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Purified ryanodine receptor of skeletal muscle sarcoplasmic reticulum forms Ca^{2+}-activated oligomeric Ca^{2+} channels in planar bilayers

(calcium release channel/ion channel/excitation–contraction coupling/reconstitution/feet structures)

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ABSTRACT **The ryanodine receptor of sarcoplasmic reticulum (SR) from fast-twitch skeletal muscle has been purified and found by electron microscopy to be equivalent to the feet structures that are involved *in situ* in the junctional association of transverse tubules with terminal cisternae of SR. We now find that when the purified receptor is incorporated into vesicle-derived planar bilayers, it forms Ca^{2+}-specific channels, which are dependent on submicromolar Ca^{2+} for activity. In the presence of 1 mM ATP, the channel shows essentially no activity at 10 nM Ca^{2+} but becomes highly activated at 50 nM Ca^{2+}. At suboptimal Ca^{2+} levels (100 nM), the channel is strongly activated by 1 mM ATP and can be blocked by ruthenium red, both effects being prevented by higher Ca^{2+} levels (1 μM). Mg^{2+}, added from the cis side at millimolar concentrations, blocks Ca^{2+} flux through the channel from trans to cis (equivalent to flux from luminal to myoplasmic compartment). Ryanodine stabilizes the open state of the channel and blocks the action of ruthenium red to close the channel. Thus, the purified ryanodine receptor incorporated into a bilayer has the Ca^{2+}-channel characteristics consistent with the calcium release observed in isolated terminal cisternae vesicles. Furthermore, ryanodine induced the appearance of a sublevel gating mode characterized by long open conductance states, which were integral multiples of the smallest observed conductance, 3.8 pS in 50 mM Ca^{2+}. The purified receptor consists essentially of a single-sized high molecular weight polypeptide (M_r, ≈360,000), which on reconstitution forms the square rectangles diagnostic of the feet structures. We conclude that the identity of the Ca^{2+}-release channel of SR is the foot structure, which consists of an oligomer of the high molecular weight polypeptide.**

The molecular basis of excitation–contraction coupling is one of the most important unsolved problems in muscle physiology and cell biology (1–3). Significant progress in this regard has been made in the past several years. (*i*) Junctional terminal cisternae of sarcoplasmic reticulum (SR) have been isolated from fast-twitch skeletal muscle (4). It consists of two types of membranes (3, 4), the calcium-pump membrane involved in energized Ca^{2+} uptake enabling muscle to relax, and the junctional face membrane with well-defined feet structures, which are involved *in situ* in junctional association with transverse tubules (5). It is by way of this triad junction that the electrical signal from transverse tubule triggers Ca^{2+} release from the terminal cisternae. (*ii*) Ca^{2+} permeability in terminal cisternae was found to be modulated by ryanodine in pharmacologically significant concentrations (6). (*iii*) Ryanodine binding in the same concentration range (K_d, ≈50 nM) has been localized to the terminal cisternae of SR (6). (*iv*) The ryanodine receptor has been isolated and found to be equivalent to the feet structures of the junctional terminal cisternae (7). (*v*) SR vesicles have been fused into black lipid films and found to contain Ca^{2+} channels (8). These channels have characteristics of the Ca^{2+} release observed in vesicles (9, 10). (*vi*) Ryanodine has been found to modulate the channel in the bilayer, thereby reinforcing its role in the Ca^{2+}-release process (11). We now find that the ryanodine receptor from fast-twitch skeletal muscle forms Ca^{2+}-activated Ca^{2+} channels in bilayers, thereby identifying the feet structures as the calcium-release channels in excitation–contraction coupling.

METHODS

Isolation and Reconstitution of the Purified Ryanodine Receptor. The ryanodine receptor was purified from rabbit fast-twitch skeletal muscle as described (7) with modification (12). Samples were shipped from Nashville, TN, to Linz, Austria, at liquid nitrogen temperature and stored in liquid nitrogen until use. The purified protein was incorporated into phospholipid vesicles according to the "fast dilution" method (13, 14). Briefly, 4 μl of purified ryanodine receptor at 100 μg/ml in 20 mM Tris·HCl (pH 7.4) buffer containing 0.3 M sucrose, 0.5 M KCl, 2 mM dithiothreitol, 0.5 μg of leupeptin per ml, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (without added lipid) was rapidly diluted with bath sonication into 20 ml of a dilute suspension (≈10 μg of lipid per ml) of crude soybean phospholipid (Sigma type II-S) in medium A or B (see below). The lipid was prepared by briefly mixing a suspension of lipid particles in medium A or B at 1 mg of lipid per ml using a Vortex mixer, filtration through a Nuclepore 0.2-μm filter, and then dilution. The polypeptide/lipid mixture was then added to a 1-liter round bottom flask containing a dried film of 18 mg of acetone-washed soybean phospholipid and 3 mg of cholesterol and protein-containing lipid vesicles were prepared by gently swirling the suspension with glass beads. Phospholipid vesicles were prepared by the same procedure using medium A or B buffer instead of the suspension of crude lipid and diluted protein.

