Extended Junctional Sarcoplasmic Reticulum of Avian Cardiac Muscle Contains Functional Ryanodine Receptors*

(Received for publication, July 29, 1993, and in revised form, September 21, 1993)

James Junker†, Joachim R. Sommer‡, Madhabananda Sarki, and Gerhard Meissner**

From the †School of Pharmacy, Campbell University, Buies Creek, North Carolina 27506, the ‡Department of Pathology, Duke University and Veterans Administration Medical Centers, Durham, North Carolina, 27710, the §Department of Cell Biology and Anatomy, and the ‡Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

The ryanodine receptor (RYR)/Ca\(^{2+}\) release channel of avian cardiac muscle was localized by immunocytochemical techniques and biochemically characterized using isolated membrane and receptor protein fractions. Monoclonal antibody C3-33 raised against the canine cardiac RYR bound to the junctional sarcoplasmic reticulum of pigeon and finch hearts, both at peripheral couplings and at extended junctional sarcoplasmic reticulum (EJSR). Immunoblots of sarcoplasmic reticulum vesicles from pigeon and finch hearts showed this antibody recognized a single high molecular weight protein, which co-migrated with the canine M\(_{r}\) 565,000 RYR/Ca\(^{2+}\) release channel polypeptide. The pigeon heart RYR bound \(^{3}H\)ryanodine with high affinity in a Ca\(^{2+}\)-dependent manner, comparable to the canine cardiac RYR. Purification of the pigeon RYR yielded a 30 S protein complex, which bound the maximum calculated amount of \(^{3}H\)ryanodine (440 \pm 60 pmol/mg protein), assuming one high affinity site/tetrameric 30 S RYR comprised of M\(_{r}\) 565,000 polypeptides. Autoradiography of isolated finch cardiac myocytes indicated \(^{3}H\)ryanodine binding throughout the cells. These results suggest that avian heart contains a single population of RYRs, and thereby support the hypothesis that avian EJSR contains functional calcium release channels which, because of the absence of transverse tubules, can be located micrometers away from the surface membrane in avian heart.

Excitation-calcium release is an early step in the process of excitation-contraction (E-C) coupling in striated muscle; an action potential propagated along the surface plasmalemma and its infoldings, the transverse (T-) tubes, triggers the release of Ca\(^{2+}\) ions stored in organelles, the junctional sarcoplasmic reticulum (JSR). JSR represents discrete differentiations of the sarcoplasmic reticulum (SR) that by definition (Sommer and Johnson, 1968) are exposed to the plasmalemma across a ~15 nm junctional gap, forming structural complexes (“couplings”) both at the cell surface (peripheral couplings) and at T-tubules (interior couplings). “Junctional processes” (JP) (Sommer and Johnson (1969) or “feet” (Frazzini-Armstrong, 1970) span the junctional gap, often incompletely. The geometry of couplings is propitious for E-C coupling by facilitating signal transmission both through direct contact (skelatal muscle; Schneider, 1981) and a diffusible transmitter such as Ca\(^{2+}\) (cardiac muscle; Fabiato, 1983). Dihydropyridine receptor antibodies in the plasmalemma at the couplings are thought to mediate SR Ca\(^{2+}\) release by acting as voltage sensors in vertebrate skeletal muscle and as voltage-dependent Ca\(^{2+}\) channels in cardiac muscle (Rios and Pizarro, 1991). The mammalian cardiac Ca\(^{2+}\) release channel has been identified as the receptor for the plant alkaloid ryanodine, localized to the JP's which decorate the membranous envelopes of JSR, and purified as a 30 S ryanodine receptor (RYR) protein complex comprised of four M\(_{r}\) 565,000 polypeptides (Inui et al., 1987; Anderson et al., 1989; Rardon et al., 1989), as determined by cDNA cloning and sequencing (Otsu et al., 1990; Nakai et al., 1990).

