Localization of Ryanodine Receptors in Smooth Muscle

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Abstract—The ryanodine receptor (RyR) in aortic and vas deferens smooth muscle was localized using immunofluorescence confocal microscopy and immunoelectron microscopy. Indirect immunofluorescent labeling of aortic smooth muscle with anti-RyR antibodies showed a patchy network–like staining pattern throughout the cell cytoplasm, excluding nuclei, in aortic smooth muscle and localized predominantly to the cell periphery in the vas deferens. This distribution is consistent with that of the sarcoplasmic reticulum (SR) network, as demonstrated by electron micrographs of osmium ferrocyanide–stained SR in the two smooth muscles. Immunoelectron microscopy of vas deferens smooth muscle showed anti-RyR antibodies localized to both the sparse central and predominant peripheral SR elements. We conclude that RyR–Ca2+-release channels are present in both the peripheral and central SR in aortic and vas deferens smooth muscle. This distribution is consistent with the possibility that both regions are release sites, as indicated by results of electron probe analysis, which show a decrease in the Ca2+ content of both peripheral and internal SR in stimulated smooth muscles. The complex distribution of inositol 1,4,5-trisphosphate and ryanodine receptors (present study) is compatible with their proposed roles as agonist-induced Ca2+-release channels and origins of Ca2+ sparks, Ca2+ oscillations, and Ca2+ waves. (Circ Res. 1998;82:175-185.)

Key Words: smooth muscle • ryanodine receptor • electron microscopy • confocal microscopy immunofluorescence

The primary, though not sole, mechanism of smooth muscle activation is an increase in [Ca2+]i, whether the stimulus for contraction is through a voltage-independent pathway (pharmacomechanical coupling) and/or membrane depolarization (electromechanical coupling) (reviewed in Reference 1). Increases in [Ca2+]i can result from either the trans–plasma membrane flux of Ca2+ through plasma membrane Ca2+ channels or the release of Ca2+ from intracellular stores, with the relative contribution from these two Ca2+ pools varying with different smooth muscles and stimuli.1 Ca2+ is stored intracellularly in the SR, which contains at least two types of Ca2+-release channels: the IP3 receptor/Ca2+-release channel and the RyR/Ca2+-induced Ca2+-release channel. The IP3 receptor is thought to be the major channel mediating pharmacomechanical coupling in smooth muscle1-5 and has been localized to both the junctional and central SR6; however, functional studies also provide evidence of a caffeine-releasable ryanodine–sensitive Ca2+ pool in smooth muscle3,7 that, as in cardiac muscle, may be subject to a Ca2+-induced Ca2+-release mechanism triggered by the trans–plasma membrane Ca2+ current.

The RyR was first isolated from skeletal muscle SR and found to be equivalent to the “foot” structures bridging the T tubules with the terminal cisternae (observed by electron microscopy8)—the major site of Ca2+ release in skeletal muscle. Similar “bridging structures” connect the junctional SR with the plasma membrane in smooth muscle,9,10 but their relationship to RyRs or IP3 receptors has yet to be established. Radioligand binding studies have shown radiolabeled ryanodine binding to the SR fraction of smooth muscle cell homogenates,11-13 but the distribution between peripheral (junctional) SR close to the plasmalemma and the central SR has not been previously determined. In the present study, we demonstrate with immunofluorescence and immuno-electron microscopy the presence of ryanodine receptors in both central and peripheral SR of aortic and vas deferens smooth muscle.

Materials and Methods

Tissue Preparation

Adult male guinea pigs (Hartley) weighing 350 g (obtained from Hilltop Farms, Scottdale, Pa) were given an overdose of halothane anesthesia and then exsanguinated by following a protocol approved by the University of Virginia Animal Experimentation Committee and in accordance with policies outlined in the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The thoracic aorta and vas deferens were dissected free and placed in HEPES-buffered normal Krebs’ solution at 37°C. The connective tissue was...
gently removed before fixation. Strips of thoracic aorta were carefully dissected with microdissection instruments under a dissecting microscope and then pinned onto dental wax strips at approximately in vivo length. The psoas muscle was prepared by freeing the muscle from the surrounding fascia and clamping it at resting length in situ with Krause muscle biopsy forceps. The clamped portion of the muscle was cut away from the muscle belly and then handled like the other specimens. Tissue for confocal microscopy was fixed overnight at 4°C in freshly prepared 3% paraformaldehyde in 10 mmol/L PBS, pH 7.4. The fixed tissue was cut into 5-mm×8-mm lengths and cryoprotected in 5% sucrose-PBS (wt/vol), pH 7.4, for 60 minutes at 4°C and then in 15% sucrose-PBS, pH 7.4, at 4°C for an additional 45 to 60 minutes. Blood vessel lumina were filled with Tissue-Tek O.C.T. compound (Miles Inc) immediately before freezing. All specimens were rapidly frozen by plunging into Freon-22 subcooled with liquid N2 and kept in Freon-22 frozen with liquid N2 until cryosectioning.

