The Brain Ryanodine Receptor: A Caffeine-Sensitive Calcium Release Channel

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Summary

The release of stored Ca\(^{2+}\) from intracellular pools triggers a variety of important neuronal processes. Physiological and pharmacological evidence has indicated the presence of caffeine-sensitive intracellular pools that are distinct from the well-characterized inositol 1,4,5-trisphosphate (IP\(_3\))-gated pools. Here we report that the brain ryanodine receptor functions as a caffeine- and ryanodine-sensitive Ca\(^{2+}\) release channel that is distinct from the brain IP\(_3\) receptor. We have recently identified the rabbit brain ryanodine receptor (Smith et al., 1988). It has been purified 6700-fold with no change in [\(^{3}H\)]ryanodine binding affinity and shown to be a homotetramer composed of an approximately 500 kd protein subunit, which is identified by anti-peptide antibodies against the skeletal and cardiac muscle ryanodine receptors. Our results demonstrate that the brain ryanodine receptor functions as a caffeine-sensitive Ca\(^{2+}\) release channel and thus is the likely gating mechanism for intracellular caffeine-sensitive Ca\(^{2+}\) pools in neurons.

Introduction

The concentration of free cytosolic Ca\(^{2+}\) is a crucial signal for a variety of neuronal processes including neurotransmitter release (Zucker and Lando, 1986) and alterations in the cytoskeleton (Bennett and Weeds, 1986). The resting free Ca\(^{2+}\) concentration in neurons is maintained by several processes, including extrusion via plasmalemmal Ca\(^{2+}\) pumps and Na\(^{+}\)-Ca\(^{2+}\) exchange, buffering by Ca\(^{2+}\)-binding proteins, and sequestration by the endoplasmic reticulum and mitochondria (for review see Blaustein, 1988). Ca\(^{2+}\) that is sequestered in the endoplasmic reticulum can be released via activation of Ca\(^{2+}\) release channels. The best studied of these types of channels is the ryanodine receptor of the skeletal muscle sarcoplasmic reticulum, which has been purified (Imagawa et al., 1987), cloned (Takeshima et al., 1989), and shown to function as the sarcoplasmic reticulum Ca\(^{2+}\) release channel (Smith et al., 1988).

The inositol 1,4,5-trisphosphate (IP\(_3\)) receptor appears to have a major role in the regulation of Ca\(^{2+}\) levels within neurons. The IP\(_3\) receptor has been purified from cerebellum (Supattapone et al., 1988) and shown to mediate Ca\(^{2+}\) flux in reconstituted lipid vesicles (Ferris et al., 1989). Interestingly, the neuronal IP\(_3\) receptor has been shown to have structural similarity with the skeletal muscle ryanodine receptor, and the two proteins have a small degree of sequence similarity at their C-termini (Mignery et al., 1989). Although the IP\(_3\) receptor is an important Ca\(^{2+}\) release channel in neurons, physiological and pharmacological evidence has indicated the presence of caffeine-sensitive intracellular Ca\(^{2+}\) pools that are distinct from the IP\(_3\)-sensitive pools. In sensory and sympathetic neurons, ryanodine can block caffeine-induced Ca\(^{2+}\) release from IP\(_3\)-insensitive pools (Thayer et al., 1988a, 1988b), and caffeine can stimulate noradrenaline release from sympathetic neurons in Ca\(^{2+}\)-free solutions (Toth et al., 1990). Caffeine-sensitive Ca\(^{2+}\) stores are also present in central neurons. In hippocampal cultures, caffeine can mobilize Ca\(^{2+}\) from intracellular pools that are distinct from IP\(_3\)-gated pools (Glaucom et al., 1990; Murphy and Miller, 1989), and caffeine can stimulate Ca\(^{2+}\) release from vesicular pools in rat brain synaptosomes (Martinez-Serrano and Sartransgut, 1989).

