Photoaffinity Labeling of the Ryanodine Receptor/Ca\(^{2+}\) Release Channel with an Azido Derivative of Ryanodine*

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Ryanodine receptors/Ca\(^{2+}\) release channels play an important role in regulating the intracellular free calcium concentrations in both muscle and nonmuscle cells. Ryanodine, a neutral plant alkaloid, specifically binds to and modulates these Ca\(^{2+}\) release channels. In the work described here, we characterize the interaction of a tritium-labeled, photoactivatable derivative of ryanodine ([\(^{3}H\)ABRY]) with the ryanodine receptor of skeletal, cardiac, and brain membranes. Scatchard analysis demonstrates that this ligand binds to a single class of high affinity sites in skeletal muscle triads. Furthermore, competition binding assays of [\(^{3}H\)ryanodine with skeletal, cardiac, and brain membranes in the presence of increasing concentrations of unlabeled ABRY illustrate that this azido derivative of ryanodine is able to specifically displace [\(^{3}H\]ryanodine from its binding site(s). Analysis of the effects of Ca\(^{2+}\), ATP, and KCl on [\(^{3}H\]ABRY binding in triad membranes shows a similar modulation of binding to that seen in these membranes with [\(^{3}H\]ryanodine. Photoaffinity labeling of triads with [\(^{3}H\]ABRY resulted in specific and covalent incorporation of [\(^{3}H\]ABRY into a 655-kDa protein that was shown to be the skeletal muscle ryanodine receptor. Digestion of the labeled ryanodine receptor revealed a [\(^{3}H\]ABRY-labeled 76-kDa tryptic fragment that was identified with an antibody directed against the COOH-terminal of the receptor. These results demonstrate that the 76-kDa COOH-terminal tryptic fragment contains the high affinity binding site for ryanodine.

The ryanodine receptor/Ca\(^{2+}\) release channel has been extensively studied in both skeletal and cardiac muscles, where it plays a central role in the regulation of the intracellular free Ca\(^{2+}\) concentration, which controls contraction. Ryanodine receptors have also been characterized in neuronal tissue, where they are also thought to perform an important role in regulating Ca\(^{2+}\) release from caffeine-sensitive Ca\(^{2+}\) stores. With the use of [\(^{3}H\]ryanodine binding assays, all three Ca\(^{2+}\) release channels have been purified and shown to be very large homotetramers with a monomer subunit molecular mass of ~655 kDa (1-6). Upon reconstitution in planar lipid bilayers, these receptors form large conductance cation channels sensitive to nanomolar concentrations of ryanodine. The primary structures of several ryanodine receptors have been determined from a number of species and tissues (7-12), and the expression of ryanodine receptor cDNA in Chinese hamster ovary or COS-1 cells has generated high molecular mass receptors for [\(^{3}H\]ryanodine (13, 14). Recently, a sulfhydryl-gated 106-kDa protein has been identified in the sarcoplasmic reticulum of skeletal muscle (15). Purification and reconstitution experiments in planar lipid bilayers suggest that this 106-kDa protein may also be a Ca\(^{2+}\) release channel sensitive to ryanodine (16).

Biochemically, ryanodine receptors/Ca\(^{2+}\) release channels have been shown to be modulated by a number of compounds including ryanodine (17-21). Other agents such as Ca\(^{2+}\), ATP, Mg\(^{2+}\), ruthenium red, caffeine, and calmodulin, for example, have been demonstrated to directly regulate the ryanodine receptor (22-26). The determination of the primary structure of the ryanodine receptor has led to further biochemical characterizations of both the ATP- and Ca\(^{2+}\)-binding sites on the skeletal ryanodine receptor (27, 28). However, little is known about the location of the ryanodine-binding site(s).

Photoaffinity labeling is a powerful tool for studying ligand-binding sites on receptors. To obtain information about the binding site of ryanodine on the calcium release channel, we have produced a novel tritium-labeled azido derivative of ryanodine that binds the ryanodine receptor with high affinity and is localized to a 76-kDa COOH-terminal tryptic fragment of the receptor.

** EXPERIMENTAL PROCEDURES

Membrane Preparation—Triads from rabbit skeletal muscle, canine cardiac sarcoplasmic reticulum vesicles, and crude rabbit brain membranes were purified as previously described (29, 30, 31).

Materials—The azido derivative of ryanodine, 10-\(\text{O}[3-(4-azidobenzenamido)propionyl]\)ryanodine (ABRY), was prepared as previously described (32). A tritiated analogue ([\(^{3}H\]ABRY) was made by Amersham International by the use of tritiated 4-azidobenzoic acid at the last synthetic step. The specific activity of [\(^{3}H\]ABRY was 45 Ci/mmol, and the radiochemical purity was >95% as determined by thin-layer chromatography on silica gel and reverse-phase chromatography. ([\(^{3}H\]Ryanodine was from DuPont NEN. Horseradish peroxidase-conjugated secondary antibodies were from Boehringer Mannheim. All other chemicals were reagent-grade.

