Target Size of the Ryanodine Receptor from Junctional Terminal Cisternae of Sarcoplasmic Reticulum†

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ABSTRACT: Target inactivation analysis was carried out on the ryanodine receptor. This receptor recently has been implicated as the channel involved in the calcium release process in excitation–contraction coupling and was localized to the junctional terminal cisternae of sarcoplasmic reticulum from skeletal muscle [Fleischer, S., Ogunbunmi, E. M., Dixon, M. C., & Fleer, E. A. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7256–7259]. Irradiation of the junctional terminal cisternae resulted in an exponential decrease in ryanodine binding with radiation dose, thereby consistent with target theory. The target molecular weight was found to be 138 000 ± 21 000, i.e., smaller than the polypeptide that binds ryanodine. The calcium pump protein in the same membrane preparation served as an internal control to validate the methodology.

Muscle contraction in skeletal muscle is triggered by the rapid release of stored calcium from the sarcoplasmic reticulum compartment. However, little is known about the molecular detail of the calcium release process [see review by Martonosi (1984)]. This release has been postulated to occur through specific calcium channels located in the junctional terminal cisternae of sarcoplasmic reticulum [see, for example, Fleischer (1985) and Ikemoto and Kim (1985)]. Two recent advances have made possible a more direct approach to identify the calcium release channels in sarcoplasmic reticulum. The first is the isolation of junctional terminal cisternae of sarcoplasmic reticulum (Saito et al., 1984). This fraction consists of two types of membranes, the calcium pump membrane and the junctional face membrane. The latter comprises ~20% of the membrane and contains well-defined feet structures. The feet, in situ, are associated with transverse tubules to form the triad junction (Franzini-Armstrong, 1983). In skeletal muscle, the signal for calcium release traverses this junction. The other ~80% of the membrane is the calcium pump membrane, which is involved in energized calcium uptake enabling muscle to relax. The longitudinal tubules (light sarcoplasmic reticulum) contain only the calcium pump membrane (Saito et al., 1984). The second advance is the finding that ryanodine at low concentrations (apparent K_I ~50 nM) acts pharmacologically on junctional terminal cisternae, appearing to lock the calcium release channels in the "open state" (Fleischer et al., 1985). Furthermore, tritiated ryanodine specifically binds to terminal cisternae vesicles with a K_D similar to the K_I (Fleischer et al., 1985; Pessah et al., 1985). By contrast, ryanodine does not bind to or affect the calcium translocating process of longitudinal tubules of sarcoplasmic reticulum (Fleischer et al., 1985). The identity of a specific ligand, tritiated ryanodine, opens the door to the isolation and characterization of the calcium release channel. In this study, target inactivation analysis is used to investigate the size of the ryanodine receptor associated with the calcium release channel localized in the junctional terminal cisternae of sarcoplasmic reticulum.

Experimental Procedures

All chemicals were of reagent grade or the best available and were prepared in deionized water. Protein was measured according to the method of Lowry et al. (1951) using bovine plasma albumin as the standard. Junctional terminal cisternae from rabbit skeletal muscle were prepared as described by Saito et al. (1984). Ryanodine was obtained from the Penick Corp. (Lyndhurst, NJ).

Irradiation Procedure. The junctional terminal cisternae vesicles were diluted to 1 mg of protein/mL in 0.3 M sucrose and 5 mM imidazole, pH 7.4. The suspension was then plated out in 1- or 1.35-mL aliquots in open aluminum trays and frozen immediately with liquid nitrogen as previously described (McIntyre et al., 1983). The freezing time was approximately 10–15 s per tray. The samples were then shipped to Buffalo in a portable liquid nitrogen refrigerator where they were irradiated under anaerobic conditions at a temperature of −40 to −50 °C in a Van de Graaff generator. The generator produced a 0.5- or 0.6-mA beam of 1.5-MeV electrons. The irradiation dose for each sample was controlled by varying the number of passes through the electron beam. The total dosage was varied between 0 and 8.16 Mrad. Sham-irradiated controls were placed in the irradiation chamber under identical conditions but with the electron beam off. These samples showed no change in activity when compared with samples that were retained in the laboratory. The radiation dose was measured at the sample irradiation temperature with the transmittance change of blue cellophane calibrated against the Fricke dosimeter. The samples were maintained at liquid nitrogen temperature until ready for analysis, then they were thawed rapidly as previously described (McIntyre et al., 1983), and the ryanodine binding assay was immediately performed.