We have recently identified the rabbit brain ryanodine receptor and shown that it is present in membranes from throughout the brain and is enriched in membranes from the hippocampus (McPherson and Campbell, 1990). Ellisman et al. (1990) used monoclonal antibodies to localize the chicken brain ryanodine receptor to Purkinje cells of the cerebellum. Here we report that the brain ryanodine receptor is a Ca\(^{2+}\) release channel that is distinct from the brain IP\(_3\) receptor. We have purified the brain ryanodine receptor 6700-fold and have demonstrated that it forms a homotetramer composed of an approximately 500 kd subunit, which is recognized by antibodies against peptides corresponding to the C-terminus of the skeletal muscle ryanodine receptor and to Purkinje cells of the cerebellum. Here we report that the brain ryanodine receptor is a Ca\(^{2+}\) release channel that is distinct from the brain IP\(_3\) receptor. We have purified the brain ryanodine receptor 6700-fold and have demonstrated that it forms a homotetramer composed of an approximately 500 kd subunit, which is recognized by antibodies against peptides corresponding to the C-terminus of the skeletal muscle ryanodine receptor and to a potential cAMP-dependent protein kinase phosphorylation site of the cardiac ryanodine receptor. When reconstituted into planar lipid bilayers, the brain ryanodine receptor forms a caffeine- and ryanodine-sensitive Ca\(^{2+}\) channel with a 107 pS slope conductance. These results indicate that the brain ryanodine receptor functions as an intracellular Ca\(^{2+}\) channel in brain and is likely responsible for releasing Ca\(^{2+}\) from caffeine-sensitive pools in neurons.

Results

Separation of the Brain Ryanodine Receptor from the Brain IP\(_3\) Receptor

We have previously shown that the rabbit brain ryanodine receptor has affinity for heparin-agarose and migrates with a characteristically large sedimentation

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Purification of the Brain Ryanodine Receptor

The combination of heparin-agarose and sucrose gradient centrifugation is able to enrich significantly for the brain ryanodine receptor. However, a small amount of \[^{3}H\]IP$_3$ receptor was still found in peak \[^{3}H\]ryanodine-binding fractions (Figure 1) as well as a major contaminant at 55 kD, which was determined to be tubulin (data not shown). The tubulin, but not the ryanodine receptor, was found to bind to DEAE-agarose in the presence of 150 mM NaCl (data not shown). Therefore, peak \[^{3}H\]ryanodine-binding fractions (fractions 11-13 of Figure 1) were pooled, diluted to a final concentration of 150 mM NaCl, and mixed with DEAE-agarose for 1 hr. The DEAE void was concentrated on heparin-agarose and recentrifuged on sucrose density gradients to remove the contaminating IP$_3$ receptor. The top left panel of Figure 2 indicates that the \[^{3}H\]ryanodine receptor again peaks in fraction 12 and is free of IP$_3$ receptor contamination. SDS-PAGE analysis (Figure 2, bottom left panel) indicates that peak \[^{3}H\]ryanodine-binding fractions are highly enriched in a large molecular weight protein of approximately 500 kD as seen by Coomassie blue staining. Fractions 9-15 were separated on SDS-PAGE, transferred to nitrocellulose membranes, and stained with a polyclonal antibody raised against the C-terminus of the skeletal muscle ryanodine receptor (top right) or with an antibody against the cardiac ryanodine receptor cAMP-dependent protein kinase phosphorylation site (bottom right).
Figure 3. $[^3H]$Ryanodine Binding to the Purified Receptor

$[^3H]$Ryanodine binding was performed on brain membranes (A) and on purified brain ryanodine receptor (B) and was characterized by Scatchard analysis. The $K_d$ and $B_{max}$ values for binding to membranes are 1.3 nM and 53 fmol/mg, respectively. For binding to the pure receptor, the $K_d$ and $B_{max}$ values are 3.4 nM and 358 pmol/mg, respectively.

500 kd. This protein is immunologically stained on nitrocellulose transfers with a polyclonal antibody raised against the C-terminus of the skeletal muscle ryanodine receptor (Figure 1, top right panel). A polyclonal antibody against the predicted cAMP-dependent protein kinase phosphorylation site of the cardiac ryanodine receptor, which immunoprecipitates PH$[^3H]$ryanodine-labeled receptor from brain membranes (data not shown), also stains this band on immunoblots (Figure 2, bottom right panel).