[\(^{3}H\]ABRY Binding Assay—Triad membranes (50 μg) were incubated with 0.5-50 nm [\(^{3}H\]ABRY for 1 h at 37 °C in the presence or absence of 10 μM ABRY in 250 μL of 10 mM sodium HEPES, pH 7.4, containing 0.5 mM KCl, 10 mM ATP, and 0.8 μM CaCl\(_{2}\) (50 μM free Ca\(^{2+}\)). Proteins were then collected on Whatman GF/B filters using a Brandel Cell Harvester. For specific experiments, the concentrations of ATP, Ca\(^{2+}\), and KCl were varied individually while keeping all other variables constant. The concentration of free divalent cations was determined using the method of Fabiato (33).

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¶ The abbreviations used are: ABRY, 10-\(\text{O}[3-(4-azidobenzenamido)propionyl]\)ryanodine; PAGE, polyacrylamide gel electrophoresis.
SDS-PAGE and Immunoblot Analysis—Proteins were analyzed by SDS-PAGE (3–12% gradient gels) using the buffer system of Laemmli (34). Gels were stained with Coomassie Blue or were transferred to nitrocellulose for immunoblot analysis as previously described (35). Specific polyclonal antibodies against the COOH-terminal 15-amino acid peptide of the skeletal ryanodine receptor (Rabbit 46) (5) were affinity-purified from Immobilon-P transfer strips of the cardiac ryanodine receptor or the COOH-terminal 15-amino acid peptide conjugated to bovine serum albumin. Both of these affinity-purified antibodies gave the same results on immunoblots. Gels used for fluorography were first stained with Coomassie Blue, destained, soaked in Enlighting (DuPont NEN), and dried under vacuum. The dried gels were then exposed to Kodak XAR-5 film with an intensifying screen at ~80 °C.

Covalent Coupling of [3H]ABrY—Skeletal muscle triads (250 μg) were incubated in the dark with 15 nM [3H]ABrY for 1 h at 37 °C in the presence or absence of 10 μM ryanodine in 1 ml of 10 mM sodium HEPES, pH 7.4, containing 0.5 mM KCl, 10 mM ATP, and 0.8 mM CaCl2 (50 μM free Ca2+). The membranes were then pelleted at 400,000 g for 15 min and resuspended in 1 ml of binding buffer with 5 mM glutathione. The samples were placed in a plastic Petri dish at 4 °C and exposed to UV light (365 nm) at a distance of 1 cm for 45 min. The membranes were then centrifuged in a microcentrifuge at maximum speed for 15 min at 4 °C. The pellets were resuspended in Laemmli sample buffer (34), and the proteins were separated by SDS-PAGE. Following electrophoresis, the gels were stained with Coomassie Blue and were processed for fluorography.

Proteolytic Digestion—Skeletal muscle triads (250 μg) were prelabeled with [3H]ABrY as described above. Membranes were then resuspended (2 mg/ml) in 50 mM ammonium bicarbonate and incubated with trypsin (1:1000 enzyme/membrane (w/w)) for 1, 10, and 15 min at room temperature. In control experiments, trypsin was omitted. Reactions were terminated by the addition of Laemmli sample buffer (34), followed by boiling for 2 min.

RESULTS AND DISCUSSION

The tritium-labeled, photoactivable ryanodine analogue ([3H]ABrY) used in this work is shown in Fig. 1A. This compound has the same structure as ryanodine, except for the addition of an azidobenzamidopropionyl group at position 10. The azido group (Fig. 1A, N3) provides it with the capability to be covalently cross-linked to proteins. [3H]ABrY also contains two tritium atoms (T) on the azidobenzene ring at positions 3 and 5, which allows detection of the specific binding of this compound to various membranes.

To characterize the binding of [3H]ABrY to the skeletal muscle ryanodine receptor, Scatchard analysis was performed on skeletal muscle triads. [3H]ABrY (0.5–50 nM) bound to skeletal muscle triads in a saturable manner (Fig. 1B). Non-specific binding was determined in the presence of 10 μM unlabeled ABrY. The values for Kd and Bmax for triads (determined from three separate experiments) were 5.5 nM and 12.7 pmol/mg, respectively. These data demonstrate that [3H]ABrY binds with high affinity to a single class of receptors in triad membranes. These Kd and Bmax values for [3H]ABrY are very similar to the values obtained for [3H]ryanodine binding in skeletal muscle triads (1).

