The Ca^{2+}-release Channel/Ryanodine Receptor Is Localized in Junctional and Corbular Sarcoplasmic Reticulum in Cardiac Muscle

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Abstract. The subcellular distribution of the Ca^{2+}-release channel/ryanodine receptor in adult rat papillary myofibers has been determined by immunofluorescence and immunoelectron microscopical studies using affinity purified antibodies against the ryanodine receptor. The receptor is confined to the sarcoplasmic reticulum (SR) where it is localized to interior and peripheral junctional SR and the corbular SR, but it is absent from the network SR where the SR-Ca^{2+}-ATPase and phospholamban are densely distributed. Immunofluorescence labeling of sheep Purkinje fibers show that the ryanodine receptor is confined to discrete foci while the SR-Ca^{2+}-ATPase is distributed in a continuous network-like structure present at the periphery as well as throughout interior regions of these myofibers. Because Purkinje fibers lack T-tubules, these results indicate that the ryanodine receptor is localized not only to the peripheral junctional SR but also to corbular SR densely distributed in interfibrillar spaces of the I-band regions. We have previously identified both corbular SR and junctional SR in cardiac muscle as potential Ca^{2+}-storage/Ca^{2+}-release sites by demonstrating that the Ca^{2+} binding protein calsequestrin and calcium are very densely distributed in these two specialized domains of cardiac SR in situ. The results presented here provide strong evidence in support of the hypothesis that corbular SR is indeed a site of Ca^{2+}-induced Ca^{2+} release via the ryanodine receptor during excitation contraction coupling in cardiac muscle. Furthermore, these results indicate that the function of the cardiac Ca^{2+}-release channel/ryanodine receptor is not confined to junctional complexes between SR and the sarcolemma.

WHILE depolarization of the sarcolemma and T-tubules in skeletal muscle is sufficient to elicit the required Ca^{2+}-release from the sarcoplasmic reticulum (SR)[1] for muscle contraction (Huxley, 1971; Schneider and Chandler, 1973), depolarization of cardiac sarcolemma and T-tubules does not elicit a Ca^{2+}-release from cardiac SR unless extracellular Ca^{2+} enters the cytosol, mainly via the slow voltage dependent Ca^{2+} channel (Reuter, 1984; Tsien, 1983). This influx of Ca^{2+} directly or indirectly induces a release of Ca^{2+} from the SR as proposed in the Ca^{2+}-induced Ca^{2+} release hypothesis (Fabiato, 1983, 1985).

Previous ultrastructural studies have demonstrated that SR in mammalian cardiac myofibers contains at least three distinct but continuous regions, namely the network SR, the interior, and peripheral junctional SR, and the corbular SR (Sommer and Johnson, 1979; Forbes and Sperelakis, 1983; Segretain et al., 1981). The network SR consists of 25–60 nm diam sarcotubules organized in an anastomosing network that surrounds the myofibrils fairly uniformly along the entire length of the sarcomere. The junctional and corbular SR are structurally specialized domains extending from the network SR and contain electron-dense material in their lumina. They are most densely distributed in the interfibrillar spaces neighboring the central region of the I-band. The prominent structural difference between these two regions of cardiac SR is that junctional SR is physically connected to either T-tubules or to sarcolemma via 'feet' structures, whereas corbular SR is not. However, it has been reported that electron dense structures similar to 'feet' structures project from the surface of corbular SR into the cytoplasm (Sommer and Johnson, 1979). Immunoelectron microscopical studies demonstrated that the SR-Ca^{2+}-ATPase (Jorgensen et al., 1982) and its regulator phospholamban (Jorgensen and Jones, 1987) are uniformly distributed in the network SR while calsequestrin is present in the lumen of junctional SR and corbular SR (Jorgensen and Campbell, 1984; Jorgensen et al., 1984, 1985, 1988). Electron microprobe analysis studies showed that the lumen of junctional SR (Jorgensen et al., 1988; Wheeler-Clark and Tormey, 1987) and corbular SR store calcium (Jorgensen et al., 1988).

The studies summarized above support the idea that Ca^{2+} is accumulated into the lumen of the SR across the entire surface of the network SR via the Ca^{2+}-ATPase. Ca^{2+} subse-

1. *Abbreviations used in this paper*: SR, sarcoplasmic reticulum; WGA, wheat germ agglutinin.

quently diffuses to the lumen of junctional and corbular SR where it is sequestered by calsequestrin and stored until the SR receives the next signal to release Ca^{2+}. The question then arises as to whether both junctional SR and corbular SR release Ca^{2+}, and if so, is Ca^{2+} released via the ryanodine receptor or does one of these domains of SR contain a different Ca^{2+} release channel such as the IP_3 receptor (Ferris and Snyder, 1992)?