Planar Bilayer Formation. Vesicle-derived planar bilayers were prepared according to the monolayer spreading method of Schindler (14, 15). Protein-containing lipid vesicles were mixed with phospholipid vesicles to obtain the desired number of high molecular weight polypeptides of the ryanodine receptor bilayer (usually 20–50), estimated from the area of the bilayer, the area occupied by a single lipid molecule (65 Å^2 at 36 dynes/cm), and the protein/lipid ratio of the vesicle suspension [usually the same within a factor of 2 as the ratio in the bilayer (14)]. The bilayers were formed on Teflon septa

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Abbreviation: SR, sarcoplasmic reticulum.

with an aperture of either 100 or 160 μm diameter, and nearly all the bilayers were obtained at the first try. Experiments were begun within 30 min of thawing the purified channel protein and conducted at 20°C–25°C for up to 3 hr thereafter.

Solutions. Vesicles and planar bilayers were prepared in either 100 mM KCl/10 mM Hepes-Tris, pH 7.4 (medium A) or 250 mM Hepes/110 mM Tris, pH 7.4 (medium B). Both media A and B also contained 2 mM dithiothreitol, 0.5 μg of leupeptin per ml, and 0.1 mM phenylmethylsulfonyl fluoride. Other components were added from concentrated stock solutions after membrane formation or when needed, such that the dilution volume was <5%. Free Ca^{2+} concentrations were calculated using the K_d for Ca-EGTA from Owen (16). Ruthenium red was obtained from Sigma and the solution concentration was corrected according to the indicated purity.

General Assays. Biochemical assays such as for protein and phospholipid, ryanodine binding, and $NaDodSO_4$/polyacrylamide gel electrophoresis were performed as described (6, 7).

Definitions. Calcium-channel openings are shown as downward deflections. The cis side is defined as the protein-containing side and corresponds to the sarcoplasmic side (see *Discussion*). The trans side, equivalent to the luminal face, contained only lipid. The potential difference across the membrane was controlled by a voltage clamp circuit with the solution on the trans side maintained at ground by an operational amplifier with a 5 or 10 GΩ feedback resistor (14). Throughout this paper, voltage is expressed as the voltage applied to the cis solution. Electrical contact with the solutions was made via Ag/AgCl electrodes. Conductance scales were calculated assuming an ohmic current–voltage relationship. The upper end of the scale always indicates the position of the baseline conductance.

Data Acquisition and Analysis. The voltage signal across the feedback resistance of the current-measuring operational amplifier was filtered at 3 kHz and stored on a pulse-code modulated audio tape recorder modified to accept dc signals. Analysis, rescaling, and plotting of recorded data were performed using a B-Scope computer system (Med-Natic, Munich), refiltering the data as described upon playback. Data were digitized at 500 Hz (macroscopic currents) or 10 kHz (single-channel data). Amplitude histograms were calculated on a point-for-point basis from raw data, not separately redefined events, and normalized to a value of 1 for the most frequently occupied class. Reversal potentials were measured under highly activating conditions (2 μM free Ca^{2+}, 1 mM ATP) by slowly varying the applied dc holding potential stepwise until the steady-state macroscopic current was reduced to 0 and finally reversed and by repeating this procedure in smaller increments around the reversal potential. It was possible with this method to determine E_{rev} with an accuracy of about ±1–2 mV.