At least three different ryanodine receptor genes are expressed in skeletal, cardiac, and brain tissues (Refs. 7, 9, and 11; reviewed in Ref. 36). Ryanodine is known to bind specifically with high affinity to the skeletal and cardiac Ca2+ release channels. This compound also binds with high affinity to the major brain form of the ryanodine receptor, which is the cardiac isoform (26, 31). To determine if [3H]ABrY bound to the same site on the Ca2+ release channel in skeletal, cardiac, and brain membranes as ryanodine, competition binding experiments were performed with [3H]ryanodine in the presence of increasing concentrations of ABrY. Fig. 2 shows that unlabeled ABrY specifically inhibited [3H]ryanodine binding to triads, with a half-maximal inhibition at ~37 nM. The half-maximal inhibition concentrations for [3H]ryanodine binding to cardiac and brain membranes were 89 and 25 nM, respectively (Fig. 2).

The competition of [3H]ryanodine binding to skeletal, cardiac, and brain membranes with unlabeled ABrY. Skeletal muscle triads (10 μg; ●), total cardiac microsomes (200 μg; ●), or crude brain microsomes (500 μg; ●) were incubated with increasing concentrations of unlabeled ABrY in the presence of 1 nM [3H]ryanodine for 1 h at 37 °C as described under "Experimental Procedures." 100% binding is defined by the amount of [3H]ryanodine specifically bound in the presence of the lowest concentration of unlabeled ABrY. All experiments were performed in triplicate, and a representative example of three separate experiments is shown.

These values are in the same range as the half-maximal inhibition concentration for [3H]ryanodine binding to triads by ryanodine (~20.0 nM), demonstrating that ABrY and ryanodine bind to the same sites on each receptor.

A number of compounds have been shown to modulate [3H]ryanodine binding to the Ca2+ release channel. Increasing concentrations of both KCl and ATP dramatically stimulate [3H]ryanodine binding to all forms of the ryanodine receptor.

FIG. 1. Structural comparison between ryanodine and azido derivative of ryanodine (ABrY) and Scatchard analysis of [3H]ABrY binding to skeletal muscle triads. A, the structure of ryanodine (left) is compared with that of ABrY (right). The locations of the tritium atoms on [3H]ABrY are designated as T in the structure of ABrY. B, triad membranes (50 μg) were incubated with various concentrations of [3H]ABrY (0.5–50 nM) for 1 h at 37 °C as described under "Experimental Procedures." Specific binding was determined in the presence of 10 μM unlabeled ABrY. All experiments were performed in triplicate, and a representative example is shown. Scatchard analysis from three separate experiments yielded apparent Kd and Bmax values for triads of 5.5 nM and 12.7 pmol/mg, respectively. B/F, bound/free.

FIG. 2. Competition of [3H]ryanodine binding to skeletal, cardiac, and brain membranes with unlabeled ABrY. Skeletal muscle triads (10 μg; ●), total cardiac microsomes (200 μg; ●), or crude brain microsomes (500 μg; ●) were incubated with increasing concentrations of unlabeled ABrY in the presence of 1 nM [3H]ryanodine for 1 h at 37 °C as described under "Experimental Procedures." 100% binding is defined by the amount of [3H]ryanodine specifically bound in the presence of the lowest concentration of unlabeled ABrY. All experiments were performed in triplicate, and a representative example of three separate experiments is shown.
To further characterize the interaction between ABry and the skeletal muscle receptor, we performed an analysis of [3H]ABry binding to skeletal muscle triads in the presence of KCl and ATP. [3H]ABry binding to skeletal muscle receptor is sensitive to changes in ionic strength as demonstrated by increased binding in the presence of increasing KCl concentrations (Fig. 3A). Binding was maximally stimulated 3-fold at a concentration of 0.5 mM KCl, an effect very similar to that observed for [3H]ryanodine binding to both triads and the purified skeletal muscle receptor (2). [3H]ABry binding to skeletal muscle triads is also sensitive to ATP, which can maximally increase binding at 4.5 mM (Fig. 3B). [3H]Ryanodine binding to the skeletal muscle receptor is also stimulated by millimolar concentrations of adenine nucleotides (37). Such concentrations of ATP stimulate the rate of Ca2+ release from both skeletal and cardiac muscle sarcoplasmic reticulum vesicles (22, 38) and increase the channel activity of both receptors in planar lipid bilayers (3, 22).