**Binding Analysis on the Pure Brain Ryanodine Receptor**

$[^3H]$Ryanodine binding was performed on the purified brain ryanodine receptor and compared with $[^3H]$Ryanodine binding to brain membranes (Figure 3). $[^3H]$Ryanodine binds to membranes with a $K_d$ of 1.3 nM and a $B_{max}$ of 56 fmol/mg protein (Figure 3A). $[^3H]$Ryanodine binds to the purified preparation with a $K_d$ of 3.4 nM and a $B_{max}$ of 358 pmol/mg (Figure 3B), representing a 6755-fold purification without affecting the affinity of the receptor. Only a single affinity for $[^3H]$Ryanodine binding was observed in brain membranes and in the purified receptor using $[^3H]$Ryanodine at concentrations between 0.1 and 20 nM.

**Structural Analysis of the Purified Brain Ryanodine Receptor Using Rotary Shadowing Electron Microscopy**

We have previously characterized the brain ryanodine receptor as an approximately 30S complex based on its identical migration on sucrose gradients as the skeletal and cardiac muscle ryanodine receptors (McPherson and Campbell, 1990), which are isolated as homotetramers (Anderson et al., 1989; Lai et al., 1989). The large sedimentation coefficient of the brain ryanodine receptor suggests that it may also be a tetramer. We therefore analyzed the purified receptor by rotary shadowing electron microscopy. The preparation reveals a population of large molecules, some of which can be seen to have 4-fold symmetry (Figure 4, top panel). Selected molecular profiles with a defined
Figure 5. Reconstitution of the Purified Brain Ryanodine Receptor in Lipid Bilayers

(A) Channel activity was monitored over a 3-5 min period and the open probability was calculated from amplitude histograms. Openings are shown as downward deflections of current. Open probability increased from <0.001 in control to 0.11 after the addition of 10 mM caffeine and to 0.21 after the subsequent addition of 3 mM ATP. Addition of 10 μM ruthenium red to the caffeine- and ATP-activated channel brought the open probability to 0.004. (B) The top panel shows traces of channel activity at indicated holding potentials, in the presence of 10 mM caffeine. Two levels of opening, representing the activity of two channels, are indicated by dotted lines. The current-voltage relationship of unitary current, illustrated below the traces, reveals a slope conductance of 107 pS. (C) The brain ryanodine receptor channel was activated with caffeine and ATP (top trace), and in five separate experiments, the addition of ryanodine at 4 μM completely blocked the channel (bottom trace).
square shape have dimensions ranging from 28 × 28 nm to 35 × 37 nm (Figure 4, bottom panels). In these selected images, the 4-fold symmetry is especially apparent. Thus, the brain ryanodine receptor is similar structurally to the ryanodine receptor isolated from skeletal and cardiac muscle (Block et al., 1988; Inui et al., 1987).

Biophysical Analysis of the Brain Ryanodine Receptor in Planar Lipid Bilayers

The purification of the brain ryanodine receptor, free from contamination by the IP₃ receptor, also permitted us to establish that this protein forms an ion channel with properties similar to those of the ryanodine receptor of striated muscle. Pure ryanodine receptor was incorporated into planar bilayers using a protocol similar to that for reconstitution of the skeletal muscle ryanodine receptor (Smith et al., 1988). K⁺ was used as the charge carrier instead of Ca²⁺ to prevent inactivation caused by large concentrations of divalent ions and because monovalent cations produce larger conductances than divalent cations (Smith et al., 1988). Similar to the channel isolated from skeletal muscle, the brain channel displayed rapid transitions between open and closed states (flickerings) that were stimulated by millimolar ATP and blocked by micromolar ruthenium red (Figure 5A). Ruthenium red at 10 μM also inhibited [³H]ryanodine binding to rabbit brain membranes to 26% of that in control conditions (data not shown).