The clear demonstration that the skeletal ryanodine receptor corresponds to feet structures confined to the junctional face of the terminal cisternae of skeletal SR (Anderson et al., 1989; Block et al., 1988; Smith et al., 1988; Inui et al., 1987; Kawamoto et al., 1986) supports the proposal that the cardiac ryanodine receptor is also associated with the junctional SR (Inui et al., 1988). On the other hand, studies by Fabiato (Fabiato, 1985) have shown that quick elevation of cytosolic Ca^{2+} can induce Ca^{2+} release from the SR of mechanically skinned canine Purkinje fibers. Because skinned Purkinje fibers presumably lack junctional SR, these results are consistent with the idea that a quick increase in cytosolic Ca^{2+} triggers Ca^{2+} release from corbular SR. Nonetheless, they do not exclude the possibility that Ca^{2+} also induces Ca^{2+} release from junctional SR in intact cardiac myofibers.

Considering the striking similarities of the physical characteristics of the tetrameric IP_3 receptor (Maeda et al., 1991) and the tetrameric ryanodine receptor (Saito et al., 1988), it is reasonable to propose that feetlike structures associated with either junctional SR or corbular SR may represent IP_3 receptors. In support of this possibility, several pharmacological studies have shown that IP_3 can trigger elevations of cytosolic free Ca^{2+} and induce contraction in skinned cardiac myofibers (Fabiato, 1990; Kentish et al., 1990; Lochner and Bester, 1989; Nosek et al., 1986; Vites and Pappano, 1990; Vites and Pappano, 1992). Furthermore, recent biochemical studies identified and characterized high and low affinity IP_3 binding sites in isolated canine cardiac SR, where they appear to be most prominent in the subpopulation of cardiac SR proposed to correspond to junctional SR (Kijima and Fleischer, 1992).

To determine directly whether junctional SR and/or corbular SR contain the ryanodine receptor, its subcellular distribution in rat papillary myofibers and sheep Purkinje fibers was determined by immunofluorescence and immunocolloidal gold labeling. The results presented show that the Ca^{2+} release channel/ryanodine receptor is localized to both the junctional SR and corbular SR but is apparently absent from the network SR in rat papillary myofibers and sheep Purkinje fibers. The presence of the ryanodine receptor in corbular SR well removed from the sarcolemma indicates that corbular SR represents a site for Ca^{2+}-induced Ca^{2+} release required for excitation contraction coupling in cardiac muscle.

Materials and Methods

Preparation of Microsomal Membranes

Cardiac microsomal membranes were prepared from rat ventricular muscle as previously described for skeletal muscle membranes (Ohlendieck et al., 1991). Protein concentrations were determined according to the procedure of Lowry (Lowry et al., 1951) as modified by Peterson (Peterson, 1977) using BSA as a standard.

Preparation of Affinity Purified Antibodies to the Ryanodine Receptor

Rabbit antiserum against a COOH-terminal peptide of the skeletal muscle ryanodine receptor (PAGDCFRKQYEDQLS;) (Takeshima et al., 1989) was prepared as described previously (McPherson et al., 1991). This COOH-terminal peptide sequence differs by only a single amino acid from that of the cardiac ryanodine receptor (Otsu et al., 1990). For affinity purification, the COOH-terminal peptide (Robey and Fields, 1989), conjugated to BSA (Parker and Hodges, 1984), was coupled to CNBr-Sepharose according to the manufacturer's instructions. Rabbit serum was diluted 1:4 in TBS (20 mM Tris-HCl, pH 7.4, 200 mM NaCl), and incubated for 16 h at 4°C with 1 ml of the peptide conjugated Sepharose. The Sepharose was loaded on a column, washed 4 × 8 ml with 50 mM Tris-HCl, pH 7.4, containing 500 mM NaCl, and washed 2 × 8 ml with Tris-HCl containing 100 mM NaCl. Antibody was eluted with 4 M $MgCl_2$, and the eluted fractions were analyzed for protein by reading OD at 280 nm. Peak fractions were pooled, dialyzed for 12 h against PBS, and then dialyzed for 12 h against PBS containing 20% sucrose, 1 mM EGTA, and .01% Na-azide.

Immunoblotting

Cardiac microsomal proteins were separated on 3–12% SDS-polyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose paper (Towbin et al., 1979), and immunoblotted with ryanodine receptor antibodies followed by HRP-coupled secondary antibodies as previously described (McPherson and Campbell, 1990).