**Anti-RyR Antibodies**

Two preparations of polyclonal antibodies were made in two different rabbits (anti–RyR 8–1 and anti–RyR 8–2) against a synthetic peptide that corresponded to the published RyR amino acid sequence (20 amino acids, 4681 to 4700; C-LEFDGLYITEQPGDDDVKGQ) as that corresponded to the published RyR amino acid sequence (20 amino acids, 4681 to 4700; C-LEFDGLYITEQPGDDDVKGQ) as previously described. Briefly, the 20–amino acid synthetic oligopeptide was linked to keyhole limpet hemocyanin (via lysines, using the bifunctional agent m-maleimidobenzoyl-N-hydroxysuccinimide) injected into adult rabbits according to the protocol of Gonatas et al, and the antisera was used to prepare affinity-purified antibodies. Immunoblots of crude homogenates from bovine heart, skeletal muscle terminal cisternae, cultured bovine aortic endothelial cell membrane preparations, and purified RyRs all showed one band of reactivity with the resulting antibody. This peptide sequence was selected because of its homology to the heart (Ryr2) 55% identity. Anti–RyR 8 has been found to react with all three RyR isoforms (L. Jeyakumar and S. Fleischer, unpublished data, 1997). The specificity of anti–RyR 8 antibodies was established by immunoblotting with membrane preparations of guinea pig heart, aorta, and vas deferens, prepared by the method of Jones et al. Briefly, tissues were homogenized in 0.75 mmol/L KCl and washed twice in the same buffer after centrifugation at 14 000g. The pellet was resuspended in 10 mmol/L NaHCO3 and 5 mmol/L histidine and washed twice in the same buffer after centrifugation at 14 000g. The resulting supernatant was centrifuged at 105 000g for 30 minutes. The pellet was resuspended in 10 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, 1 mmol/L 4-(2-aminoethyl)-benzenesulfonylfluoride, and 84 μmol/L leupeptin. Proteins from membrane preparations were fractionated by SDS-PAGE on a 6% gel and transferred to PVDF membranes (Immobilon-P, Millipore). After blocking in a 10% (wt/vol) solution of nonfat dry milk/PBST, the membranes were incubated with anti–RyR 8–2 antibody (diluted 1:660) for 2 hours at room temperature, followed by incubation in a secondary anti-rabbit IgG coupled to horseradish peroxidase (Amersham Life Science). Antibody binding to the RyR bands was visualized using enhanced chemiluminescence (Amersham Life Science) according to the manufacturer’s instructions. Control studies were performed with anti-RyR 8–2 by preadsorbing the antibody with synthetic antigen peptide (20–mer) at a molar ratio of ≈1:10 (antigen/antibody). Antibody and antigen were incubated together for 30 minutes, on ice, before application to tissue sections or blot membranes.

A murine monoclonal anti-ryanodine antibody (mAb110E), prepared to avian skeletal muscle foot protein polypeptides, was also used for both immunohistochemistry and Western blotting. This antibody has been shown to bind selectively to a variety of avian and mammalian RyR isoforms.

The reactivity of mAb110E for guinea pig RyR isoforms was determined by Western blotting. Homogenates of guinea pig aorta, ileum, cardiac muscle, and psoas muscle were size-fractionated by SDS-PAGE on 7.5% gels and transferred to PVDF membranes. Non-specific immunoreactive sites on the PVDF membranes were blocked with a solution of 10% (wt/vol) nonfat dry milk in PBST, pH 7.3, at room temperature for ~1 hour. The membranes were incubated in mAb110E (diluted 1:20 000) in PBST for 3 hours at room temperature, washed in three changes of PBST (5 minutes each), and then incubated for 1 hour at room temperature in a peroxidase-conjugated goat anti-mouse IgG (Goldmark Biologicals) diluted 1:65 000 in PBST. After incubation in the secondary antibody, the membranes were washed in three changes of PBST (5 minutes each) and then in PBS (without Tween 20) to remove residual detergent and developed using an enhanced chemiluminescence protocol (Amersham Life Sciences) according to the manufacturer’s instructions.

**Anti-Vimentin Antibody Binding Studies**

A monoclonal anti-vimentin antibody (clone V9, Sigma Immunochemicals) was used for both immunocytochemical localization of vimentin in cultured smooth muscle cells and for immunodetection of vimentin on Western blots. The specificity of this anti-vimentin antibody has previously been established24–26; the sensitivity of the V9 antibody for vimentin was determined by Western blotting against guinea pig smooth muscle homogenates known to contain predominantly vimentin (aorta) or desmin (vas deferens). Purified pig aorta vimentin (a gift from Dr David Harshorne, University of Arizona) was initially included as a positive control on Western blots.