The brain channel was different from the skeletal channel in a variety of ways. The brain receptor exhibited a slope conductance of 107 pS (Figure SB), which represented approximately one-fourth of the peak conductance of the skeletal receptor in the same solutions. In contrast, the brain IP₃ receptor was determined to have a slope conductance of 27 pS under identical conditions (Valdivia et al., 1991, Biophys. Soc. abstract). Ashley (1989) reported that IP₃ modulated the same channel as ryanodine. However, Ashley's recordings were made from brain microsomes incorporated into lipid bilayers, and it is possible that separate ryanodine- and IP₃-sensitive channels were present in the preparation. Fluctuations of brain receptor channels among several conductance states were not observed, whereas the skeletal receptor displays multiple transitions between "monomeric," "dimERIC," and "tetramERIC" conductance states (Smith et al., 1988). Furthermore, increased openings of the brain receptor channel required the constant presence of caffeine. For instance, 10 mM caffeine increased open probability from undetectable levels to 0.11 ± 0.04 (Figure 5A). In skeletal muscle, caffeine is not required for channel opening, and the same concentration of caffeine increased channel activity by 2-fold or less (Valdivia et al., 1990, J. Neuro. Sci., abstract). A concentration of 4 μM ryanodine completely blocked activity of the brain ryanodine receptor channel (Figure 5C; n = 5). The blockade was complete and occurred at all membrane potentials. Lower concentrations (10-100 nM) of ryanodine failed to induce the modification seen in skeletal muscle of decreased unitary channel current and increased open probability and mean open time (Rousseau et al., 1987).

Effects of Caffeine on [³H]Ryanodine Binding to Brain Membranes

The caffeine sensitivity of the release channel led us to investigate the ability of caffeine to affect [³H]ryanodine binding to its receptor in rabbit brain membranes. Caffeine increases [³H]ryanodine binding in a manner that is both dose dependent and saturable (Figure 6). This result is similar to what is observed for [³H]ryanodine binding to rat cardiac sarcoplasmic reticulum under identical conditions (Pessah et al., 1990).

Discussion

We have purified a high affinity [³H]ryanodine receptor from rabbit brain membranes that is distinct from the IP₃ receptor and that forms a caffeine- and ryanodine-sensitive Ca²⁺ channel when incorporated into planar lipid bilayers. The protein is purified 6700-fold by utilizing its affinity for heparin-agarose and its extremely large sedimentation coefficient on sucrose density gradients (approximately 30S). Scatchard analysis of the purified receptor indicates that it has a similar affinity for [³H]ryanodine as the receptor in brain membranes. Rotary shadowing electron microscopy indicates that the large size of the brain ryanodine receptor is probably due to its isolation as a tetramer, similar to the "foot" structures of the junctional face of the sarcoplasmic reticulum of skeletal muscle (Block et al., 1988). Henkart et al. (1976) demonstrated...
that junctions between the plasma membrane and subsurface cisterns of endoplasmic reticulum in mouse cerebellar neurons are similar to the transverse tubular sarcoplasmic reticulum junctions in skeletal muscle. This similarity includes the presence of large particles in the endoplasmic reticulum that span the junction with the plasma membrane. It is conceivable that the brain ryanodine receptor could be present at such neuronal junctions and could function to couple excitatory events at the surface membrane with intracellular activities.