RESULTS

The purified ryanodine receptor, incorporated into vesicle-derived planar bilayers, reproducibly induced Ca^{2+}-selective conductance. Activity was observed in virtually every membrane, and the amount of activity was roughly proportional to the protein/lipid ratio in the vesicle suspension used to form the bilayer over the range from ≈20 to several hundred polypeptides per bilayer. The appearance of "single-channel" activity was quite heterogeneous, even when extreme dilutions were used (10–20 molecules of M_r 360,000 protein expected per bilayer). The foot structure is composed of an oligomeric association of 12–16 high molecular weight polypeptides (7, 12). As shown in Fig. 1, the smallest conductance observed (traces a and b), 3.8 pS, was also the most well defined, demonstrating a classical burst pattern. Well-behaved channels of twice this conductance, 7.6 pS (trace c),

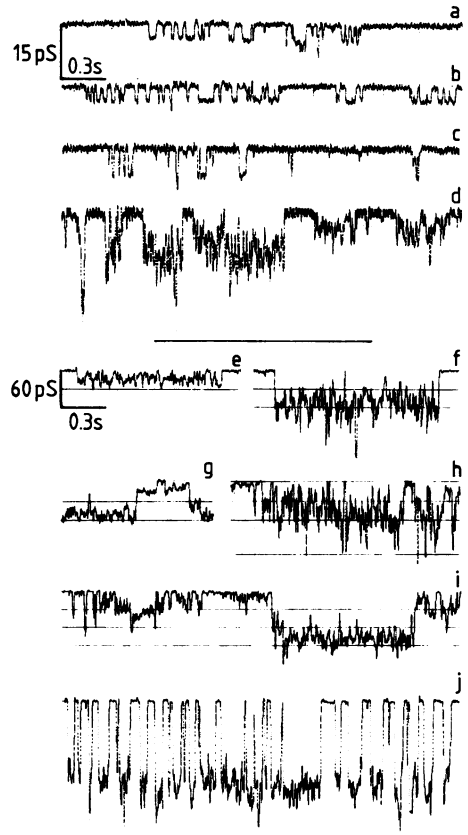

FIG. 1. Single-channel conductance states of the purified ryanodine receptor. Selected traces of single-channel activity in trans 50 mM $CaCl_2$ and medium B (traces c, h, and i) or medium A (other traces), activated with cis 1 mM ATP and cis 0.05–2.0 μM free Ca^{2+}. Holding potential was −50 (traces d and j), −100 (traces a–c and e), or −150 mV (traces f–i). The data were filtered at 100 Hz.

were also observed. Larger events were more frequent, however, and usually less well defined, but often formed stabilized bursts with combinations of various conductance levels (traces d and e could be considered "most typical"). Fast noisy flickering in the open state was characteristic of the larger bursts (e.g., trace f). Stabilized levels or sudden jumps of about 15, 30, and 60 pS were often seen, occasionally becoming stable for longer periods (traces g–i). Large homogeneous bursts of 120 pS (trace J) or larger were more seldom. Mean conductance of channel events increased with time (range of several minutes; data not shown), although fusion of vesicles with the bilayer does not occur under these conditions. The protein profile of the purified ryanodine receptor used in this study is shown in Fig. 2. The purified receptor showed essentially a single polypeptide band of M_r 360,000 when protease inhibitors were included during the SR preparation and receptor purification. Therefore, the multiple conductance states revealed by the ryanodine receptor/Ca^{2+} channel are referrable to this single polypeptide (7, 12).

Channel activity was observed at any voltage applied from −200 mV to well beyond the reversal potential, including zero potential difference. There was no indication for a threshold type of voltage gating. For a quantitative analysis of open-channel states, different approaches will be required because of the particular form of channel conductances in multilevel events.

The channel activity obtained for the purified ryanodine receptor was specific for Ca^{2+} as shown by experiments using bi-ionic gradients. Competition between Ca^{2+} (50 mM, trans) and $Tris^+$ (110 mM, cis and trans) yielded a reversal potential of +110 mV (cis), and between Ca^{2+} (50 mM, trans) and K^+

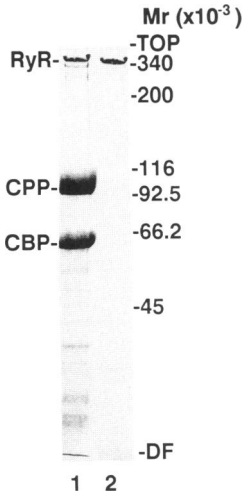

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis of the purified ryanodine receptor. The purified receptor preparation used in this study is shown in lane 2, to be compared with junctional terminal cisternae of fast-twitch skeletal muscle SR (4) (lane 1). Electrophoresis was carried out in NaDodSO$_4$ on a 7.5% polyacrylamide gel using the buffer system of Laemmli (17). The gel was stained with Coomassie blue. The purified receptor revealed the protein band of M_r 360,000 with a minor band of M_r 330,000. The relative intensity of the bands, determined by densitometry, was 92:8 (M_r 360,000/330,000). Molecular weight standards were run in parallel gel lanes. RyR, ryanodine receptor protein; CPP, Ca^{2+}-pump protein; CBP, Ca^{2+}-binding protein (calsequestrin); TOP, the top of the gel; DF, the dye front.