An established rat aortic smooth muscle cell line (RS1393V9) was grown to confluence on glass microscope coverslips (these cells were a gift from Dr Gary Owens, University of Virginia). The formation of intermediate filament “cables” was induced in the cultured cells by the addition of 1 μmol/L colcemid (Sigma) to the growth medium for 24 hours.27 Cells were then removed from culture, rinsed in PBS, and treated for 2 minutes with methanol precooled to ~−20°C. Non-specific immunoreactive sites were blocked by a 20- to 30-minute incubation of the permeabilized cells in PBS-ALB, pH 7.4, at room temperature. The cells were then exposed to the either the anti-vimentin antibody (diluted 1:50 [vol/vol] in PBS-ALB) or to either of the two lots of anti-ryanodine antibodies (diluted 1:50 [vol/vol] in PBS-ALB), as previously described for tissue sections.25 Primary antibody binding sites were detected indirectly with an affinity-purified, species-specific, TRITC-conjugated F(ab′)2 fragment, Jackson ImmunoResearch Laboratories, Inc). Cells were incubated for 1 hour in secondary F(ab′)2 fragment at a final concentration of ~10 μg/mL in PBS-ALB for 1 hour at room temperature, washed with PBS-ALB, and mounted in a buffered glycerol–containing medium for examination in the confocal microscope.

**Immunolabeling for Confocal Microscopy**

Specimens frozen in Freon-22 were allowed to warm to ~−20°C in the cryochamber and then affixed to a cryosectioning chuck, with

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<th>Selected Abbreviations and Acronyms</th>
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<tr>
<td>IP_3 = inositol 1,4,5-trisphosphate</td>
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<td>mAb (with number) = monoclonal antibody</td>
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<td>PBS-ALB = PBS containing 3% bovine serum albumin</td>
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<td>PBST = PBS with 0.05% Tween 20</td>
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<td>PVDF = polyvinylidene fluoride</td>
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<td>RyR = ryanodine receptor</td>
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<td>SR = sarcoplasmic reticulum</td>
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Tissue-Tek O.C.T. compound, in either a longitudinal or transverse orientation. Cryosectioning was performed on a Leica Frigocut 2800 E (Leica Instruments GmbH). Sections, ∼5 to 10 μm thick were cut and collected on gelatin-coated Superfrost Plus glass microscope slides (Fisher Scientific). An affinity-purified, species-specific, TRITC-conjugated F(ab′)2 (Jackson ImmunoResearch Laboratories, Inc) was used for indirect fluorescent labeling. Sections were preincubated in PBS–ALB to block nonspecific immunoreactive sites. The solution was aspirated from the slide, and the sections were incubated with either anti–RyR 8–1 or anti–RyR 8–2 diluted 1:1000 or 1:2000 (vol/vol) in PBS–ALB solution. Primary antibody was then aspirated from the slide, and the sections were washed with four changes of PBS–ALB (∼250 μL) for 5 minutes each. Nonspecific immunoreactive sites were blocked for 5 to 10 minutes with a 5% solution (vol/vol) of preimmune donkey serum diluted in PBS–ALB. This was aspirated and replaced with the TRITC-labeled F(ab′)2 fragment at a final concentration of ∼10 μg/mL in PBS–ALB solution. Tissue samples were incubated in the secondary F(ab′)2 fragment for 1 hour, washed as before with four changes of PBS–ALB, and mounted with phosphate-buffered glycerol containing antifade (Ted Pella Inc) or with PBS containing 100 mg/mL DABCO (Sigma). In several double-labeling experiments, psoas sections were labeled with both 35 nmol/L BODIPY-phallolidin (which binds to F-actin, located in the I band) and anti–RyR 8–1 antibody. Tissue sections of guinea pig aorta were also labeled with anti–RyR 8–1 antibody that was previously incubated with purified pig aorta vimentin. These competition experiments were performed to absorb antibodies that could have cross-reacted with vimentin epitopes (see “Results”). Anti–RyR 8–1 was incubated for 30 minutes at room temperature in PBS–ALB containing vimentin at a 10:1 molar ratio of vimentin to RyR 8–1, and then the antibodies were handled as previously described for tissue labeling. Confocal images were obtained with a Bio-Rad MRC–1000 Zeiss Axiosvert 35 laser scanning confocal imaging system equipped with a krypton-argon laser and an oil-immersion lens (X40; numerical aperture, 1.3) or a water-immersion lens (X40; numerical aperture, 1.2). The laser was fitted with either a blue (excitation, 488 nm) or a yellow (excitation, 568 nm) filter block.

