could be concluded that no endogenous antibodies or contaminants had an effect on channel activity. Since the anti-JFM and anti-RYR antibodies are polyclonal, it is possible that binding of several large immunoglobulins at several locations could cause gross deformations in channel structure. However, since antibody-bound channels remain functional and are modulated by ATP and Mg^{2+} , it is not likely that gross structural deformations are present. The antibodies appear to stabilize subconducting states of the channel (Fig. 5). Thus re-activation of antibody-bound channels by ATP (Fig. 6) to the highest conductance level confirms that the conductive pathway of antibody-bound channels is unaltered and is not physically obstructed by the bound antibody. Since the single-channel properties of the SR K^+ and Cl^- channels were not altered by the antibodies, it is unlikely that the antibodies alter the physical properties of the lipid bilayer. These observations suggest that the anti-JFM and anti-RYR antibodies specifically modify SR release-channel gating.

Actions of the anti-JFM and anti-RYR antibodies

It is interesting that the anti-JFM antibody preparation, containing antibodies recognizing several junctional SR proteins, produced a variety of effects, whereas the anti-RYR antibodies had only one. The variety of anti-JFM antibody effects may reflect (1) a combination of effects due to antibodies specific for different regions of the channel, (2) effects due to antibodies bound to junctional SR proteins which may modulate or be part of the channel protein complex, or (3) a combination of these actions.

The SR Ca^{2+} -release channel, under our control conditions, is activated by micromolar myoplasmic (*cis*) Ca^{2+} . Therefore anti-RYR antibody modification of channel activity in control solutions (Figs. 5 and 6) suggests that this antibody interferes with the Ca^{2+} -activation mechanism. Since antibody-bound channels remain sensitive to Mg^{2+} and ATP, the primary action of the antibody is to disrupt the Ca^{2+} activation mechanism. This observation supports the conclusion of Zorzato *et al.* (1989) that the antibodies interrupt the Ca^{2+} -activated Ca^{2+} -release mechanism. Our data demonstrate that modified single-channel gating properties, decreased P_o and stabilized subconducting states underlie the action of the antibody. Anti-RYR antibody data (Figs. 5, 6 and 7) demonstrated that the antibodies act without obstructing or altering the conduction pathway. If the antibody does disrupt a ligand-binding site (Ca^{2+} activation site), our data suggest that the site is not in the conductive pathway. These data are not consistent with the results of Ma *et al.* (1988), which suggested the Ca^{2+} -activation site is actually in the aqueous pore.

Kinetic analysis provided an insight into the action of the anti-RYR antibody and the interaction between the Ca^{2+} and ATP activation mechanisms. Although ATP can still bind and modulate channel activity, ATP-re-activated antibody-bound channels have altered open time kinetics. Since the primary action of the anti-RYR antibody is thought to be the disruption of the Ca^{2+} activation mechanism, these results support the hypothesis that the Ca^{2+} and ATP activation mechanisms may interact.

These results demonstrate that antibodies can be used to gain important information into the gating mechanism of the SR Ca^{2+} -release channel. This type of information is central to developing a reliable working model of this Ca^{2+} -release channel. Additional studies using antibodies directed specifically towards

each of the putative binding sites of the Ca^{2+} -release channel will provide invaluable information concerning the ligand-gating mechanisms which regulate this channel *in vivo*.

Tentative localization of the Ca^{2+} activation site in the RYR primary sequence

Recently, workers in two laboratories have elucidated the primary amino acid sequence of the RYR (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990). Although the actual sequences are virtually identical, the two groups have interpreted the sequence differently. Takeshima *et al.* (1989) located three putative Ca^{2+} -binding sites and one putative ATP-binding site. Zorzato *et al.* (1990) argued that no clear consensus sequences for high-affinity Ca^{2+} -binding sites are evident. Zorzato *et al.* (1990) do, however, identify a putative ATP-binding site. Perhaps the most striking difference in the interpretations of these two groups is in the number of transmembrane segments; Takeshima *et al.* (1989) specify four, whereas Zorzato *et al.* (1990) propose up to twelve. Since arguments concerning the interpretation of the sequence data are outside the scope of this paper, we present simple representations of the interpretations of both Takeshima *et al.* (1989) (Fig. 8a) and Zorzato *et al.* (1990) (Fig. 8b).

Two antigenic regions, recognized by the anti-RYR antibodies, were located, and are depicted by thick lines in each representation. Putative Ca^{2+} (●) or ATP (■) sites are also shown. To identify the antigenic regions, anti-RYR antibodies were used to isolate partial cDNA clones from a cDNA expression library in λ gt11. Analysis of the DNA sequence of the immunoprotein clones show that some epitopes recognized by anti-RYR anti-

bodies lie between residues 4760–4877 and 4445–4586 in the primary structure of the RYR (Zorzato *et al.*, 1990). If these regions are recognized by the population of antibodies which affects the gating mechanism of the Ca^{2+} -channel, it is suggested that the Ca^{2+} -activating domain is localized near the transmembrane spanning segments (M1, M2, M3, Fig. 8a; M5, M7, M8, M9, Fig. 8b). Such a Ca^{2+} -binding site might be formed by residues 4489–4499, which share some structural features with a high-affinity Ca^{2+} -binding site of the EF-hand type (Takeshima *et al.*, 1989). Another possibility is that antibody binding to epitopes in the defined regions (Figs. 8a and 8b) may influence Ca^{2+} -binding at a distant site. These interpretations are, at present, speculative, since the anti-RYR antibodies may interact with other regions of the RYR. It is clear that exact localization of the epitopes of the anti-RYR antibodies will be useful in understanding the structure–function relationship of the SR Ca^{2+} -release channel.

In this paper we show that the Ca^{2+} -activation site is functionally separate from the ATP-activation site; it is likely that the distance from each to the other corresponds to at least the size of an immunoglobulin. These results do not support a hypothesis that these sites are close to each other in the modulatory binding region of the protein (Takeshima *et al.*, 1989).

Conclusion

In these experiments, we have introduced an immunological approach in order to study structural and functional properties of the SR Ca^{2+} -release-channel gating at the single-channel level. Our results indicate that: (1) the majority of reconstituted SR Ca^{2+} -release channels are oriented in one direction, (2) antibodies act by stabilizing subconducting states, (3) Ca^{2+} and ATP have functionally separate binding sites, and (4) the myoplasmic Ca^{2+} activation site may be located in the putative modulatory binding region identified by Takeshima *et al.* (1989). Further, our results demonstrate that antibodies can be used to induce abnormal channel gating (decreased P_o and stabilization of subconducting states) of the SR Ca^{2+} -release channel without directly disrupting the conduction pathway. Thus antibodies with defined sites of action can be used to study the structural and functional properties of the SR Ca^{2+} -release channel gating at the single-channel level.

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Fig. 8. Locations of the antigenic regions in the primary sequence of the RYR

Diagrams are not drawn to scale. Transmembrane segments, shown as hatched rectangles, are referred to in text as M1, M2, M3, etc. (left to right). Antigenic regions are depicted as thick lines. Putative binding sites are indicated along the sequence as ● (Ca^{2+}) or ■ (nucleotide). (a) The interpretation of Takeshima *et al.* (1989) includes four transmembrane segments, three putative Ca^{2+} -binding sites, and one putative nucleotide-binding site. (b) The interpretation of Zorzato *et al.* (1990) includes ten transmembrane segments and one putative nucleotide-binding site.

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