In avian hearts, JSR has a dual location; it is both attached to plasmalemma as part of the peripheral couplings (JSR proper (~25% of JSR) and unattached in the form of EJSR (~75% for review, see Sommer et al., 1979, 1991). Although removed from the plasmalemma by several micrometers (avian hearts have no T-tubules), EJSR, like JSR proper, is decorated with JP's. A homologous, much less numerous organelle also occurs in mammalian cardiac muscle as so-called corbular SR (Dolber and Sommer, 1984), especially in cells without T-tubules (e.g. conduction cells). Anti-ryanodine receptor antibodies localized to the JP's have corroborated the structural homology of corbular SR with JSR proper in mammalian cardiac muscle (Jorgensen et al., 1993).

Here, we have characterized and localized avian cardiac RYRs biochemically and by immunocytochemical and radioligand binding techniques, respectively. Our results indicate that avian hearts display 1) a uniform RYR population, 2) localization of RYRs to EJSR, and 3) \(^{3}H\)ryanodine binding properties similar to those established in mammalian hearts. These results support the idea that EJSR is a functional homologue of JSR proper. Some of the results of this study have been presented in abstract form (Junker et al., 1992).

**EXPERIMENTAL PROCEDURES**

Chemicals—\(^{3}H\)ryanodine (54.7 Ci/mmol) was obtained from Du Pont-NEC, \(\text{Ca}^{2+}\) was from ICN Radiochemical (Irvine, CA), and unlabeled ryanodine from AgriSystems International (Wind Gap, PA). Protease inhibitors and SDS gel molecular weight standards were purchased from Sigma and Boehringer Mannheim, phospholipids from Avanti Polar Lipids (Birmingham, AL) and ruthenium red from Fluka. All other reagents were of analytical grade.

**Animals**—Pigeons were purchased from Walker Farms, Dunn, NC, and Palmetto Pigeon Plant, Sumter, SC. Following anesthetization with...
**Fig. 1.** Light microscopic immunolocalization of RYR using canine cardiac RYR monoclonal antibody C3-33. Antibody staining occurs as rows of dots running transversely across the cells (arrows). As shown in Fig. 2, this labeling occurs at Z lines. Little staining is evident elsewhere. Control sections show no staining. a, pigeon heart (0.1 µg C3-33/ml); b, pigeon heart-preabsorbed antibody control (0.1 µg C3-33 + 6.8 µg RYR/ml); c, finch heart (1 µg C3-33/ml); d, rat heart (1 µg C3-33/ml). Bars = 10 µm.

For electron microscopy, the coverslips and mounting medium were removed by soaking the slides in normal saline. The sections were fixed for 15 min in 2% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4, and postfixed by flooding the slides with 2% OsO4 in water. The sections were dehydrated with alcohol and propylene oxide, embedded by invert-OM-U3 ultramicrotome, mounted on copper grids, and examined on a Zeiss 10B electron microscope.

**Preparation of Isolated Cells—**Finch heart cells were isolated similarly as described (Bendukidze et al., 1985). Briefly, the heart of an anesthetized finch (60 µl of pentobarbital, 65 ng/ml, injected intraperitoneally) was excised from the thorax while still beating. Cell dissociation was begun by perfusing the coronary arteries for 5 min with an enzyme solution containing 1 mg/ml collagenase (Sigma, type I), 0.07 mg/ml Pronase (Sigma protease, type XIV), 0.1 M NaCl, 0.05 M taurine, 0.01 M KCl, 0.0012 M KH2PO4, 0.1 mM CaCl2, 0.004 M MgSO4, 0.02 M dextrose, 0.01 M HEPES, pH 7.4 (with KOH). The ventricles were then chopped into small pieces and stirred in enzyme solution containing 0.2 mM CaCl2 at 35 °C. Isolated cells were collected from the solution, placed into Eppendorf tubes, and centrifuged for 5 s in a microfuge. The cells were resuspended and stored up to 1 h in the above solution with 0.2 mM CaCl2, but without enzymes.

**Autoradiography—**Isolated cells were rinsed in solution A (0.15 M NaCl, 0.1 M EGTA, 1.5 mM CaCl2, 5 mM dextrose, 20 mM K-HEPES, pH 7.4). They were then incubated for 1 h at room temperature in solution A containing either 50 nM [3H]ryanodine or 50 nM [3H]ryanodine + 50 µM unlabeled ryanodine. Following incubation, the cells were rinsed three times with solution A. Aliquots (20 µl) were spread over dried emulsion precoated slides (Kodak NTB-2, Eastman Kodak, Rochester, NY) and stored in light-tight black desiccator boxes at -20 °C (Stumpf and Sar, 1975). After 30 days of exposure, the slides were developed in Kodak D-19 for 1 min, fixed with Kodak fixing solution for 5 min, and stained with methyl green-pyronin.