(100 mM, cis and trans) one of +47 mV. According to the constant-field equation for mixtures of divalent and monovalent ions (18), P_{Ca}/P_{Tris} = 2900 and P_{Ca}/P_K = 19.0. No chloride currents (upward deflections; e.g., using medium B with trans 50 mM $CaCl_2$) were observed. It is important to note that, despite the heterogeneous nature of the channel conductance, all conductance levels were reduced to zero at the respective reversal potentials (data not shown), even for macroscopic currents, indicating a strongly homogeneous ion selectivity among the various sublevels.

The most noteworthy characteristic of the reconstituted channel activity is its sensitivity for and absolute dependence on low Ca^{2+} levels at the cis (protein) side of the membrane. At a tightly buffered free-Ca^{2+} concentration of 10 nM, very little activity could be measured, even in the presence of 1 mM adenine nucleotide (Fig. 3*A*). However, increasing the free-Ca^{2+} concentration to only 50 nM produced an immediate (within the mixing time) activation of channel activity at every attempt (n = 5) (Fig. 3*B*). Visual inspection shows a dramatic change in channel activity, especially in the occurrence of smaller conductance events (≈15 pS). Further increase in the Ca^{2+} concentration stepwise (up to 1 μM) yielded less dramatic increases in activity (data not shown). In many cases, the Ca^{2+}-induced activation was only transient, declining after several minutes.

At suboptimal concentrations of Ca^{2+}, a clear activation could also be demonstrated for ATP and ryanodine (three experiments each), two other activators of calcium channels from native SR vesicles (Fig. 4). The effect of ATP required some 45 sec to become optimal. Addition of ryanodine often produced effects other than that shown in Fig. 4*B*. For example, the staircase activation reported for calcium channels from native terminal cisternae vesicles (11) was occasionally observed, but not so cleanly as with the native channels. In addition (see below), ryanodine often induced a dramatic prolongation of the open state, which allows clear visualization of sublevels (the "S" or "sublevel" mode, see Fig. 6).

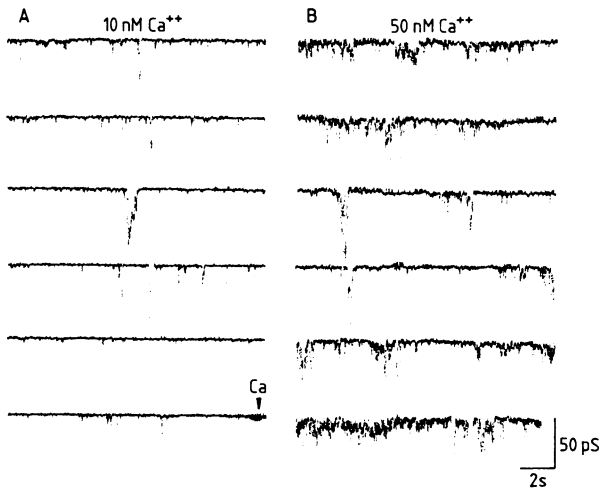

FIG. 3. Ca^{2+} activation of the Ca^{2+} release channel. (*A*) Continuous recording (left to right, top to bottom) in medium A plus trans 50 mM $CaCl_2$, and cis 1 mM ATP and 10 nM free Ca^{2+} (10 mM EGTA, 1.59 mM $CaCl_2$). (*B*) Same bilayer 15 sec after addition of $CaCl_2$ to give cis 50 nM free Ca^{2+} (10 mM EGTA, 4.86 mM $CaCl_2$). The holding potential was −50 mV (*A* and *B*).