Ryanodine Binding. Tritiated ryanodine, radiolabeled as previously described (Fleischer et al., 1985), was diluted with carrier to approximately 8000 cpm/pmol. Scatchard plots of

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ryanodine binding to junctional terminal cisternae were performed, and the dissociation constant (K_D) and maximum number of binding sites (B_{max}) for the ryanodine receptor were determined by analyzing the data with the program LIGAND (Munson & Rodbard, 1980). Specific binding was subsequently measured in the irradiated samples and controls by using one of two near-saturating concentrations of ryanodine. For measurement of ryanodine binding (Fleischer et al., 1985), the assay in 1 mL contained the binding buffer (1 M KCl, 10 mM Hepes,[1] pH 7.4; modification by Dr. Makaoto Inui of our laboratory), 0.1 mg of junctional terminal cisternae protein, and [^{3}H]ryanodine at either 90 or 270 nM concentrations. Nonspecific binding was measured by including a 100-fold excess of unlabeled ryanodine in the binding medium. In general, nonspecific binding was 10% or less of the total binding. Specific binding was determined for each radiation point at both [^{3}H]ryanodine concentrations by subtracting the nonspecific from the total binding. Each total binding point was determined in triplicate and each nonspecific binding point in duplicate. The samples, after being mixed, were incubated at room temperature for 30 min. Following incubation, samples were filtered in an Amicon filtration apparatus at reduced pressure equivalent to 25 mmHg with 0.22-μm filters (GSWP, Millipore Corp., Bedford, MA), which had been presoaked for 30 min in the binding buffer. The filtered samples were then washed 2 times with 2-mL aliquots of the binding buffer and 4 times with 2-mL aliquots of 10% ethanol in water. After being washed, the filters were collected and placed in 12 mL of ACS scintillation fluid (Amersham Corp., Arlington Heights, IL). Radioactivity was measured to 0.5% of σ accuracy in a Searle Analytic 81 scintillation counter with quench correction. Counting efficiency was approximately 55%.

Scatchard analysis of ryanodine binding was carried out for control and selected radiation dose samples. For such analysis, the ryanodine concentration was varied from 30 to 240 nM; the assay for specific binding was otherwise as described above. Each total binding point was measured in duplicate, and each nonspecific point was a single determination.

The target size of the calcium pump protein in junctional terminal cisternae was also measured and served as an internal standard in these samples. Aliquots from each irradiated sample and sham control were solubilized and separated by SDS–PAGE according to the method of Laemmli (1970). The amount of calcium pump protein (M_r ~110 000) was quantitated by densitometry after being stained with Coomassie blue as previously described (McIntyre et al., 1983; Hymel et al., 1984). The gels were scanned at 633 nm with an LKB Ultrascan XL densitometer. The major band referable to the calcium pump protein was cut out from the scan, and the area was determined by weighing. The amount of calcium pump protein remaining was determined in duplicate for each irradiation dose.

Data Presentation and Calculation. The data were analyzed with a single-target, single-hit model of radiation inactivation (Pollard, 1953). Plots of the logarithm of the percentage of surviving activity (ryanodine binding or polypeptide remaining) were linear over 1 order of magnitude with correlation coefficients of ≥0.96. Apparent molecular weights were calculated by using the formula: molecular weight (M_r) = $(6.4 \times 10^{11})/D_{37}$ (rad), where D_{37} is the dose of absorbed radiation required to reduce the activity to 37% of the original (Kepner & Macey, 1968). The data, with 90 and 270 nM ryanodine, were compared by using a paired, two-tailed Student's t test.