The slides were examined with an Olympus photomicroscope. To determine the percentage of cell clusters clearly labeled with [3H]ryanodine, randomly selected clusters were visually inspected. If the num-
Avian Ryanodine Receptor

I.

A

in Fig. 1 were processed for electron microscopy. The antibody labels EJSR (arrows) located between myofibrils (brackets), and JSR proper at FIG. 2. Electron microscopic localization of RYR using canine cardiac RYR monoclonal antibody C3-33. Sections such as those seen peripheral couplings (double arrows) located between myofibrils and plasmalemma, all at the level of Z lines. Use of osmium tetroxide results in enhancement of the diaminobenzidine reaction product as part of the immunocytochemical procedure, as well as a weak background staining of Z lines (arrowheads), mitochondria (asterisks), and free SR (open arrows) useful for general orientation. Although these structures are visible in the controls (cf. Fig. 2, c and g), prominent staining of EJSR and JSR proper is not. For comparison, the appearance of EJSR and JSR proper prepared for conventional transmission electron microscopy after contrasting with osmium tetroxide, uranyl acetate, and lead citrate is shown in b. Solid circles = extracellular space. a, pigeon heart 0.1 pg C3-33/ml; b, pigeon heart-conventional transmission electron microscopy; c, pigeon heart-preabsorbed antibody control; d, rat heart (1 µg C3-33/ml); e, finch heart (1 µg C3-33/ml); f, finch heart (1 µg C3-33/ml); g, finch heart-nonimmune serum control (1 µg normal mouse IgG/ml). Bars = 1 µm.

ber of grains over the cells was visibly greater than background, the cluster was classified as labeled. Photographs of labeled clusters and controls were taken for grain counting, and the number of grains from two randomly selected regions over a cell cluster, each 90 µm², was counted. Background levels were determined by counting the number of grains found in two regions of the surrounding extracellular space. The net grain count was determined by subtracting the background from the cluster grain count for each cluster. Statistical analysis was performed using a two sample test of means (SPCC System, Walmyr Publishing, Tempe, AZ).

Isolation of SR Vesicles and 30 S RYR Complex—Cardiac SR vesicles were prepared from pigeon and finch hearts similarly as described for mammalian heart (Meissner and Henderson, 1987). The hearts of three pigeons (~4 g each) were rapidly excised and immediately immersed in ice-cold 0.3 M sucrose. Minced hearts were added to 10 volumes of an ice-cold 20 mM Tris-HCl, pH 7.4, buffer containing 0.3 M sucrose, 0.5 mM EDTA, and protease inhibitors (1 mM diisopropyl fluorophosphate, 100 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin, 1 mM benzamidine, 1 mM iodoacetamide) and homogenized at 4°C for 60 s using an ultra torrex homogenizer (Tekmar). A crude SR membrane fraction was obtained as a 10,000-100,000 × g pellet which was resuspended in 0.4 M KCl medium and layered at the top of a linear 20-40% (w/w) sucrose gradient in 0.4 M KCl. After centrifugation for 2 h at 240,000 × g in a Beckman SW41 rotor, membranes sedimenting at 25-33% (Fraction 1) and 35-40% (Fraction 2) sucrose were collected, diluted with two volumes of 0.4 M KCl, sedimented, resuspended in 0.3 M sucrose, 5 mM potassium PIPES, pH 7.0, and stored in 0.25-ml aliquots at -135°C. Finch hearts (~0.2 g) were frozen on dry ice following dissection, and a crude SR membrane fraction was prepared from five frozen hearts as described above.

The CHAPS-solubilized 30 S pigeon and canine heart RYRs were isolated by rate density gradient centrifugation in the presence of protease inhibitors as described (Anderson et al., 1989).