Staining of the SR for Electron Microscopy
Guinea pig aorta and vas deferens smooth muscle were fixed overnight at 4°C in 2% glutaraldehyde and postfixed in osmium ferrocyanide to stain selectively the SR network, as previously described by Nixon et al.6 Cryosectioning was performed on a Leica Frigocut 2800 E (Leica Instruments GmbH) at −100°C. Sections (∼100 nm thick) were then transferred from the knife edge using a wire loop containing a droplet of 2.3 mol/L sucrose in 0.1 mol/L phosphate buffer. The droplet was removed from the chamber, thawed completely at room temperature, and touched to a Formvar (Ernest F. Fullam, Inc)–coated gold 1.2-μm mesh (diluted 1:1000) overnight at 4°C. Control sections were exposed only to secondary antibody. Grids were washed in six changes of PBS/BSA for a total of 30 minutes and incubated for 1 hour with a goat anti-rabbit F(ab′)2 fragment covalently linked to a 1.4-nm gold particle (diluted 1:100, Nanoprobes, Inc). After 1 hour of incubation at room temperature with the secondary antibody, grids were washed once in PBS–ALB and then in three additional changes of PBS for a total of 20 minutes. Sections were postfixed in PBS with 2% glutaraldehyde for 10 minutes and washed in three changes of distilled H2O (total time, 15 minutes). Gold particles were silver-enhanced using an HQ silver enhancement kit (Nanoprobes, Inc) and then washed in three changes of distilled deionized H2O. Sections were simultaneously stained and embedded by incubating them in 1% polyvinylalcohol in a 2% organotungsten stain (Nanoprobes, Inc) for 10 minutes (modified from Reference 24). Grids were removed from the droplet with a wire loop, and excess solution was removed by touching the grid edge to Whatman’s No. 50 filter paper and examined in a Philips CM12 electron microscope at 120 kV.

Gold particles were counted in sections incubated with anti–RyR 8 antibody to quantify their location within the cell. Cell profiles were divided into six distinct areas: peripheral SR, central SR, mitochondria, nucleus, cytoplasm, and extracellular space. The number of gold particles within each of these areas was recorded. SR of more than three caveolar lengths from the cell membrane was regarded as central SR.25 Only profiles of whole cells with the entire cell area visible (25 cells for each antibody) were used for particle counting.

Results

Anti–RyR 8 Antibody Specificity
The specificity of anti–RyR 8–1 for the ryanodine receptor has previously been determined15 using immunoblots of purified skeletal RyR, skeletal muscle terminal cisternae, and crude membrane preparations of rabbit skeletal and cardiac muscle and bovine endothelial cells. However, in the course of subsequent immunoelectron microscopic experiments, later preparations of anti–RyR 8–1 were found to also label, in addition to SR, intermediate filaments. Therefore, Western blot and dot blot membranes containing purified vimentin (the major intermediate filament protein in aortic smooth muscle) and RyR were probed with anti–RyR 8–1; this antibody bound to both RyRs and vimentin. To explore this cross-reactivity further, Western blots of cell homogenates of aorta and vas deferens were probed with anti–vimentin antibody (Fig 1B). Aortic smooth muscle proteins showed one band of reactivity at ∼60 kD with several lower molecular weight bands (presumably breakdown products of vimentin). The anti–vimentin antibody failed to detect any proteins in homogenates of vas deferens, which is consistent with reports that 10-nm filaments in nonvascular smooth muscle (from gut and urogenital sources) are composed of desmin rather than vimentin.26 Therefore, anti–RyR 8–1 could be used for selectively labeling RyR in the vas deferens, but not aortic smooth muscle or endothelial cells, which express vimentin.26–28

The second antibody, anti–RyR 8–2, was also tested for specificity. Western blots of membrane preparations from guinea pig vas deferens, aortic, and cardiac muscle revealed a single high–molecular-weight band at ∼400 kD when probed with anti–RyR 8–2 (Fig 1A). Preadsorption of anti–RyR 8–2 with peptide antigen blocked the recognition of the ∼400-kD band found on blots of smooth muscle membrane fractions from guinea pig aorta, vas deferens, and cardiac muscle. Two low–molecular-weight bands (∼80 and 100 kD) on blots of aortic smooth muscle membrane preparations presumably represent degradation products of the ryanodine receptor, as they were not seen on other immunoblots. Anti–RyR 8–2
bound to the RyR protein from skeletal muscle terminal cisternae but showed no cross-reactivity with vimentin on dot blots containing purified pig lens vimentin.

The specificity of a third RyR antibody, mAb110E, was tested on whole tissue homogenates from guinea pig skeletal muscle (psoas), cardiac muscle, aorta, and ileum by Western blotting. Because of the large amount of guinea pig tissue required to prepare membrane fractions, we determined the reactivity of the mAb110E on whole homogenates, rather than membrane preparations, of skeletal, cardiac, and smooth muscle of the aorta and ileum. A single high-molecular-weight (≈400-kD) protein band was present in skeletal muscle, and a typical doublet was seen in the cardiac lane. There was weak signal in the same region in the lane containing homogenate of ileum smooth muscle; however, no signal was detected in the lane containing aorta homogenate. The abundant signal in the striated compared with the smooth muscle lanes (at similar total protein loading) reflects the much higher content of junctional SR, in skeletal and cardiac muscle.