Because Ca2+ is an important regulator of Ca2+ release from the ryanodine receptor, we have also examined the effect of Ca2+ concentrations on the binding of [3H]ABry to skeletal muscle triads. Ca2+ increases [3H]ABry binding to the skeletal muscle ryanodine receptor at concentrations between 100 nM and 100 μM (Fig. 3C). The half-maximal stimulation of [3H]ABry binding occurs at ~5 μM. The resultant bell-shaped Ca2+ response curve (Fig. 3C) is very similar to the effect of Ca2+ on [3H]ryanodine binding to skeletal, cardiac, and brain ryanodine receptors (28, 37).

Since [3H]ABry contains a photoactivable azido group, this compound was used to covalently label skeletal muscle triads following equilibrium binding. In the presence of UV light, [3H]ABry covalently binds to the skeletal muscle ryanodine receptor (Fig. 4, right). Covalent incorporation of [3H]ABry into the skeletal muscle ryanodine receptor was abolished with excess unlabeled ryanodine (10 μM) (Fig. 4, right). The identity of the labeled protein as the skeletal muscle ryanodine receptor was confirmed by immunoblot analysis (Fig. 4, center) and by immunoprecipitation with affinity-purified polyclonal antibodies against the ryanodine receptor (data not shown). The smaller band just below the 565-kDa protein (~500 kDa) that was identified by photocoupled [3H]ABry and affinity-purified antibodies against the ryanodine receptor is a previously recognized COOH-terminal proteolytic fragment of the receptor.

Photoactivating experiments performed in the presence of magnesium and ruthenium red, at concentrations known to inhibit ryanodine binding, abolished the [3H]ABry labeling of the skeletal muscle ryanodine receptor (data not shown).

To further define the ryanodine-binding site, tryptic digestion of the [3H]ABry-photolabeled skeletal muscle ryanodine receptor was performed to identify proteolytic fragments containing the ABry-binding site. Fig. 5 demonstrates the appearance of a 76-kDa fragment after tryptic digestion that bound [3H]ABry and was also identified with the affinity-purified COOH-terminal polyclonal antibody against the ryanodine receptor. Furthermore, a-chymotryptic digestion of the [3H]ABry-labeled skeletal muscle ryanodine receptor also showed that the antibody staining pattern of the COOH-terminal ryanodine receptor fragments was nearly identical to the pattern obtained from the fluorogram of [3H]ABry-labeled ryanodine receptor fragments (data not shown). Recently, it has been demonstrated that one of the major tryptic fragments resulting from a partial digestion of the ryanodine receptor is a 76-kDa fragment that contains the carboxyl-terminal portion of
the receptor (39). Our data are consistent with this observation and also demonstrate that the 76-kDa COOH-terminal fragment, which is likely to contain transmembrane domains, is critical for the formation of the high affinity ryanoidine-binding site. Since a known modulator of ryanoidine binding, ATP, has also been demonstrated to bind to a similar 76-kDa fragment of the ryanoidine receptor after partial tryptic digestion (27), it is possible that the same 76-kDa trypic fragment that is critical in forming the high affinity ryanoidine-binding site also contains the ATP-binding site.

Salama et al. (40) have suggested that ryanoidine also interacts with a 106-kDa protein present in the sarcoplasmic reticulum of skeletal muscle. In planar lipid bilayers, ryanoidine was thought to affect this sulphydryl-gated protein at concentrations (nanomolar) that would suggest a high affinity interaction between ryanoidine and the 106-kDa protein (16). While this protein may be involved in the function of the sarcoplasmic reticulum, our data demonstrate that ABRy, which shares high structural homology with ryanoidine, is capable of binding only to the 565-kDa ryanoidine receptor/Ca$^{2+}$ release channel.

The results presented here indicate that the tritiated photoactivatable derivative of ryanoidine, ABRy, interacts directly and specifically with the active ryanoidine-binding site on the Ca$^{2+}$ release channel. Our results also demonstrate that the binding of [3H]ABRy to the skeletal muscle ryanoidine receptor is allosterically regulated by KCl, ATP, and Ca$^{2+}$, compounds known to directly affect Ca$^{2+}$ release from the ryanoidine receptor. The identification of the 76-kDa COOH-terminal tryptic fragment of the skeletal muscle ryanoidine receptor covalently binding [3H]ABRy illustrates that this region of the Ca$^{2+}$ release channel is critical in the formation of the high affinity ryanoidine-binding site. Localization of the high affinity ryanoidine-binding site will provide new information on the structure and functional relationship between ryanoidine and the Ca$^{2+}$ release channel. This information should be useful in determining if this binding site is conserved among the different ryanoidine receptor gene products. The identification of the ryanoidine-binding site could also provide information on how ryanoidine and other modulators of the Ca$^{2+}$ release channel affect channel function and could possibly help to determine the structure of the Ca$^{2+}$ channel pore.

REFERENCES