45Ca²⁺ Efflux Measurements—Pigeon heart SR vesicle fractions (5-10 mg protein/ml) were passively loaded with 5 mM 45Ca²⁺ in a medium containing 0.1 M KCl and 5 mM 45Ca²⁺ (Meissner and Henderson, 1987). 45Ca²⁺ efflux was initiated by diluting vesicles 200-fold into isosmolar efflux media and stopped by placing 0.4-ml aliquots at various times on 0.45-µm filters (Type HA, Millipore Co, Bedford, MA). Filters were washed with a quench solution containing 0.2 mM EGTA, 10 mM Mg²⁺, and 20 µM ruthenium red. Radioactivity remaining with the vesicles on the filters was determined by liquid scintillation counting.
Fig. 3. Autoradiography following incubation of isolated finch cardiac cells with \(^{3}H\)ryanodine. Cell clusters containing two to three cells are shown. \(a\), labeling with \(^{3}H\)ryanodine in the absence of an excess of unlabeled ryanodine. Nearly two-thirds of the cell clusters were labeled as in \(a\), with the remaining one-third of the clusters showing labeling close to background. \(b\), labeling with \(^{3}H\)ryanodine in the presence of a 1000-fold excess of unlabeled ryanodine. In this condition, very few grains were seen over clusters. Bars = 20 \(\mu m\).

### Table I

Autoradiographic grain counts of finch myocytes labeled with \(^{3}H\)ryanodine

| Cell clusters | \[^{3}H\]Ryanodine | \[^{3}H\]Ryanodine + unlabeled ryanodine |
|---------------|---------------------|----------------------------------------|
| Total grain count/100 \(\mu m^2\) | 26.5 ± 9.2 | 2.6 ± 1.0 |
| Background grain count/100 \(\mu m^2\) | 3.3 ± 1.2 | 1.1 ± 0.6 |
| Net grain count/100 \(\mu m^2\) | 23.3 ± 8.6 | 1.5 ± 1.1* |

* The difference between the net grain counts over \[^{3}H\]ryanodine-labeled and control (\[^{3}H\]ryanodine plus unlabeled ryanodine) cell clusters is highly significant \((p < 0.0001)\).

**Results**

** Autoradiography—**The presence of a functional RYR in the EJSR was tested by incubating isolated finch heart cells with \(^{3}H\)ryanodine in the absence and presence of an excess of unlabeled ryanodine. In the autoradiographs, the cells were typically observed as small rectangular clusters comprised of two to three cells. The boxy appearance of the clusters (Fig. 3), as opposed to the elongated appearance of freshly isolated cardiac myocytes, indicated a state of contracture. Light microscopic examination showed that most clusters (131 out of 202) incubated with \(^{3}H\)ryanodine alone were clearly labeled (Fig. 3a). By contrast, when cells were incubated with \(^{3}H\)ryanodine in the presence of a 1000-fold excess of unlabeled ryanodine (Fig. 3b), nearly two-thirds of the cell clusters were labeled as in \(a\), with the remaining one-third of the clusters showing labeling close to background. In this condition, very few grains were seen over clusters.

** Autoradiography—**The presence of a functional RYR in the EJSR was tested by incubating isolated finch heart cells with \(^{3}H\)ryanodine in the absence and presence of an excess of unlabeled ryanodine. In the autoradiographs, the cells were typically observed as small rectangular clusters comprised of two to three cells. The boxy appearance of the clusters (Fig. 3), as opposed to the elongated appearance of freshly isolated cardiac myocytes, indicated a state of contracture. Light microscopic examination showed that most clusters (131 out of 202) incubated with \(^{3}H\)ryanodine alone were clearly labeled (Fig. 3a). By contrast, when cells were incubated with \(^{3}H\)ryanodine in the presence of a 1000-fold excess of unlabeled ryanodine (Fig. 3b), nearly two-thirds of the cell clusters were labeled as in \(a\), with the remaining one-third of the clusters showing labeling close to background. In this condition, very few grains were seen over clusters.
Avian Ryanodine Receptor