Immunofluorescent Localization of Anti-RyR Binding Sites

Labeling of skeletal muscle with either anti–RyR 8–1 or anti–RyR 8–2 produced periodic double rows of punctate fluorescence (Fig 2). The distance from the center of one double row of fluorescence to the next was ≈3.0 to 3.6 μm. Double-labeling experiments with anti–RyR 8–1 and phalloidin-FITC (the latter to identify F-actin in the I band) established that the punctate fluorescence, from TRITC-conjugated secondary antibody bound to anti-RyR, was located at the A-I junction where the T tubules form triad junctions with the terminal cisternae. Sections labeled with either anti–RyR 8–1 or anti–RyR 8–2 revealed a similar pattern of fluorescent signal; however, the double row of fluorescence was more clearly defined with anti–RyR 8–2 (see Fig 2).

Anti–RyR 8–2 binding was also apparent in longitudinal sections of thoracic aorta smooth muscle (Fig 3a and 3b). Fluorescence was present in both the vascular endothelial cells, as previously shown with anti–RyR 8–1,15 and in subendothelial smooth muscle cells that alternate with rows of elastic

Figure 1. A, Western blot of membrane preparations from guinea pig cardiac (C), vas deferens (Vas), and aortic (Ao) smooth muscle. Membrane preparations were size-fractionated by SDS-PAGE on 6% gels, transferred to PVDF membranes, and blotted with anti–RyR 8–2. These blots demonstrate the presence of immunoreactive proteins in both Vas and Ao smooth muscle membrane preparations. B, Western blot analysis of homogenates from guinea pig Vas and Ao smooth muscle. The homogenates were fractionated by SDS-PAGE, transferred to PVDF membranes, and probed with anti-vimentin antibody. Vimentin is present in the Ao homogenates but absent in the Vas.

Figure 2. Photomicrograph of guinea pig psoas muscle labeled with either anti–RyR 8–1 (left panel) or anti–RyR 8–2 (right panel) and then a TRITC-conjugated secondary antibody. Both images show the presence of anti-RyR antibody binding sites in skeletal muscle and confirm previous studies demonstrating the presence of RyRs in skeletal muscle at the A-I interface. Labeling experiments of skeletal muscle consistently demonstrated double rows of punctate fluorescence separated from each other by ≈3.0 to 3.6 μm.
lamellae in the media. Fluorescent labeling of cytoplasmic structures was varied, inhomogeneous, and seen in every cell. Frequently, on close inspection, the staining pattern was meshlike (arrows in Fig 3a), suggestive of the SR network. Nuclei were largely void of signal, but in some cells, there was increased labeling at the nuclear poles in both endothelial and aortic smooth muscle cells (see Fig 3a). Control tissue sections labeled with either antigen-preadsorbed antibody or TRITC-conjugated donkey anti-rabbit F(ab')2 revealed no intracellular fluorescence when imaged under identical conditions (pinhole, laser intensity) of confocal signal detection.

Immunoelectron micrographs of aortic smooth muscle cells labeled with anti–RyR 8–1 revealed that the anti–RyR 8–1 used in our initial experiments,15 but not the anti–RyR 8–2, also labeled (in addition to SR-associated epitopes) intermediate (10-nm) vimentin filaments. In control experiments, similar patterns of anti–RyR 8–1 and anti-vimentin labeling of intermediate filaments were present in cultured rat aortic smooth muscle cells treated with colcemid to induce the formation of intermediate filament cables.22 Sections of thoracic aorta labeled with anti-vimentin antibody showed intense cytoplasmic fluorescence in the endothelial cells and weaker signal in the cytoplasm of the smooth muscle cells. We were unable to adsorb vimentin-binding epitopes in the anti–RyR 8–1 by preincubating the antibody with purified pig lens vimentin before labeling tissue sections.

Figure 3. a, Confocal photomicrograph of guinea pig aorta labeled with anti–RyR 8–2 and then with a TRITC-conjugated secondary antibody. There is cytoplasmic labeling of both endothelial and aortic smooth muscle cells, and the label correlates with the distribution of the SR in both cell types. Arrows indicate a meshlike staining pattern in two separate cells, suggestive of the SR network. b, Higher magnification view of guinea pig aorta showing similar patterns of immunolabeling with anti–RyR 8–2.
The possibility that the smaller (difference contrast microscopy is not confocal, we cannot exclude differential interference contrast microscopy. However, because differential interference contrast microscopy is not confocal, we cannot exclude the possibility that the smaller (~3-μm-diameter) fluorescence profiles were due to perinuclear staining rather than labeling at the periphery of tapered cells in transverse sections. Asterisks are placed at the centers of selected cells. Arrows show punctate fluorescence at the cell periphery.

