Inositol 1,4,5-Trisphosphate Receptor Expression in Cardiac Myocytes

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Abstract. Calcium release from intracellular stores is the signal generated by numerous regulatory pathways including those mediated by hormones, neurotransmitters and electrical activation of muscle. Recently two forms of intracellular calcium release channels (CRCs) have been identified. One, the inositol 1,4,5-trisphosphate receptors (IP3Rs) mediate IP3-induced Ca^{2+} release and are believed to be present on the ER of most cell types. A second form, the ryanodine receptors (RYRs) of the sarcoplasmic reticulum, have evolved specialized functions relevant to muscle contraction and are the major CRCs found in striated muscles. Though structurally related, IP3Rs and RYRs have distinct physiologic and pharmacologic profiles. In the heart, where the dominant mechanism of intracellular calcium release during excitation-contraction coupling is Ca^{2+}-induced Ca^{2+} release via the RYR, a role for IP3-mediated Ca^{2+} release has also been proposed. It has been assumed that IP3Rs are expressed in the heart as in most other tissues, however, it has not been possible to state whether cardiac IP3Rs were present in cardiac myocytes (which already express abundant amounts of RYR) or only in non-muscle cells within the heart. This lack of information regarding the expression and structure of an IP3R within cardiac myocytes has hampered the elucidation of the significance of IP3 signaling in the heart. In the present study we have used combined in situ hybridization to IP3R mRNA and immunocytochemistry to demonstrate that, in addition to the RYR, an IP3R is also expressed in rat cardiac myocytes. Immunoreactivity and RNAse protection have shown that the IP3R expressed in cardiac myocytes is structurally similar to the IP3R in brain and vascular smooth muscle. Within cardiac myocytes, IP3R mRNA levels were ~50-fold lower than that of the cardiac RYR mRNA. Identification of an IP3R in cardiac myocytes provides the basis for future studies designed to elucidate its functional role both as a mediator of pharmacologic and hormonal influences on the heart, and in terms of its possible interaction with the RYR during excitation–contraction coupling in the heart.

INOSITOL 1,4,5-trisphosphate (IP3)[1] is an important second messenger regulating intracellular Ca^{2+} concentration (Berridge and Irvine 1984; 1989). Membrane receptor activation leading to G-protein–coupled phosphoinositide hydrolysis results in the generation of IP3 and subsequent release of Ca^{2+} from ER stores. Significant advances in understanding the molecular basis of IP3-stimulated intracellular Ca^{2+} release have been made in recent years. Chief among these has been the identification of the inositol 1,4,5-trisphosphate receptors (IP3R) which are intracellular calcium release channels on the ER. In many cell types the IP3 signaling pathway is the primary mechanism governing intracellular calcium release. In contrast, in the heart the major pathway for intracellular calcium release involves the ryanodine receptor (RYR) on the sarcoplasmic reticulum (reviewed in Bers, 1991). The RYR is a member of the same gene family as the IP3R, but has evolved specialized functions relevant to excitation–contraction coupling in striated muscles (Fleischer and Inui, 1989; Catterall, 1991).

It has been proposed that hormonal regulation of cardiac contractility may be mediated by IP3-induced intracellular calcium release. However, in the two types of cells which already contain a major non-IP3–responsive pathway for intracellular calcium release (the RYRs in cardiac and skeletal muscle) it has not previously been established that IP3Rs are expressed, nor has the structure of the IP3Rs in striated muscle been characterized. Indeed, the significance of IP3-induced intracellular calcium release in the heart has been hotly debated in recent years. This debate has focused on conflicting data (summarized below) regarding the effects of IP3 on cardiac muscle contraction and calcium release.

In the present study we have used combined in situ hybridization and immunocytochemistry to demonstrate conclu-

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1. *Abbreviations used in this paper*: CRC, calcium release channel; E-C, excitation–contraction; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; IP3, inositol 1,4,5-trisphosphate; IP3R, IP3 receptor; RYR, ryanodine receptor; SR, sarcoplasmic reticulum.

sively that an IP3R is expressed in rat cardiac myocytes. We have shown that a single cell type, cardiac myocytes, express both forms of calcium release channels characterized to date, IP3R and RYR. On the basis of immunoreactivity and RNAse protection we have demonstrated that the cardiac IP3R is structurally similar to that expressed in the brain and vascular smooth muscle. These findings form the basis for further studies designed to elucidate the molecular events associated with IP3 signaling in cardiac myocytes.

Materials and Methods

cDNA Cloning and Sequencing

A 946-bp mouse aortic smooth muscle IP3R cDNA (Marks et al., 1990) was used for Northern blot analyses (see below) and to isolate a 1.7-kb rat aortic smooth muscle IP3R cDNA (R-IP3R-1) from a random primed rat aortic smooth muscle cDNA library constructed in λ ZAP II (a gift from Dr. Mark Taubman, Mount Sinai School of Medicine, New York, NY). This 1.7-kb cDNA, R-IP3R-1, was sequenced using the dideoxy chain termination methodology and an automated sequencer (model 373; ABI Adv. Biotechnologies, Inc., Columbia, MD). Sequences were edited and compared to the Genbank database using MacVector software (v3.5.3). Compared to the mouse aortic smooth muscle IP3R (Marks et al., 1990) R-IP3R-1 corresponded to nucleotides 987-2705. The procedures used for library screening and isolation of cDNA clones were performed as described previously (Marks et al., 1989).