Immunofluorescence Labeling of Papillary Muscle Cryosections

Small bundles of papillary muscle tissue were dissected from anaesthesized rats and sheep and quickly cryofixed in liquid nitrogen cooled isopentane. Longitudinal and transverse cryostat sections (6–8 μm thick) were cut as previously described (Jorgensen et al., 1982; Jorgensen and Jones, 1987).

Single Labeling. Indirect immunofluorescence labeling of cryostat sections of unfixed rat and sheep papillary muscle was carried out as previously described (Jorgensen and Jones, 1987). Briefly, affinity purified rabbit antibodies to a COOH-terminal peptide of the ryanodine receptor (25 μg/ml) were used as the primary reagent. Affinity purified $F(ab)_2$ fragments of donkey anti-rabbit gamma globulin-conjugated to fluorescein (20 μg/ml; Jackson Immunoresearch Laboratories Inc., Westgrove, PA) were used as the secondary reagent.

Double labeling. Some cryostat sections containing bundles of sheep Purkinje fibers were double labeled with antibodies to the ryanodine receptor and wheat germ agglutinin (WGA) as previously described (McLeod et al., 1991). Briefly, the double labeling procedure composed of the single labeling procedure for the ryanodine receptor followed first by labeling with WGA conjugated to biotin (5 μg/ml; Vector Laboratories, Burlingame, CA) and next by streptavidin conjugated to Texas Red (10 μg/ml; Vector Laboratories, Burlingame, CA).

Cryosections of sheep papillary muscle were double labeled with antibodies to the ryanodine receptor and to the dog cardiac SR CA^{2+}-ATPase. Briefly, the double labeling procedure was composed of the single labeling procedure for the ryanodine receptor followed first by labeling with Mab IID8 to canine cardiac SR Ca^{2+}-ATPase (Jorgensen et al., 1988) and next by affinity purified $F(ab)_2$ fragments of goat antimouse IgG_1 conjugated to Texas Red (20 μg/ml; Sera Laboratories, Copthorne, England).

Controls. For adsorption, 25 μg/ml of affinity purified antibodies to the COOH-terminal peptide of the ryanodine receptor were incubated with 0 and 5 μg/ml of the COOH-terminal peptide, conjugated to rabbit serum albumin (40:1) (Robey and Fields, 1989) as previously described (Jorgensen and Jones, 1987). Thus, the molar ratio of peptide/antibody was ~4 for the adsorption. The supernatants obtained by centrifugation were used as the primary reagent in the indirect immunofluorescence and immunoelectron microscopical labeling procedure.

Imaging. Conventional fluorescence microscopy was carried out with a photomicroscope (Carl Zeiss, Inc. Thornwood, NY) provided with an epifluorescence attachment and a phase-contrast condenser. Confocal microscopy was carried out with a photomicroscope (Nikon, Inc., Garden City, NY) provided with a confocal fluorescence imaging system (Lasersharp MRC Bio-Rad Laboratories Ltd., Richmond, CA) using a krypton argon laser for illumination (White et al., 1987).

tion of the total Ca^{2+} storage and release sites in cardiac myofibers.

The functional significance of the presence of two structurally distinct Ca^{2+}-release sites, both of which presumably release Ca^{2+} via the ryanodine receptor is presently unknown. The structural analogy of cardiac junctional SR and terminal cisternae of skeletal SR has favored junctional SR as the site of Ca^{2+} release relevant to excitation-contraction coupling in cardiac muscle (Fleischer and Inui, 1989; Wier, 1992). The possibility that corbular/extended junctional SR also plays a significant role in this process was originally proposed by Sommer (Jewett et al., 1973; Sommer and Waugh, 1976) on the basis of ultrastructural studies in avian cardiac myofibers. Its proposed potential role has been extended to include mammalian myocardial myofibers on the basis of more recent ultrastructural (Segretain et al., 1981), immunocytochemical (Jorgensen and Campbell, 1984; Jorgensen et al., 1984, 1985, 1988; Jorgensen and McGuffee, 1987) and electronmicroprobe analytical studies (Jorgensen et al., 1988). Regarding the specific role of corbular SR, it is noteworthy that it has not been observed in skeletal muscle. Thus, the presence of corbular SR at variable distances from junctional SR and from the SL may be relevant to features of excitation-contraction coupling in cardiac myofibers distinct from those in skeletal muscle. We have previously proposed that Ca^{2+} release from these two structurally distinct but continuous regions of the SR occurs in response to different stimuli during excitation-contraction coupling (Jorgensen and Campbell, 1984; Jorgensen et al., 1984, 1985). Thus, it was suggested that Ca^{2+} release from junctional SR might be triggered by signals depending on the physical contact via feet structures between junctional SR and sarcolemma, while corbular SR which is not in physical contact with the SL might be triggered to release Ca^{2+} by a diffusable agent. Considering our finding that the Ca^{2+} release channel/ryanodine receptor is present in both corbular SR and junctional SR, is it still reasonable on the basis of present evidence to propose that Ca^{2+} release from these two storage sites is triggered by different mechanisms?