Ca\(^{2+}\) has a central role in the regulation of neuronal function. Ca\(^{2+}\) is the trigger for neurotransmitter release, it can alter membrane excitability by direct activation of K\(^{+}\) or Cl\(^{-}\) channels, and it can indirectly affect channels and other proteins through Ca\(^{2+}\)-dependent protein kinases and phosphatases (Kennedy, 1989). The resting free cytosolic Ca\(^{2+}\) concentration in neurons is maintained at approximately 10\(^{-7}\) M, and it must be increased severalfold to act as a signal (Miller, 1988). Increased free cytosolic Ca\(^{2+}\) concentrations result from activation of voltage-sensitive Ca\(^{2+}\) channels or release of Ca\(^{2+}\) from intracellular stores (Kostyuk and Tepikin, 1991). The intracellular Ca\(^{2+}\) release can be triggered from IP\(_3\)-sensitive Ca\(^{2+}\) pools after receptor activation. Caffeine can also stimulate Ca\(^{2+}\) release from intracellular pools (Lipscombe et al., 1988) that are distinct from IP\(_3\)-sensitive pools. Such caffeine-sensitive pools have been studied in peripheral neurons (Thayer et al., 1988a, 1988b) as well as central neurons (Glaum et al., 1990; Murphy and Miller, 1989; Martinez-Serrano and Satrustegui, 1989). Here, we have demonstrated that the brain ryanodine receptor functions as a caffeine- and ryanodine-sensitive Ca\(^{2+}\) channel. We also show that caffeine is able to stimulate \(^{3}H\)ryanodine binding to brain membranes. It is therefore likely that the brain ryanodine receptor is the gating mechanism and release channel for intracellular caffeine-sensitive pools in neurons. Although the physiological significance of such pools is unknown, they likely serve to help maintain the narrow range of free cytosolic Ca\(^{2+}\) concentrations between resting and stimulated neurons.

The brain ryanodine receptor appears to be more similar to the cardiac ryanodine receptor than to the skeletal receptor. The brain and cardiac receptors migrate to an identical position on an SDS-polyacrylamide gel and are slightly smaller than the skeletal receptor (data not shown). Monoclonal antibodies against the cardiac receptor (Imagawa et al., 1989) identify the brain receptor on Western blots, whereas monoclonal antibody XA7 against the skeletal muscle receptor (Campbell et al., 1987) does not cross-react with the brain receptor (data not shown). A polyclonal antibody against a CAMP-dependent protein kinase phosphorylation site from the predicted sequence of the cardiac ryanodine receptor (Otsu et al., 1990) recognizes the brain (Figure 2) and cardiac (data not shown) ryanodine receptors, but not the skeletal muscle ryanodine receptor (data not shown). Northern analysis using cDNA clones against the cardiac receptor, but not the skeletal receptor, detects a message of 17 kb in rabbit brain (Otsu et al., 1990; Nakai et al., 1990). It is conceivable that the brain ryanodine receptor operates in a manner identical to that of the cardiac ryanodine receptor, which appears to operate by a mechanism of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Nabauer et al., 1989). This form of stimulation may play a role in amplifying Ca\(^{2+}\) signals within neurons. The smaller conductance state of the brain ryanodine receptor compared with the skeletal muscle receptor (one-fourth the conductance under identical conditions) agrees with the lower levels of free cytosolic Ca\(^{2+}\) in stimulated neurons versus stimulated muscle cells.

Ca\(^{2+}\) oscillations and Ca\(^{2+}\) waves in response to agonist stimulation have been observed in a wide variety of tissues, including neurons (Kuba and Takeshita, 1981) and astrocytes (Cornell-Bell et al., 1990). It is hypothesized that the cycles of Ca\(^{2+}\) may be produced by exchange between an IP\(_3\)-sensitive pool and a second pool of Ca\(^{2+}\) that is caffeine sensitive and IPs insensitive and that displays Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Berridge, 1990; Dupont et al., 1990; Wakui et al., 1990). Although the physiological significance of such phenomena is unclear, the observation that the frequency of oscillations can vary with the ligand concentration indicates that oscillations may be part of a frequency-encoded signaling system (Berridge and Galione, 1988). Thus, the ryanodine receptor is likely to be the "gate" for the caffeine-sensitive, IP\(_3\)-insensitive Ca\(^{2+}\) pools observed in neurons and may play a role in Ca\(^{2+}\) oscillations in neurons or glia as well as a wide variety of cells.