Sections of thoracic aorta labeled with mAb110E showed patterns of fluorescence in endothelium and smooth muscle that were similar to sections labeled with the polyclonal anti–RyR 8–2. Confocal microscopy with ~1-μm optical slices through the center of a cell showed no significant nuclear labeling with any of the three RyR antibodies. Control sections exposed only to secondary antibody showed no significant fluorescent signal when imaged at similar conditions (pinhole and laser intensity) of confocal signal collection.

Guinea pig vas deferens cut in transverse orientation and labeled with anti–RyR 8–2 revealed inhomogeneous circumferential fluorescence (see Fig 4). The periphery of some cells showed a punctate pattern of fluorescence (arrows in Fig 4), similar to the predominantly peripheral distribution of SR seen in osmium ferrocyanide–treated cells (see Fig 6). When viewed with similar image-detection parameters, the quantity of fluorescence seen in these images was significantly less than in sections of aorta, consistent with the smaller volume of SR in the vas deferens.

Electron Microscopy of Aortic and Vas Deferens Smooth Muscle Postfixed With Osmium Ferrocyanide

Electron microscopy of guinea pig aorta treated with osmium ferrocyanide showed an extensive network tubular staining throughout the cytoplasm. As seen in Fig 5, there is an interconnecting reticulum that is continuous with the outer nuclear envelope, extends throughout the cytoplasm frequently encircling the mitochondria, and forms apparent surface couplings with the plasma membrane. Onemicron-thick sections, imaged at 200 keV to visualize the same tissue volume imaged with confocal microscopy (see Fig 5c and 5d), showed that the superimposition of the SR tubules in the thick sections gives rise to a dense, patchy, meshlike network, which, when viewed at a magnification similar to the fluorescent images (insert, Fig 5d), resembles the fluorescence in Fig 3a and 3b.

Electron micrographs of guinea pig vas deferens postfixed with osmium ferrocyanide, in contrast to those of the aorta, showed a less dense reticular network located predominantly at the periphery of the cells and at the nuclear poles (see Fig 6). Similar to aortic smooth muscle, the reticulum is continuous with the outer envelope and is present in the cytoplasm. Interestingly, the outer mitochondrial membranes were often very close (<20 nm) to elements of the SR network, which at times encircled an entire mitochondrion (arrows in Fig 6c).

**Immunogold Labeling**

In cryosections of vas deferens (which contains no detectable vimentin) immunolabeled with anti–RyR 8–1, silver-enhanced gold particles were seen on SR membranes, predominantly near the plasma membrane (Fig 7A and 7B). Occasional immunogold labeling was also seen in central areas of the cell and on vesicular membranes of the perinuclear SR (Fig 7C), consistent with the sparse central SR in this muscle. Anti–RyR 8–1 did not label caveolae, mitochondria, nuclei, or the nuclear membrane. The extracellular space contained only occasional gold particles. Paired control sections, which were incubated with primary antibody or treated with preimmune rabbit serum, also contained a negligible number of gold particles. Attempts to immunogold label cryosections of vas deferens with anti–RyR 8–2 were unsuccessful, even at low dilutions of this primary antibody.

The number of gold particles counted in each area of the vas deferens cells labeled with anti–RyR 8–1 is shown in Fig 8. Not all elements of the SR were labeled, but gold particles on the SR (central plus peripheral) accounted for ~90% of the total particles counted. The majority of particles per cell cross section were peripheral, consistent with the distribution of the SR. Binding of gold particles to central SR was ~50% to 60% of that found on peripheral SR. The cytoplasm, extracellular space, mitochondria, and nuclei all contained a much lower density of gold particles.

**Discussion**

Cloning studies have identified a family of RyRs encoded by three distinct genes (ryr1, ryr2, and ryr3).29–31 The peptide sequence used to generate anti–RyR 8 (C-LEFDGLYITE-QPGDDDDVKGQ) is a skeletal muscle sequence (ryr1)32 that shares ~85% homology with both ryr2 (cardiac)33 and ryr3 (brain and smooth muscle).34 Monoclonal antibody mAb110E recognizes one or more of the RyR isoforms present in cardiac tissue and brain, although its epitope has not been mapped.18 Vascular smooth muscle is reported to contain predominantly ryr334; however, mRNA transcripts for all three isoforms have been identified in various smooth muscles using reverse transcription–polymerase chain reaction.35 mRNA transcripts for ryr3 and small amounts of ryr2 have been found in aortic smooth muscle.36