Studies in skinned mammalian and avian myofibers have demonstrated that graded Ca^{2+}-induced Ca^{2+} release from internal SR occurs in cardiac but not skeletal muscle (Fabiato, 1983, 1985). These studies also showed that the size of the Ca^{2+} transient is determined by the rate of change of the free cytosolic $[Ca^{2+}]$. As skinned cardiac myofibers lack T-tubules, and thus functional junctional SR, it is highly likely that the corbular SR is the prominent site of Ca^{2+} induced Ca^{2+} release in these fibers (Fabiato 1983, 1985). However, these studies do not exclude the possibility that Ca^{2+} induced Ca^{2+} release also occurs at the junctional SR. On the basis of more recent studies of isolated rat and guinea pig ventricular myofibers it was concluded that Ca^{2+} induced Ca^{2+} release from the SR is a prominent component of the Ca^{2+}-transient leading to contraction in these myofibers (Wier, 1990; Nabauer et al., 1989; Cleemann and Morad, 1991; Wier, 1992; Beuckelmann and Wier, 1988). Furthermore, the Ca^{2+} release from SR was demonstrated to be triggered directly by the Ca^{2+} current entering the cytosol via the voltage gated L-type Ca^{2+} channel (Nabauer et al., 1989; Cleemann and Morad, 1991). Although this Ca^{2+} entry is triggered by depolarization of the SL and T-tubules, Ca^{2+} release from SR and the ensuing contraction does not occur without the Ca^{2+} current, implying that the depolarization dependent mechanism leading to Ca^{2+}-release from skeletal SR (Schneider and Chandler, 1973; Bean and Rios, 1989) does not operate in cardiac muscle. Nonetheless, it has been observed that early repolarization of the SL shortens the $[Ca^{2+}]$ transient in cardiac myofibers (Beuckelmann and Wier, 1988; Cannell et al., 1987), a finding that does not support the hypothesis of Ca^{2+} induced Ca^{2+} release. Thus, the possibility remains that a depolarization-dependent mechanism distinct from that required for excitation-contraction coupling in skeletal muscle may modulate Ca^{2+} induced Ca^{2+} release from cardiac SR (Wier, 1992).

These considerations, combined with our findings that the Ca^{2+} release channel/ryanodine receptor in cardiac SR is present in both junctional SR and corbular SR, makes it tempting to speculate that a combination of depolarization and Ca^{2+} current first induces Ca^{2+} release via the ryanodine receptor from junctional SR, which is closer to the SL and the site of Ca^{2+} entry via the L-type Ca^{2+} channel. This Ca^{2+} release then in turn, possibly by a regenerative mechanism, induces further release of Ca^{2+} via ryanodine receptors from corbular SR located further away from the SL and junctional SR. While a sufficiently large Ca^{2+} current might directly trigger Ca^{2+} release from corbular SR, the proposal that the initial Ca^{2+} release from junctional SR is an essential step in cardiac E-C coupling are consistent with the idea that Ca^{2+}-release from both corbular and junctional SR is triggered by Ca^{2+} but modulated only at the junctional SR by a yet unknown depolarization-dependent mechanism. Because the size of the Ca^{2+} induced Ca^{2+} release in skinned fibers is graded and determined by the rate of change of the free cytosolic Ca^{2+} concentration at the Ca^{2+} release site (Fabiato, 1983, 1985), an increase in the Ca^{2+} current could provide a steeper Ca^{2+} gradient triggering Ca^{2+} release from corbular SR located further into the fiber thus resulting in a larger Ca^{2+} transient and thereby an increase in contraction as observed in response to various inotropic agents such as epinephrine (Callewaert et al., 1988). To test this hypothesis it will be important in future studies to determine whether the spatial distribution of gradients of cytosolic free Ca^{2+} during the earliest stages of excitation-contraction coupling is consistent with this model. Although this is not feasible with presently available techniques (Wier, 1992), it is likely to be feasible with improved techniques in the near future.

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