[1] Abbreviations: SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Da, dalton.

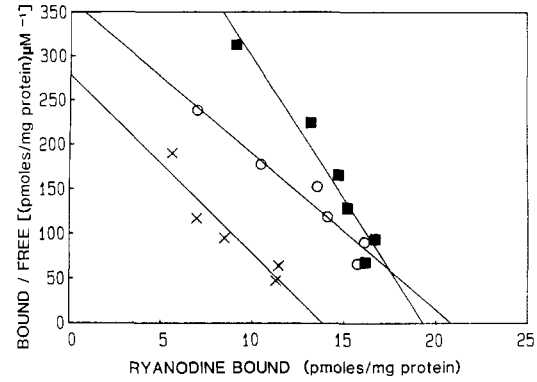

FIGURE 1: Scatchard analysis of ryanodine binding to control and irradiated junctional terminal cisternae. The same preparation was subjected to zero radiation (○), 1.15 Mrad (■), or 4.13 Mrad (×). Specific binding of ryanodine was calculated as described under Experimental Procedures. Correlation coefficients of the straight-line Scatchard plots were −0.97 or better. The results for the zero radiation plot represent results from two separate experiments with the mean of each point displayed. The results for 1.15- and 4.13-Mrad radiation doses are from one experiment each. The K_D (dissociation constant) and B_{max} (maximum number of ryanodine binding sites) are tabulated in Table I.

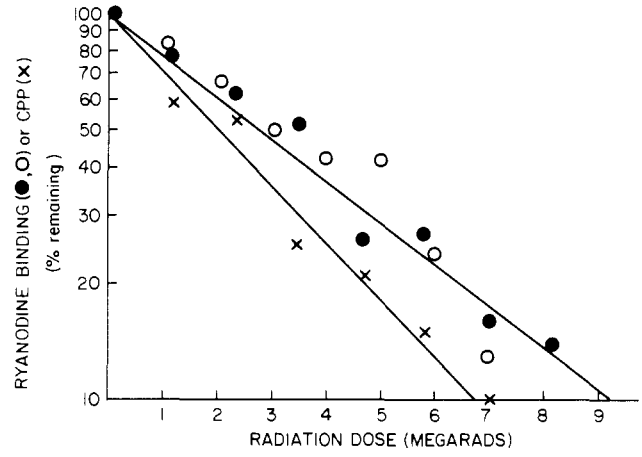

FIGURE 2: Semilog plot of ryanodine binding as a function of irradiation dose. The junctional terminal cisternae were irradiated in the frozen state at different doses, and ryanodine binding was carried out as described under Experimental Procedures. The 100% values for ryanodine binding, obtained from the sham-irradiated control (electron beam off), were the same as those for each sample prior to dilution and preparation for irradiation. For different membrane preparations, the maximum ryanodine binding ranged between 14 and 24 pmol/mg of protein. Data are shown for binding of both 90 (○) and 270 nM (●) ryanodine. The linear regression line for the 270 nM experiment is drawn. The data for the 90 nM experiment closely approximated this line. The slope and D_{37} of the inactivation profile were determined by linear regression (correlation coefficients ≥0.97), and the target molecular weights, calculated by the established relationship (Kepner & Macey, 1968) $M_r = (6.4 \times 10^{11})/D_{37}$, were 153 000 and 157 000 for 90 and 270 nM ryanodine, respectively. D_{37} is the amount of radiation in rads required to reduce activity to 37% of the original. The target molecular weight for the calcium pump protein (×) in the same preparation, as determined by densitometry of the irradiated samples separated by SDS–PAGE, was 222 000 and served as an internal control. For these data, the D_{37} values for the ryanodine receptor and for the calcium pump protein were 4.08 and 2.88 Mrad, respectively. The data from 10 experiments are summarized in Table II.