Ca^{2+} current induced by the purified ryanodine receptor could be completely blocked by known inhibitors of calcium-induced Ca^{2+} release from SR terminal cisternae. Ruthenium red (30 μM) totally and irreversibly blocked the purified channel within seconds of addition (n = 5) (Fig. 5*A*), while 20 μM resulted in an incomplete block (data not shown). Subsequent addition of 10 μM ryanodine had no effect, but prior addition prevented the block, as did higher Ca^{2+} concentrations (e.g., 1 μM; data not shown). Millimolar concentrations of Mg^{2+} (cis) could also block the flow of Ca^{2+} in the trans to cis direction, representative of three experiments (Fig. 5 *B* and *C*), but this effect could usually be reversed by application of a high driving force (e.g., −200 mV) for a brief period, in which case subsequent increases in Mg^{2+} would again block the channel. Ryanodine (10 μM cis) did not prevent the Mg^{2+} block.

In the presence of ryanodine (and rarely in its absence) a qualitatively different mode of the calcium release channel was observed (Fig. 6). The S mode was characterized by open times of several hundred milliseconds to minutes, where the

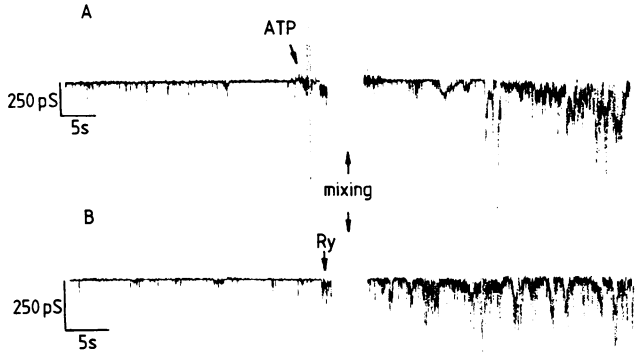

FIG. 4. Activation of Ca^{2+}-channel activity by ATP and ryanodine. (*A*) Macroscopic conductance in medium A with trans 50 mM $CaCl_2$ and cis 100 nM free Ca^{2+} (100 mM EGTA, 6.55 mM $CaCl_2$). ATP addition, 1 mM, was cis. The 15-sec mixing interval is not shown. The holding potential was −50 mV. (*B*) As in *A*, with cis 1 mM ATP already present, ≈5 min after activation with 100 nM free Ca^{2+}. After the activity had spontaneously decreased, 10 μM ryanodine was added to the cis side; the 45-sec mixing interval is not shown. The holding potential was −100 mV.

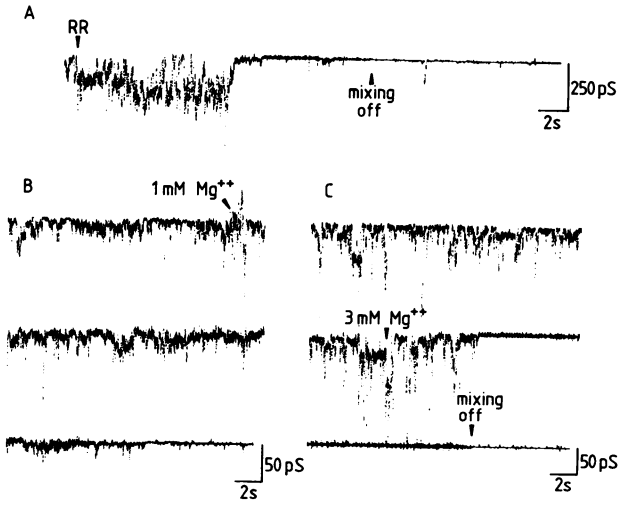

FIG. 5. Ruthenium red and Mg^{2+} block of the Ca^{2+} channel. (*A*) Macroscopic channel activity in medium A in the presence of trans 50 mM $CaCl_2$, cis 1 mM ATP and 100 nM free Ca^{2+}. Ruthenium red (30 μM) was added to the cis side. The holding potential was −100 mV. (*B*) Ca^{2+}-channel activity in medium A plus trans 50 mM $CaCl_2$, and cis 1 mM ATP, 100 nM free Ca^{2+}, and 10 μM ryanodine. The holding potential was −100 mV. $MgCl_2$ (1 mM) was added to the cis side. (*C*) Same membrane as in *B* (1 mM Mg^{2+} present), but after brief reactivation at −200 mV. The holding potential was −150 mV. At the arrowhead, $MgCl_2$ was added cis to a final concentration of 3 mM.

sublevels of a channel associate became stabilized. As indicated in the amplitude histograms, the sublevels were increments of the smallest observed conductance state, 3.8 pS. The channel events in this mode switched between sublevels for minutes at a time, later to be replaced with the usual more heterogeneous gating mode, implying the action of a single large oligomeric channel associate. Conductance jumps corresponding to instantaneous switching of 20 or more increments were not uncommon.