The fraction of Ca\(^{2+}\)-permeable vesicles was obtained as shown in Fig. 4 by determining the amounts of \(^{45}\)Ca\(^{2+}\) remaining with the vesicles at 20 s in the quench (10 mM Mg\(^{2+}\) and 20 \(\mu\)M ruthenium red) as well as the 30 s in the Ca\(^{2+}\) release channel inhibiting medium (Fig. 4). As observed with a rat skeletal RYR antisem (Meissner et al., 1989) (not shown), taken together, data of Table I and Figs. 5 and 6 suggest that avian and mammalian cardiac muscle ex-

| Yield (mg protein/mg muscle) | 4.4 ± 1.3 | 1.0 ± 0.3 | 0.6 ± 0.3 |
|-------------------------------|-----------|-----------|-----------|
| \(^{45}\)Ca\(^{2+}\) release (%) | 35 ± 5 | 32 ± 2 | 37 ± 16 |
| \(^{3}H\)Ryanodine binding | 4.6 ± 0.6 | 4.5 ± 0.6 | 9.9 ± 4.5 |
| \(K_D (\mu M)\) | 2.9 ± 0.8 | 2.1 ± 0.7 |

\(^{45}\)Ca\(^{2+}\) Release and \(^{3}H\)Ryanodine Binding to SR-enriched Membrane Fractions—The functionality of the RYR in EJSR was further tested by determining the biochemical properties of the isolated avian EJSR and RYR. Since at present EJSR and JSR proper cannot be separated to test their individual activities, these studies were based on the observation that EJSR accounts for a large portion (75%) of total JSR in birds (Bossh et al., 1978). Therefore, \(^{3}H\)Ryanodine binding properties of isolated membrane fractions should be reflected to a large extent by those of the EJSR. Because of their larger size, pigeon rather than finch hearts were used as the source for the isolation and characterization of avian cardiac RYR. A crude microsomal membrane fraction was prepared and subfractionated on a sucrose gradient (see "Experimental Procedures"). Table II shows the yields for the crude and 25–33 and 35–40% sucrose gradient membrane fractions.

The presence of a Ca\(^{2+}\)-activated \(^{45}\)Ca\(^{2+}\) efflux pathway in pigeon heart membrane fractions was assessed by diluting passively loaded vesicles into efflux media that contained the two SR Ca\(^{2+}\) release channel inhibitors Mg\(^{2+}\) and ruthenium red, and either <10\(^{-5}\) M or 20 \(\mu\)M free Ca\(^{2+}\). As previously observed for canine cardiac SR vesicles enriched in Ca\(^{2+}\) release activity (Meissner and Henderson, 1987), \(^{45}\)Ca\(^{2+}\) efflux was slow in the Ca\(^{2+}\) release channel inhibiting medium (Fig. 4). Omission of Mg\(^{2+}\) and ruthenium red from the <10\(^{-5}\) M Ca\(^{2+}\) efflux medium resulted in an increased \(^{45}\)Ca\(^{2+}\) efflux rate. A further increase in the \(^{45}\)Ca\(^{2+}\) efflux rate was observed when vesicles were placed into a medium containing 20 \(\mu\)M free Ca\(^{2+}\). About half of the radioactivity remained with the vesicles for longer times (Fig. 4), indicating the presence of a subpopulation of vesicles lacking a Ca\(^{2+}\)-gated Ca\(^{2+}\) release pathway (Meissner and Henderson, 1987). Table II lists the fraction of vesicles that contained a Ca\(^{2+}\)-gated release pathway in crude SR membrane and sucrose gradient Fractions 1 and 2.

\(^{3}H\)Ryanodine binding experiments (see "Experimental Pro-

FIG. 4. \(^{45}\)Ca\(^{2+}\) release from pigeon heart SR vesicles. SR vesicles (Fraction 2 of Table I) were passively loaded with 5 mM \(^{45}\)Ca\(^{2+}\) and then diluted 200-fold into isosmolar efflux media containing 0.2 mM EGTA, 10 mM Mg\(^{2+}\), and 20 \(\mu\)M ruthenium red (○), 2 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N"-tetraacetic acid (<10\(^{-6}\) M free Ca\(^{2+}\)) (□), or 20 \(\mu\)M free Ca\(^{2+}\) (□). Amounts of \(^{45}\)Ca\(^{2+}\) remaining with the vesicles at 20 s (Table I) were measured as described under "Experimental Procedures."
FIG. 5. Ca\textsuperscript{2+} dependence of \([\textsuperscript{3}H]\)ryanodine binding to SR membranes from pigeon and canine hearts. Specific \([\textsuperscript{3}H]\)ryanodine binding to pigeon (○) and canine (□) heart 32%–40% sucrose gradient fractions was determined at the indicated Ca\textsuperscript{2+} concentrations in the presence of 5 mM Mg\textsuperscript{2+} and 5 mM AMP-PCP as described under “Experimental Procedures,” using a \([\textsuperscript{3}H]\)ryanodine concentration of 4 nM. Data points are the average of two different experiments carried out in duplicate. Values (100%) of \([\textsuperscript{3}H]\)ryanodine binding correspond to 5.7 and 3.1 pmol/mg pigeon and canine heart microsomal protein, respectively.