RESULTS

Binding isotherms were performed on nonirradiated control samples and two irradiated samples. Scatchard analysis of

Table I: Ryanodine Binding Characteristics in Normal and Irradiated Junctional Terminal Cisternae[a]

radiation dose (Mrad)	K_D (nM)	B_{max} (pmol/mg of protein)
0	59 ± 11	19.8 ± 1.7
1.15	36 ± 17	20.9 ± 3.2
4.13	53 ± 21	14.7 ± 2.5

[a] The results are tabulated from the data in Figure 1. The data were analyzed by the nonlinear method of Munson and Rodbard (1980), utilizing the program LIGAND to obtain K_D and B_{max} values and their standard errors. Comparable values for K_D and B_{max} were obtained by using linear regression of the straight-line Scatchard plots (see Figure 1). The correlation coefficients determined by linear regression were typically −0.97 for 5–12 points each.

Table II: Target Molecular Weight of Ryanodine Binding Receptor[a]

component	M_r
(A) ryanodine receptor	
(1) binding 90 nM ryanodine	151 000 ± 15 000 (3)
(2) binding 270 nM ryanodine	142 000 ± 22 000 (3)
(3) av of all values with both 90 and 270 nM ryanodine	138 000 ± 21 000 (10)
(B) calcium pump protein by densitometry of SDS–PAGE	258 000 ± 42 000 (10)

[a] Ryanodine binding was measured as a function of radiation dosage with 90 and 270 nM [^{3}H]ryanodine concentrations for each preparation of junctional terminal cisternae (A1 and A2). The target size was determined in each experiment for both ryanodine concentrations with the formula $M_r = (6.4 \times 10^{11})/D_{37}$. D_{37} values were calculated by using linear regression analysis of the inactivation plot of log activity vs. irradiation dose (see Figure 2). In (A3) the combined result for the experiments in (A1) and (A2) plus four additional studies using only 270 nM ryanodine is presented. Correlation coefficients for the inactivation curves varied between −0.96 and −0.99. Data are tabulated as the mean molecular weight and standard deviation, with the number of experiments given in parentheses. The target size of the calcium pump protein for each sample was also determined by densitometry (see Figure 2 legend).

ryanodine binding data indicates a single high-affinity binding site with a dissociation constant (K_D) of approximately 50 nM (Table I). Irradiation decreased the number of binding sites (B_{max}) (Figures 1 and 2) and within experimental error did not alter the K_D (Table I).

Ryanodine binding was initially measured in paired irradiated samples with 90 and 270 nM [^{3}H]ryanodine. Semilog plots of inactivation of ryanodine binding as a function of irradiation dose were linear for measurements made with either concentration (Figure 2). Mean target molecular weights, determined by using either of the two different concentrations of [^{3}H]ryanodine, were experimentally the same (Table II); i.e., at 90 nM [^{3}H]ryanodine, the target molecular weight was 151 000 ± 15 000, and at 270 nM [^{3}H]ryanodine, the target molecular weight was 142 000 ± 22 000 ($p > 0.2$). In the subsequent experiments, ryanodine binding was measured as a function of radiation dose with 270 nM [^{3}H]ryanodine. The combined results from 10 experiments averaged 138 000 ± 21 000 Da for the target size of the ryanodine receptor (Table II).

The calcium pump protein in the junctional terminal cisternae served as an internal standard for our experimental conditions. The target size obtained by measuring the amount of polypeptide remaining after irradiation was 258 000 ± 42 000. This is in agreement with previous target inactivation studies for the calcium pump protein of 213 000 ± 31 000 (Hymel et al., 1984), in which the calcium pump protein in the membrane was found to be a dimer. Each of the individual calcium pump protein molecular weight determinations was within one standard deviation of this mean and is within expected range of variability for target inactivation assays. Therefore, there was no need to correct the molecular weight of the ryanodine receptor on the basis of the internal standard.

Discussion

This study investigates the size of the ryanodine receptor that previously has been localized to the junctional terminal cisternae of sarcoplasmic reticulum (Fleischer et al., 1985). The target molecular weight was found to be 138 000 ± 21 000.