**Experimental Procedures**

**Preparation of Membranes**

Whole-rabbit brains minus the medullas were homogenized in 50 mM Tris-Cl (pH 7.4) containing protease inhibitors as described (McPherson and Campbell, 1990). Membranes were pelleted at 35,000 x g for 15 min, resuspended in 50 mM Tris-maleate (pH 7.0), 0.303 M sucrose containing protease inhibitors, and frozen at -135°C. Protein was determined by the method of Lowry et al. (1951) as modified by Peterson (1977).

**Purification of the Brain Ryanodine Receptor**

Rabbit brain membranes were solubilized using CHAPS-PC and chromatographed on heparin-agarose as previously described (McPherson and Campbell, 1990). Proteins eluted from the heparin-agarose column with 0.6 M NaCl were centrifuged on 10%-30% linear sucrose density gradients in a Beckman VTi 50 rotor for 2 hr and 20 min at 238,000 x g using slow acceleration and deceleration profiles. The gradients were composed of buffer A (10 mM Na-HEPES [pH 7.4], 0.8 mM CaCl\(_2\), protease inhibitors) containing 0.5 M NaCl, 0.36% CHAPS, and 0.14% PC. Gradient fractions were analyzed by postlabeling with \(^{3}H\)ryanodine or \(^{3}H\)IP\(_3\) receptor as described below. For further purification of the ryanodine receptor, fractions 11-13 (Figure 1) were pooled, diluted to 0.15 M NaCl, and applied to 10 ml of DEAE-agarose, equilibrated in buffer A containing 0.15 M NaCl, 0.36% CHAPS, and 0.14% PC. After 1 hr of gentle mixing at 4°C, the DEAE void was applied onto a 7 ml heparin-agarose column, equilibrated
in buffer A containing 0.3 M NaCl, 0.36% CHAPS, and 0.14% PC, and eluted in the same buffer containing 0.6 M NaCl. The eluate was centrifuged on sucrose gradients as described above.

**Postlabeling of [3H]IP$_3$ Receptor and [3H]Ryanodine Receptor**

Aliquots (200 μl) of gradient fractions were added to binding mixtures to a final solution of 2.5 nM [3H]ryanodine in 10 mM Na-HEPES (pH 7.4), 1.5 M KCl, 10 mM ATP, 0.8 mM CaCl$_2$ (100 μM free Ca$^{2+}$), 0.18% CHAPS, and 0.07% PC in the presence or absence of 1 μM unlabeled ryanodine. After 1 hr at 37°C, 50 μl of carrier protein (0.5% BSA, 0.5% γ-globulin in 50 mM Tris-HEPES [pH 7.4]) and 3.5 ml of polyethylene glycol glycol buffer (50 mM Tris-HEPES [pH 7.4], 1 mM MgCl$_2$, 10% polyethylene glycol) were added. Samples were collected after 15 min on ice by membrane filtration as previously described (Lattanzio et al., 1987) using polyethylene glycol buffer to wash the filters. Aliquots (250 μl) of column fractions were also added to binding mixtures to a final solution of 2.0 nM [3H]ryanodine in 50 mM Tris-HEPES (pH 8.3), 0.07% β-mercaptoethanol, 0.4 mM CaCl$_2$, 5 mM EGTA (0 free Ca$^{2+}$), 0.18% CHAPS, and 0.07% PC in the presence or absence of 1 μM unlabeled IP$_3$. After 30 min at 4°C, 50 μl of carrier protein (50 mM Tris-HEPES [pH 8.3], 0.5% γ-globulin) and 500 μl of polyethylene glycol glycol buffer (50 mM Tris-HEPES [pH 8.3], 1 mM EGTA, 0.07% β-mercaptoethanol, 25% polyethylene glycol) were added. Samples were collected after 15 min on ice as described for the ryanodine receptor using 50 mM Tris-HEPES (pH 8.3), 1 mM EGTA, 0.07% β-mercaptoethanol, 10% polyethylene glycol as the wash buffer.