Our results indicate that RyRs are present in guinea pig aorta and vas deferens smooth muscle and are localized to both the central and peripheral SR. The labeling of the triadic region in guinea pig skeletal muscle with anti–RyR 8 con-
Figure 5. Electron micrograph of guinea pig aorta treated with osmium ferrocyanide to selectively stain the sarcoplasmic and endoplasmic reticulum, showing an extensive network of reticulum throughout the cytoplasm. a, A 70-nm-thick section showing portions of two longitudinally oriented guinea pig smooth muscle cells lying between elastic lamellae (el). The densely stained reticulum is continuous (small arrows) with the nuclear envelope and extends through the cytoplasm to the plasma membrane, forming surface couplings (large arrows). Nuc indicates nucleus. b, Obliquely oriented 70-nm-thick section of an aortic cell showing tubules of reticulum in the central regions of the cell extending to the periphery, forming surface couplings with the plasma membrane (small arrows). Mitochondria (m) are frequently surrounded by closely apposed elements of reticulum. c and d, Longitudinal views from the same osmium ferrocyanide–stained blocks of aorta shown in panels a and b, but in this case, sections were cut at a 1-μm thickness for comparison with the 1-μm optical sections used for the immunofluorescence studies. Note the high density of reticulum membranes, which appear connected throughout the cell and surround a cluster of mitochondria (m) in panel c. The image of the cells in panel d has been reduced to a size comparable to that shown in the confocal immunofluorescence images. Note that immunolabeling of ryanodine receptors associated with this dense complex network of reticulum could give rise to the patchy meshlike staining seen in the confocal fluorescence images.
firmed previous studies localizing the foot structures (RyR-1) to the terminal cisternae of the SR in the triad (References 37 and 38 and review in Reference 39) and also established the efficacy of these antibodies for immunolabeling. We now show that with the RyR-specific antibodies (anti–RyR 8–2 and mAb110E), labeling occurs at the nuclear poles and in irregular patches in the cytoplasm of endothelial cells, in agreement with our earlier results that were produced with the less specific anti–RyR 8–1.15 These results are consistent with the distribution of the endoplasmic reticulum.

The preponderance of the label at the plasma membrane of vas deferens cells, the patchy cytosolic distribution of immunofluorescence in 1-μm-thick optical slices of aortic smooth muscle labeled with either polyclonal anti–RyR 8–2 or monoclonal mAb110E antibody, and immunoelectron microscopic analysis of the vas deferens using anti–RyR 8–1 were consistent with the known distribution of SR. This has been shown with selective SR staining by osmium ferrocyanide in the aorta and vas deferens (the present study) and with conventional electron microscopy of the aorta.25 The SR in smooth muscle (≈5% of the fractional volume of the cytoplasm in the aorta) forms a continuous network, is contiguous with the nuclear envelope, frequently associates with mitochondria, and approaches the plasma membrane to form surface couplings.25,26 These data strongly suggest that the location of the RyRs in smooth muscle reflects the different distribution of SR in the two tissues examined. Furthermore, they also demonstrate that RyRs are not confined to surface couplings but are distributed over most of the SR network. Surface couplings in smooth muscles consist of junctional SR separated from the plasma membrane by an ≈18-nm gap traversed by periodic bridging structures. These electron-dense structures are reminiscent of RyRs localized to “foot processes” connecting the SR and plasma membranes/T tubules in skeletal and cardiac muscle.10,40,41 However, on close examination, the bridging structures are different, in that the spacing of feet between bridging structures is greater and the density is less than in striated muscles.42,43 Considering the localization of RyRs to terminal cisternae, it may seem surprising that in smooth muscle RyRs are present on both central and peripheral SR. However, this need not imply similar excitation-contraction coupling mechanisms and raises the possibility of different mechanisms gating the RyRs in peripheral SR and central SR, respectively.25 Electron probe x-ray microanalysis has shown that Ca²⁺ is sequestered in both central and peripheral SR and that in norepinephrine-stimulated muscles the Ca²⁺ content is decreased, compared with unstimulated muscles, in both regions.44,45 The presence of RyRs on the central SR of smooth muscles, which lack transverse tubules, is also consistent with some findings in skeletal and cardiac muscle. Dulhunty et al.46 using immunogold labeling techniques, detected RyRs in rat skeletal muscle not only on the junctional face of the terminal cisternae but also on the extrajunctional SR, and Jorgensen et al.47 found RyRs in corbular SR, a bulbous protrusion of cardiac muscle SR containing calsequestrin and Ca²⁺, but distant from transverse
tubules. They suggested that these receptors may release Ca\(^{2+}\) directly into the myoplasm rather than into the triad junction. In rat papillary muscle, Jorgensen et al\(^{47}\) also demonstrated the presence of ryanodine receptors in both junctional SR and surface couplings, both of which also store Ca\(^{2+}\).\(^{48}\) The extended junctional SR of avian cardiac muscle also contains functional RyRs.\(^ {49}\)

IP\(_3\) receptors are also present in both peripheral and central SR in vas deferens,\(^ {5}\) suggesting a structural overlap between the IP\(_3\)-sensitive Ca\(^{2+}\) pools and the ryanodine-sensitive Ca\(^{2+}\) pools in both types of smooth muscle, as in cerebellar Purkinje neurons.\(^ {50}\) Analytical subfractionation has also detected significant colocalization of both IP\(_3\) and RyR\(_{s}\) in the SR of intestinal smooth muscle.\(^ {11}\) Well-controlled double-labeling experiments of the IP\(_3\) receptor and RyR\(_{s}\) at electron microscopic resolution would, however, be necessary to define their proximity.