Disocclusion by stereology the total cell volume fraction of JSR in mouse cardiac myocytes (cell diameter 15 μm) and of JSR proper is considered to be the site of Ca\textsuperscript{2+} release in mammalian skeletal and cardiac muscle. In skeletal muscle, the mechanical coupling hypothesis suggests that the SR RYR/Ca\textsuperscript{2+} release channel is physically linked to a voltage-sensing dihydropyridine receptor located in the T-tubule (Rios and Pizarro, 1991). In contrast, in cardiac muscle E-C coupling is thought to be triggered by a diffusible transmitter molecule. A voltage-dependent dihydropyridine receptor/Ca\textsuperscript{2+} channel localized in the surface membrane and T-tubules mediates the entry of Ca\textsuperscript{2+} ions that trigger SR Ca\textsuperscript{2+} release (Cannell et al., 1987; Nabauer et al., 1989). A close apposition of the surface membrane and SR Ca\textsuperscript{2+} channel proteins favors local control of the release channels by the trigger Ca\textsuperscript{2+}, thereby supporting a rapid and graded translation of the action potential into SR Ca\textsuperscript{2+} release (Stern, 1992; Gyorke and Palade, 1992). The presence of putative Ca\textsuperscript{2+} release structures, micrometers away from any direct contact.
Fig. 7. Sedimentation profile and analysis of pigeon heart RYR. A, pigeon heart SR vesicles (Fraction 2 of Table I, 1.5 mg protein/ml) were solubilized with CHAPS (1.6%) in a medium containing 1.0 M NaCl, 20 mM NaPIPES, pH 7.0, 200 μM Ca²⁺, 5 mM AMP, 5 mg/ml phosphatidylincholine, 1 mM dithiothreitol, 1 mM diisopropyl fluorophosphate, 5 μM leupeptin, and 30 mM [³H]ryanodine. The solubilized proteins were loaded onto a linear 5–20% sucrose gradient in the above medium containing 1% CHAPS and centrifuged at 2 °C in a Beckman SW28 rotor for 16 h at 26,000 revolutions/minute. Eighteen fractions of 2 ml each were collected and analyzed for [³H]radioactivity. The arrow indicates the position of bound [³H]ryanodine peak fraction of gradients containing the canine cardiac RYR. B, SDS-PAGE and immunoblot (right lanes) analysis of selected gradient fractions. SDS gel was stained with Coomassie Blue (fraction 7, left lane). After transfer onto Immobilon polyvinylidene difluoride membranes, gradient fractions 3, 5, 7, 9, 11, 13, 15, and 17 were probed with canine cardiac RYR monoclonal antibody C3-33 (right lanes).

with the cell surface membrane, suggests that the emphasis on a juxtaposition of Ca²⁺ trigger and release structures may need to be reconsidered. Furthermore, the presence, especially in avian heart, of a large number of putative Ca²⁺ release organelles with and without plasmalemmal contacts suggests that both may play an important yet, perhaps, physiologically different role in regulating intracellular Ca²⁺ in cardiac muscle. Several observations support the idea of a Ca²⁺-sensitive Ca²⁺ release mechanism by the EJSR of avian heart. First, evidence for such a mechanism is provided by our observation...
that $[^{3}H]$ryanodine binding to the avian RYRs is a Ca$^{2+}$-dependent process. A similar ligand dependence of $[^{3}H]$ryanodine binding and Ca$^{2+}$ release channel activities has suggested that ryanodine is a sensitive ligand for probing mammalian release channel function (Chu et al., 1990; Meissner and El-Hashem, 1992). Second, mammalian cardiac muscle exhibits structural equivalents (corbular SR) (Dolber and Sommer, 1984; Jorgensen et al., 1993) that have been found to store Ca$^{2+}$ and to contain calcequstrin, a Ca$^{2+}$-binding protein characteristic of JSR (Jorgensen et al., 1988). Third, as recently reported (Airey et al., 1993) and confirmed in this study, avian heart appears to contain only a single RYR and not two RYRs as observed for avian skeletal muscle.