DISCUSSION

A clear assignment of the molecular machinery responsible for calcium release in excitation–contraction coupling in skeletal muscle is now possible. This study establishes the identity of the ryanodine receptor as the calcium-release channel, which is localized to the junctional face membrane of SR terminal cisternae. The ryanodine receptor has recently been purified and shown to consist of a single polypeptide of M_r 360,000 (7, 12). The identity has been confirmed also by immunoprecipitation (19). The polypeptide self-associates to oligomers (7) to form the major if not the entire portion of the "feet" structures of the terminal cisternae, which join the junctional face membrane of terminal cisternae to the adjacent transverse tubule. The channels formed by the isolated receptor are Ca^{2+} selective, and submicromolar concentrations of Ca^{2+} at the cis side stimulate activity (Fig. 3). Channel activity is stimulated by millimolar concentrations of ATP and inhibited by micromolar concentrations of ruthenium red and millimolar concentrations of Mg^{2+} (Figs. 4 and 5). Both properties were previously described for $^{45}Ca^{2+}$ flux from native SR vesicles (6, 9, 10) and for channels incorporated directly from the native SR membrane into bilayers (20, 21). In addition, ryanodine was shown to stabilize the open state of the channel and to prevent the effect of ruthenium red, similar to studies with junctional terminal cisternae vesicles (6) and to recently demonstrated effects on the native channel in the bilayer (11).

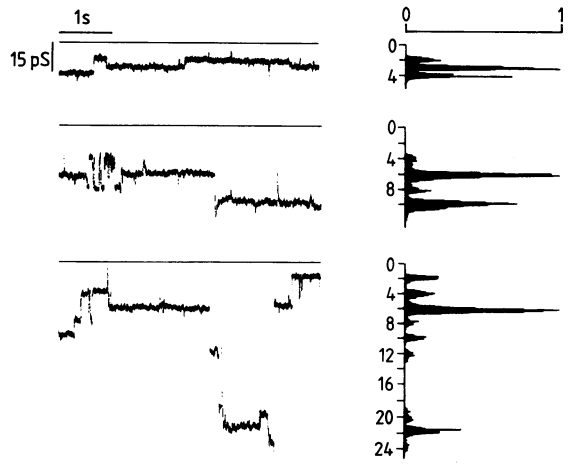

FIG. 6. The sublevel gating mode induced by ryanodine. Selected single-channel activity in medium A with trans 50 mM $CaCl_2$, and cis 1 mM ATP, 2 μM free Ca^{2+} (10 mM EGTA, 9.75 mM $CaCl_2$), and 10 μM ryanodine. The holding potential was −50 mV. Histograms have been scaled to the same conductance amplitude scale as channel traces. Multiples of the monomer conductance of 3.8 pS are indicated.

The Ca^{2+} channels described here are distinct from those located in the transverse tubule membrane by at least three lines of evidence. First, the purified ryanodine receptor (7, 12) consists of an oligomer of a high molecular weight protein (M_r, 360,000), whereas the purified dihydropyridine receptor from transverse tubule membranes consists of multiple polypeptide subunits (M_r, 165,000, 155,000, 65,000, and 32,000) (22). Second, the ryanodine receptor/Ca^{2+} channel shows quite different channel properties from those of the dihydropyridine receptor/Ca^{2+} channel in the bilayer. For example, the SR Ca^{2+} release channel has a unitary conductance of 3.8 pS (50 mM Ca^{2+}), while the purified transverse tubule Ca^{2+} channel has a unitary conductance of 0.9 pS (100 mM Ba^{2+}) and requires phosphorylation by the cAMP-dependent protein kinase for activity (L.H., J. Striessnig, H. Glossmann, and H.S., unpublished data). Third, the effects of activators and inhibitors reported here coincide with similar effects on Ca^{2+} release from isolated SR terminal cisternae vesicles (6, 9) and Ca^{2+} channels incorporated from these vesicles into planar bilayers (11, 20, 21).