Although IP\(_3\) is thought to be the physiologically important mediator of pharmacomechanical Ca\(^{2+}\) in smooth muscle (reviewed in References 1 and 51), vascular and other smooth muscles also possess a caffeine-sensitive intracellular Ca\(^{2+}\) store that contains sufficient Ca\(^{2+}\) to produce, when released, tension development.\(^ {45,52}\) Ca\(^{2+}\)-induced Ca\(^{2+}\) release from ryanodine receptors, similar to that seen in cardiac\(^ {53,54}\) and skeletal muscle (reviewed in Reference 39), also occurs in various smooth muscles.\(^ {7,55-58}\) Localized domains of high [Ca\(^{2+}\)] may exist near surface couplings of junctional SR\(^ {59}\) sufficient to raise local Ca\(^{2+}\) to the \(\approx 1-\mu\)mol/L level required to trigger Ca\(^{2+}\)-induced Ca\(^{2+}\) release.\(^ {7,55,59}\) Caffeine and ryanodine-sensitive Ca\(^{2+}\) spikes originating from the peripheral SR of vascular smooth muscle.

**Figure 7.** Immunoelectron micrographs of guinea pig vas deferens cryosections labeled with anti–RyR 8–1 and negatively stained with organotungsten. In panel A, silver-enhanced gold particles (marked with large arrows) can be seen adherent to internal membranes structures located close to the plasma membrane (marked with arrowheads of portions of two adjacent cells). For comparison, a mitochondrion (M) can be seen in this micrograph. A similar distribution of gold label in the peripheral SR membranes is noted in the micrograph in panel B of portions of two adjacent cells. In some sections, vesicular membranes of the perinuclear SR were labeled, as in panel C. A large cluster of silver-enhanced gold particles is marked by an arrow, and individual particles associated with membranes are indicated by arrowheads.
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have been observed and are, in many cells, coupled to the activation of transmembrane cation currents.\(^1\) Ca\(^{2+}\) sparks contribute to relaxation of cerebrovascular smooth muscle via activation of a plasma membrane Ca\(^{2+}\)-sensitive K\(^+\) channel and plasma membrane hyperpolarization.\(^6\) Activation of Ca\(^{2+}\) release from peripheral RyRs could, then, be gated by dihydropyridine receptors, as in skeletal muscle, or by Ca\(^{2+}\), as in cardiac muscle cells.\(^6\) Activation of RyRs in the central SR, however, would require a diffusible second messenger like Ca\(^{2+}\) or cADPR-ribose.\(^6\) During excitation-contraction coupling, other factors, such as phosphorylation\(^6,66\) and/or modulators of Ca\(^{2+}\) release from the RyRs, including Mg\(^{2+}\), Ca\(^{2+}\), pH, calmodulin, and adenine nucleotides,\(^6\) may also alter the physiological responsiveness of these channels in vivo.

Acknowledgments

This study was funded by Public Health Service grant 1K11 AR01871 (Dr Lesh) and National Institutes of Health grants HL-48807 (Drs A.P. and A.V. Somlyo) and HL-32711 (Dr Fleischer). The authors wish to acknowledge Dr Anthony Timmerman for affinity-purifying anti–RyR antibodies (Dr Lesh) and National Institutes of Health grants HL-48807 (Drs A.P. and A.V. Somlyo) and National Institutes of Health grants HL-48807 (Drs A.P. and A.V. Somlyo). This study was funded by Public Health Service grant 1K11 AR01871 (Dr Lesh) and National Institutes of Health grants HL-48807 (Drs A.P. and A.V. Somlyo) and National Institutes of Health grants HL-48807 (Drs A.P. and A.V. Somlyo). This study was funded by Public Health Service grant 1K11 AR01871 (Dr Lesh) and National Institutes of Health grants HL-48807 (Drs A.P. and A.V. Somlyo). This study was funded by Public Health Service grant 1K11 AR01871 (Dr Lesh) and National Institutes of Health grants HL-48807 (Drs A.P. and A.V. Somlyo).

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Circ Res. 1998;82:175-185
doi: 10.1161/01.RES.82.2.175

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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