There is now good reason to believe that the ryanodine receptor is associated with the calcium release channel and mediates calcium release in excitation–contraction coupling in muscle. Ryanodine, a neutral alkaloid, has been known for some time to be toxic in low doses (~20–300 μg/kg) to both vertebrates and invertebrates and leads to irreversible skeletal muscle contractures and death (Jenden & Fairhurst, 1969; Sutko et al., 1985). The earlier studies with ryanodine on subcellular muscle membrane fractions used concentrations in the range of 100 μM or greater (Jones et al., 1979; Chamberlain et al., 1984; Feher & Lipford, 1985), which is far in excess of the pharmacologically significant range (nanomolar). Recently, we described a junctional terminal cisternae preparation from skeletal muscle with poor calcium pumping activity (Saito et al., 1984), which could be increased by the addition of ruthenium red or Mg^{2+}; the calcium loading rate, normalized for calcium pump protein, then became comparable to that of light sarcoplasmic reticulum (Saito et al., 1984; Chu et al., 1986). The working hypothesis was that the leak was due to calcium release channels that could be closed by ruthenium red or Mg^{2+} [see Ohnshi (1979) and Miyamoto and Racker (1982)]. We then found that pretreatment of junctional terminal cisternae with ryanodine in the nanomolar concentration range blocked the action of ruthenium red on calcium loading. Furthermore, ryanodine binding could be measured in this pharmacologically significant range and was localized to junctional terminal cisternae in contrast to the longitudinal cisternae of sarcoplasmic reticulum (Fleischer et al., 1985). Those studies buttress the interpretation that a calcium release channel is involved; ruthenium red closes the calcium release channels, and pretreatment with ryanodine locks them in the open state (Fleischer et al., 1985). Recent studies by Meissner (1986) also showed that ryanodine in the nanomolar concentration range modulates the calcium permeability of a "heavy" sarcoplasmic reticulum fraction. The calcium release channel was further characterized in that study by fusing the vesicles into a planar lipid bilayer and obtaining single-channel recordings. The probability of the single channel to remain open increased by adding calcium ions to the same side (cis) as vesicle insertion and was inhibited by cis Mg^{2+}. The channels are also nucleotide activated (Smith et al., 1985, 1986).

Accurate determination of molecular weight by target analysis requires fulfillment of several criteria. First, the assay must be specific for the receptor being measured. The ryanodine binding assay used in this study appears to be specific for the calcium release channels of junctional terminal cisternae (Fleischer et al., 1985). Second, a single-hit model must pertain. Third, the damaged target should result in complete loss of the measured activity. Two findings validate premises 2 and 3 for our study: (1) ryanodine binding exhibits an exponential decay with radiation dose yielding a single molecular weight inactivation profile to 10% of residual activity; (2) within experimental error, the dissociation constant for the ryanodine binding site was found to be essentially unchanged, whereas B_{max} is lowered with increasing radiation dose. The calcium pump protein, which served as an internal standard of the radiation inactivation analysis methodology, yielded

accurate molecular weight predictions, further validating our techniques.