Although it now seems clear which molecular component is involved in release of Ca^{2+} from the junctional face membrane of the SR, many questions remain regarding the exact nature of the release mechanism and its modulation. The high sensitivity to Ca^{2+} of the Ca^{2+} release channel (Fig. 3) suggests that Ca^{2+}-induced Ca^{2+} release (23) may be operative in excitation–contraction coupling in skeletal muscle. If so, the source of this Ca^{2+} and the coupling of its release to depolarization of the transverse tubule membrane must be elucidated. Interestingly, recent studies have confirmed that the purified dihydropyridine receptor from skeletal muscle forms Ca^{2+} channels in planar bilayers (22, 24, 25–27) and has strong sequence homology with the Na^+ channel from brain (28), and that perhaps as many as 50% of the dihydropyridine binding sites observed in intact skeletal muscle fibers (29) are indeed active Ca^{2+} channels (L.H., J. Striessnig, H. Glossmann, and H.S., unpublished data). Since the Ca^{2+} release channel protein is morphologically referable to the feet structures (7), which are involved *in situ* in junctional association of transverse tubules with the terminal cisternae, another possibility is that the Ca^{2+} release channel could be directly regulated by a voltage-sensing molecule in the transverse tubule membrane (30), perhaps part of the dihydropyridine receptor itself (31). Although our results demonstrate that the voltage sensor is not obligatory for Ca^{2+}-channel activity under the conditions of our mea-

surements, conformational coupling might be essential for channel activity or its modulation under physiological conditions—e.g., allowing graded contraction in relation to graded depolarization of the transverse tubule membrane (32, 33). Still another possibility would be that transient depolarization of the junctional face membrane could contribute to the Ca^{2+} release mechanism. Although we observed Ca^{2+}-channel activity at a holding potential of 0 mV (data not shown), the voltage dependence of the Ca^{2+} release channel remains to be investigated in detail.

An intrinsic problem in any reconstitution experiment is the loss of some attributes of the native system. In our experiments with the purified Ca^{2+} release channel, we observed more heterogeneity of channel conductance states (Fig. 1) than reported from experiments with "native" channels. Dissociation with detergent during the purification of the channel protein could disrupt the natural biosynthetically induced order of the system, which is not restored by simply removing the detergent or during protein reassociation in the planar bilayer to oligomers as reflected by the multilevel conductance events. Another possibility is the removal of a component that modulates channel behavior.

The appearance of substate gating with integral multiples of the smallest conductance (3.8 pS) which we observed to be promoted by the presence of ryanodine underscores the need to study the association of purified Ca^{2+} release channel in the membrane. From the present data, a clear structural assignment of the 3.8 pS conductance level is not yet possible, although it appears likely that it corresponds to a single M_r 360,000 polypeptide rather than to an oligomer of this peptide. The effect of channel-subunit association on cooperative gating and conductance states has been so far described for three systems: the acetylcholine receptor (13), bacterial porin (34–36), and K^+ channels from cardiac sarcolemma (37). In this regard, recent evidence indicates that the Ca^{2+} channel from transverse tubule exists in the native membrane as a similarly sized aggregate (L.H., J. Striessnig, H. Glossmann, and H.S., unpublished data), inviting speculation of direct interaction between the two oligomeric channel complexes.

The foot structure serves a key role in excitation–contraction coupling. It is directly involved in junctional association of transverse tubule with terminal cisternae of sarcoplasmic reticulum. The precise nature of how depolarization of the transverse tubule is transduced to the release of Ca^{2+} from the terminal cisternae remains to be elucidated. The foot structure consists mainly of an oligomeric association (12–16 polypeptides) of the high molecular weight polypeptide (7, 12). This small number of polypeptides of the purified ryanodine receptor when incorporated into a single bilayer exhibits Ca^{2+}-channel activity. The incorporation of the ryanodine receptor into the bilayer was $\approx$100% $\pm$ a factor of 2, assuming that the 3.8 pS conductance is equivalent to one polypeptide. The sidedness of Ca^{2+} flux from the Ca^{2+} release channel is from luminal to cytoplasmic (cis) sides. The activation of the channel by Ca^{2+}, ATP, and ryanodine and complete inhibition by ruthenium red is obtained at the face equivalent to the cytoplasmic side of the junctional face membrane. Thus, the Ca^{2+} release channel in the bilayer has similar characteristics to those of the native channels as deduced from modulation of calcium permeability in the isolated junctional terminal cisternae vesicles. It is now clear that the molecular machinery of the Ca^{2+} release channel and of the foot structure are one and the same.

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