**[3H]Ryanodine Binding to Rabbit Brain Membranes**

Aliquots (500 μg) of rabbit brain membranes were added to a final volume of 250 μl in 10 mM Na-HEPES (pH 7.4) containing 1.5 M KCl, 0.8 mM CaCl$_2$ (100 μM free Ca$^{2+}$), 10 mM ATP, and 0.120 nM [3H]ryanodine in the presence or absence of 1 μM ryanodine. Samples were incubated for 1 hr at 37°C and were collected by membrane filtration as previously described (Lattanzio et al., 1987) using 10 mM HEPES (pH 7.4), 150 mM KCl as the wash buffer. For the caffeine dose-response curve (Figure 6), the binding assay was identical except that the buffer was 10 mM Na-HEPES (pH 7.4) containing 250 mM KCl, 15 mM NaCl, 1 mM MgCl$_2$, 0.32 mM CaCl$_2$, 0.22 mM EGTA (100 μM free Ca$^{2+}$), 2.5 mM [3H]ryanodine, and 1-20 mM caffeine.

**SDS-PAGE and Immunoblot Analysis**

Gradient fractions were analyzed on 3%-12% gradient SDS-polyacrylamide gels by the method of Laemmli (1970) and either stained with Coomassie blue or transferred to nitrocellulose paper according to Towbin et al. (1979). Proteins on nitrocellulose membranes were immunologically stained with anti-peptide antibodies against the skeletal or cardiac muscle ryanodine receptors using secondary antibodies coupled to horseradish peroxidase as described (McPherson and Campbell, 1990).

**Production of Antibodies against Peptide Sequences of the Skeletal and Cardiac Muscle Ryanodine Receptors**

Peptides against the C-terminus of the skeletal muscle ryanodine receptor (PAGDCFRRKQYEDQLS) (Takeshima et al., 1989) and the cAMP-dependent phosphorylation site of the cardiac ryanodine receptor (LYNTRRISQTSQV) (Otsu et al., 1990) were obtained from the University of Iowa Protein Structure Facility and the Howard Hughes Medical Institute Peptide Facility (Washington University), respectively. The C-terminal peptide was bromoacetyl and coupled to keyhole limpet hemocyanin by reacting the peptide with the protein thiol (Roby and Fields, 1989). The phosphorylation site peptide was supplied as a C-terminal photobase (Gorka et al., 1989) and was conjugated to keyhole limpet hemocyanin by dissolving 2 mg of peptide and 2.5 mg of keyhole limpet hemocyanin in 1 ml of PBS and exposing the solution to a 365 nm light source at 1 cm for 3 hr. Both peptides were mixed with Freund's incomplete adjuvant (1:1) and injected into rabbits by intramuscular and subcutaneous routes. Rabbits were boosted using peptide in incomplete Freund's adjuvant for 6 weeks to 2 week intervals. Bleeds were tested for reactivity on immunoblots against purified skeletal muscle and cardiac muscle ryanodine receptors.

**Rotary Shadowing Electron Microscopy**

Isolated preparations at a protein concentration of approximately 0.02 mg/ml were placed on a freshly cleaved mica surface, washed with 50 mM ammonium acetate, fixed with 1% 2-urenyl acetate for 30 min, rinsed with 30% methanol or 3% glycerol, and frozen in liquid nitrogen. Following freeze drying, the molecules were rotary shadowed with platinum at 15°C.

**Single-Channel Recordings in Planar Lipid Bilayers**

Planar bilayers, composed of an equimolar mixture of brain phosphatidylethanolamine and phosphatidylserine, were brought to a final concentration of 20 mg/ml in decane and were formed in a 0.42 mm diameter hole in a Delrin cup. Isolated brain ryanodine receptor (4-10 μg) was added to the cis solution composed of 0.25 M KCl and 10 mM HEPES-Tris (pH 7.2). The trans solution was the same. Solutions were connected via an Ag-AgCl electrode and an agar-KCl bridge to a List L/M EPC 7 amplifier. The trans solution was held at ground, and recordings were filtered through a low-pass Bessel filter at a front panel setting of 500 Hz and digitized at 2 kHz. Acquisition, storage, and analysis were done on a POAT computer using PClAMP software.

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