Several ionic channels from excitable membranes have been isolated and characterized (Hille, 1984; Talvenheimo, 1985). These include the acetylcholine receptor channel from several sources (Karlin et al., 1983; Montal et al., 1986), the sodium channel from several sources (Barhanin, 1983; Hartshorne & Catterall, 1984), and the calcium channel from the transverse tubule of skeletal muscle (Borsotto et al., 1984; Curtis & Catterall, 1984). Each of these channels is composed of three or more different polypeptides. The acetylcholine receptor from torpedo electric tissue consists of four types of polypeptides [64, 58, 48, and $(39)_2$ kDa] (Karlin et al., 1983). The sodium channel from rat brain and skeletal muscle consists of three different subunits (260, 39, and 37 kDa) (Hartshorne & Catterall, 1984). However, only the 260-kDa polypeptide appears to be present in the receptors from electric eel and chick cardiac muscle [see review by Talvenheimo et al. (1983)]. The calcium channel from transverse tubule consists of three subuntis [160 (or 130 under reducing conditions), 50, and 33 kDa (Curtis & Catterall, 1984) or 142, 33, and 32 kDa (Borsotto et al., 1984)].[2] The key to the isolation of these channels was to find a specific ligand, e.g., α-bungarotoxin for the acetylcholine receptor, tetrodotoxin or saxitoxin for the sodium channel, and the dihydropyridines for the calcium channel from transverse tubule. For each, the radiolabeled specific ligand also made possible studies to obtain a molecular weight using target inactivation analysis. The sodium channel from brain and electric eel, measuring tetrodotoxin binding, gave a target molecular weight of 224 000–260 000, approximately the size of the α-subunit (Levinson & Ellory, 1972; Barhanin, 1983; Angelides et al., 1985). The target molecular weight for the acetylcholine receptor was 300 000, with α-bungartotoxin as the ligand (Lo et al., 1982). Target analysis of the calcium channel from brain and transverse tubule of skeletal muscle using nitrendipine binding reflects the molecular weight of the sum of the three different subunits (Norman et al., 1983; Ferry et al., 1983). Direct photoaffinity labeling of the nitrendipine receptor in a cardiac sarcoplasmic reticulum membrane fraction yielded 32 kDa, comparable in size to the smallest subunit of the calcium channel of transverse tubule (Campbell et al., 1984).

The target molecular weight that is measured can reflect the entire mass of the oligomeric structure or any subunit or combination thereof. Different specific ligands are known to bind to different polypeptides in the same complex and/or more than one polypeptide of the complex and thereby reflect the target size of the polypeptide(s), which is essential for the measured function [see Kempner and Fleischer (1988)]. The target size can be less than the oligomeric structure when the measured characteristic is localized to a single polypeptide [see, for example, Goll et al. (1984)]. An additional factor relevant to the target molecular weight obtained is whether the energy from a hit is transferred from one polypeptide in the complex to another. When this occurs, the oligomeric size of the complex is obtained [McIntyre et al., 1983; Hymel et al., 1984; see Kempner and Fleischer (1988)].

The ryanodine receptor has recently been isolated in our laboratory. It contains three polypeptide subunits, 360, 330, and 175 kDa, and, when reconstituted, forms the feet structures that are involved in junctional association of transverse tubule and terminal cisternae of SR (Inui et al., 1987a). The target size for ryanodine binding obtained here approximates that of or is smaller than the smallest polypeptide. We now find that, when the ryanodine receptor is purified in the presence of protease inhibitors, the amount of the minor bands (330 and 175 kDa) becomes vanishingly small (Inui et al., 1987b). A recent abstract reports ryanodine binding to a purified receptor containing the 360-kDa component (Imagawa et al., 1987). These studies taken together indicate that the target size for ryanodine binding reflects a domain of the high molecular weight component. This study of the ryanodine receptor is an example of three reported cases in which the target size represents a portion of the functional structure and only a domain of the active polypeptide. Target sizes referable to a protein domain, i.e., a portion of the polypeptide, have been reported previously for nitrate reductase and DNA polymerase I (Solomonson et al., 1986) and for binding of inhibitor to the anion channel in erythrocytes (Verkman et al., 1986). In this context, the target size using ryanodine binding represents a minimum molecular weight.

It should be noted that the calcium release channel from the junctional terminal cisternae of sarcoplasmic reticulum is distinct from that of the calcium channel in the transverse tubule or plasma membrane in terms of sensitivity to specific ligands. The former is modulated by ryanodine, ruthenium red, Mg^{2+}, Ca^{2+}, and nucleotides (Ohnishi, 1979; Miyamoto & Racker, 1982; Fleischer et al., 1985; Smith et al., 1985, 1986; Meissner, 1986), whereas the latter is sensitive to dihydropyridines, diltiazem, and verapamil (Ferry et al., 1983; Gengo et al., 1983). It is now clear that the subunit composition is also distinct.

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[2] Added in proof: Recent studies revise the subunit composition of the nitrendipine receptor. It consists of four major polypeptides of 165, 155, 65, and 32 kDa as revealed by nonreducing SDS–PAGE (Glossmann et al., 1987).

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