It can be argued that Ca$^{2+}$ ions move too slowly in cells (Allbritton et al., 1992; Nowicky and Pinter, 1993) to support a Ca$^{2+}$-induced Ca$^{2+}$ release mechanism by the EJSR. Birds such as finch and pigeon have a heart rate of about 5–10 beats/s. Accordingly, the SR Ca$^{2+}$ release phase during one contraction-relaxation cycle is probably limited to 25–50 ms. In the absence of Ca$^{2+}$ buffers, this time is sufficient for Ca$^{2+}$ to nearly equilibrate throughout a cell with a diameter of 10 μm (Nowicky and Pinter, 1993).

On the other hand, the presence of intracellular Ca$^{2+}$ buffers slows the movement of Ca$^{2+}$ even as mononuclear increases in [Ca$^{2+}$] can speed it up (Allbritton et al., 1992; Nowicky and Pinter, 1993). At present, precise quantitative information about parameters that determine the diffusion rate of Ca$^{2+}$ such as the amounts of Ca$^{2+}$ released by JSR and buffered by, or bound to, the cytosol, is largely missing.

Other conditions supporting a Ca$^{2+}$-induced Ca$^{2+}$ release mechanism may exist in avian hearts. One of these could involve the creation of a Ca$^{2+}$ release wave expanding from JSR to EJSR. Such a mechanism would be attractive since the maintenance of a high Ca$^{2+}$ concentration between adjacent EJSRs can provide a pathway for Ca$^{2+}$-induced Ca$^{2+}$ release mechanism by the EJSR. Birds such as finch and pigeon have a heart rate of about 5–10 beats/s. Accordingly, the SR Ca$^{2+}$ release phase during one contraction-relaxation cycle is probably limited to 25–50 ms. In the absence of Ca$^{2+}$ buffers, this time is sufficient for Ca$^{2+}$ to nearly equilibrate throughout a cell with a diameter of 10 μm (Nowicky and Pinter, 1993).

It should be kept in mind that in the absence of unambiguous proof of Ca$^{2+}$-induced Ca$^{2+}$ release by EJSR in avian cardiac muscle, other diffusible effectors must be considered such as cyclic ADP-ribose (Meszaros et al., 1993) or lipid metabolites (Sabbadin et al., 1992).

In conclusion, in this study we have described the first direct observations of a functional RYR without any plasmamlemal contact. Radioligand labeling of isolated cells, SR membrane, and RYR fractions suggested that RYRs in avian heart were capable of binding $[^{3}H]$ryanodine in a manner characteristic of mammalian JSR RYRs, implying the presence of an intrinsic Ca$^{2+}$ channel activity in all RYRs of avian heart.

Acknowledgments—We thank Dr. Neal Shepherd for help in isolating cardiac myocytes and Anita Cohn for excellent technical assistance.

REFERENCES

Airey, J. A., Grissell, M. M., Jones, L. R., Sutko, J. L., and Witcher, D. (1993) Biochemistry 32, 5739-5745

Allbritton, N. L., Meyer, T., and Stryer, L. (1992) Science 258, 1812-1815

Anderson, K., Lai, A., Liu, Q.-Y., Rousseau, E., Erickson, H. P., and Meissner (1989) J. Biol. Chem. 264, 1329-1335

Bendukidze, Z., Isenberg, G., and Klockner, U. (1985) Basic Res. Cardiol. 80 (Suppl. 1), 513-517

Bossen, E. H., Sommer, J. R., and Waugh, R. A. (1978) Tissue Cell 10, 773-784

Cannell, M. B., Berlin, J. R., and Lederer, W. J. (1987) Science 238, 1419-1423

Chu, A., Diaz-Munoz, M., Harkies, M., Brush, K., Hamilton, S. L. (1990) Mol. Pharmacol. 37, 735-741

Dolber, P. C. and Sommer, J. R. (1994) J. Ultrastruct. Res. 87, 190-196

Fabbiato, A. (1983) Am. J. Physiol. 245, C1-C14

Franzini-Armstrong, C. (1970) J. Cell Biol. 47, 488-499

Györke, S., and Fill, M. (1993) Science 260, 807-809

Györke, S., and Palade, P. (1992) J. Physiol. 447, 195-210

Inui, M., Suzuki, A., and Fleischer, S. (1987) J. Biol. Chem. 262, 15637-15642

Jorgensen, A., O. Broderick, R., Somlyo, A. P., and Somlyo, A. V. (1988) Circ. Res. 63, 1060-1069

Jorgensen, A. O., Shen, A. C. Y., Arnold, W., McPherson, P. S., and Campbell, K. P. (1993) J. Cell Biol. 129, 969-980

Junker, J., Meissner, G., Sar, M., and Sommer, J. R. (1992) PASEB J. 4, (abstr.) 1560

Lai, F. A., and Meissner, G. (1989) Bioenerg. Biomembr. 21, 227-246

Lai, F. A., Miura, M., Xu, L., Smith, H. A., and Meissner, G. (1989) J. Biol. Chem. 264, 16776-16785

Lai, F. A., Liu, Q.-Y., Xu, L., El-Hashem, A., Kramarzy, N. R., Sealock, R., and Meissner, G. (1992) Am. J. Physiol. 263, C365-C372

Meissner, G., and El-Hashem, A. (1992) Mol. Cell. Biochem. 114, 119-123

Meissner, G., and Henderson, J. S. (1987) J. Biol. Chem. 262, 2065-2073

Meissner, G., Rousseau, E., and Lai, F. A. (1989) J. Biol. Chem. 264, 1715-1722

Meszaros, L. G., Bak, J., and Chu, A. (1993) Nature 364, 76-79

Nabauer, M., Callewaert, G., Cleemann, L., and Morad, M. (1989) Science 244, 809-803

Nakai, J., Imagawa, T., Nakamata, Y., Shigekawa, M., Takeshima, H., and Numa, S. (1990) FEBS Lett. 271, 169-177

Nowicky, M. C., and Pinter, M. J. (1994) Biophys. J. 64, 77-91

Oou, K., Willard, H. F., Khanna, V. K., Zorato, F., Green, N. M., and MacLennan, D. H. (1990) J. Biol. Chem. 265, 13472-13483

Rardon, D. P., Cafali, D. C., Mitchell, R. D., Seler, S. M., and Jones, L. R. (1989) Circ. Res. 64, 779-789

Rios, E., and Pizarro, G. (1991) Physiol. Rev. 71, 849-860

Sabbadini, R. a., Betto, R., Teresi, A., Fachechi-Cassano, G., and Salvagiotti, G. (1992) J. Biol. Chem. 267, 15475-15484

Sar, M. (1985) In Techniques in Immunohemistry (Bullock, G. R., and Petrusz, P., eds) Vol. 3, pp. 43-54, Academic Press, New York

Schneider, M. F. (1981) Annu. Rev. Physiol. 43, 507-517

Sommer, J. R., and Johnson, E. A. (1969) J. Cell Biol. 38, 497-502

Sommer, J. R., and Johnson, E. A. (1969) J. Cell Biol. 38, 497-502

Sommer, J. R., and Johnson, E. A. (1979) In Handbook of Physiology, Section 2 The Cardiovascular System (Berne, R. M., ed) Vol. I, pp. 113-186, American Physiological Society, Bethesda, MD

Sommer, J. R., Bossen, E., Dalen, H., Dolber, P., High, T., Lewet, P., Johnson, E. A., Junker, J., Leonard, S., Nasser, B., Scherer, B., Spach, M., Spray, T., Taylor, L., Wallace, N. R., and Waugh, R. (1991) Acta Physiol. Scand. 143 (Suppl. 599), 5-21

Stern, M. D. (1992) Biophys. J. 63, 497-517

Stumpf, W. E., and Sar, M. (1975) Methods Enzymol. 36, 135-156