RNA Preparation, RNAse Protection, and Northern Blot Analysis

RNA was prepared from rat tissue ground with a tissue-mizer (Tekmar, Cincinnati, OH) then purified using standard guanidinium-thiocyanate lysis buffer and centrifugation through a cesium chloride cushion as previously described (Marks et al., 1989). RNA was size separated on formaldehyde/agarose gels and Northern blot transfer was carried out overnight using 10× SSC. Northern blot analysis was as previously described (Marks et al., 1989); hybridization was performed at 42°C overnight and washing was at 55°C in 0.2× SSC. Northern blot analysis was performed with an IP3R cDNA probe corresponding to the sequence of the cRNA probe used for in situ hybridization to demonstrate that this probe only hybridized to a single mRNA of 10 kb in size. Films were autoradiographed with a single intensifying screen at −70°C.

RNAse protection was performed using the same cRNA probes (sense and antisense) as those used for in situ hybridization (see below) except that the radionucleotide was ^{32}P instead of ^{35}S. Total RNA samples from adult rat were hybridized overnight at 45°C with 8×10^4 cpm of the respective radiolabeled riboprobe using reagents from a ribonuclease protection assay kit (Ambion Inc., Austin, TX) following the recommended procedures. Samples were resuspended in 50% deionized formamide, heated at 95°C and electrophoresed in 6–8% denaturing polyacrylamide gels.

Northern blot analyses were performed with the following probes: (*a*) IP3R1, a 946-bp mouse aortic smooth muscle IP3R cDNA corresponding to nucleotides 817 to 1763 of the mouse brain IP3R sequence (Marks et al., 1990); (*b*) HCRC1, a 580-bp rabbit cardiac RYR cDNA corresponding to nucleotides 5027-5647 of the cDNA encoding the rabbit cardiac RYR (Brillantes et al., 1992); (*c*) the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) probe was a 1.3-kb PstI fragment from clone pUC-GAPDH13 containing the entire coding region and part of the 3′-untranslated region of the rat GAPDH (Fort et al., 1985). All probes were uniformly labeled with random primers using Klenow and α-P^{32} dCTP to a specific activity of $>10^9$ cpm per μg. mRNA levels were determined by Northern blot analyses using laser densitometry and the Image 1.36 software for data analysis on a MacIntosh computer.

Antibody Preparation and Western Blot Analysis

A synthetic peptide, IP3R2690, based on the sequence of amino acid residues 2690-2700 ([Cys]-Glu-Gln-Asn-Glu-Leu-Arg-Asn-Leu-Gln-Glu-Lys-Leu, with an amino terminal cysteine added) of the murine brain IP3R (Furuichi et al., 1989) was synthesized using an ABI peptide synthesizer. 10 mg of this synthetic peptide were coupled to keyhole lympet hemocyanin in the presence of 0.3% glutaraldehyde following standard protocols (Harlow and Lane, 1988). A New Zealand White rabbit was immunized with 1 mg of peptide–keyhole lympet hemocyanin conjugate in complete Freund's adjuvant, followed by 0.5-mg booster injections subcutaneously at 3, 5, and 7 wk in incomplete Freund's adjuvant. The rabbit was bled and serum obtained 12 d after the final injection. IgG was purified from the antiserum by passing through Econopac IgG purification columns (Pharmacia Fine Chemicals, Piscataway, NJ). To affinity purify the IP3R antibody, synthetic IP3R peptide (used as antigen) was coupled to CNBr-activated Sepharose as per manufacturer's instructions (Pharmacia Fine Chemicals). 5 mg of synthetic peptide was coupled to 1 g of CNBr-activated Sepharose and equilibrated in PBS. Purified IgG was then passed through the peptide-CNBr–activated Sepharose columns, washed with PBS, BBS-Tween (0.1 M boric acid, 25 mM sodium borate, 1 M sodium chloride, and 0.1% Tween 20), and then PBS again. The bound antibody was eluted with 4 M guanidine-HCl, 10 mM Tris, pH 8.0, and the eluate was dialyzed overnight against 50 mM Hepes, pH 7.4, 100 mM NaCl at 4°C.

The specificity of the resulting anti-IP3R antibody was determined by immunoblots of rat tissue homogenates. SDS-PAGE was performed on 1.0-mm thick 6% gels. Size fractionated proteins were transferred to polyvinylidene difluoride membrane using a semi-dry blotting apparatus from Bio-Rad Laboratories (Cambridge, MA) at 25 mV for 1 h. Membranes were prewetted with 100% methanol, washed in distilled water, and equilibrated with Tris-glycine buffer, pH 8.3. Membranes were blocked with 5% dry milk in TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) at 23°C for 1 h. Blots were incubated with affinity-purified antibodies overnight at 4°C in the same buffer diluted at 1:50. Membranes were washed four times for 10 min each in PBST (PBS with Tween-20) then incubated with a 1/2,500 dilution of peroxidase-conjugated horse anti-rabbit Ig (Bio-Rad Laboratories) for 1 h. Immunodetection was carried out using ECL (chemiluminescence reagent; Amersham Corp., Arlington Heights, IL) followed by exposure to autoradiographic film.

In Situ Hybridization Combined with Immunocytochemistry

IP3R (rat) cDNAs were subcloned into pBluescript (Stratagene, La Jolla, CA) using standard methods (Sambrook et al., 1989). RNA probes for in situ hybridization were synthesized using either the T3 and T7 RNA polymerases (Ambion Inc.). Antisense and sense probes were transcribed from linearized plasmids in the presence of 50 μCi [^{35}S]CTP (New England Nuclear, Boston, MA). A 1.0-kb antisense-strand cRNA probe was synthesized by linearizing R-IP3R-1 with HindIII and using T3 RNA polymerase; a 1.3-kb sense-strand probe was synthesized by linearizing R-IP3R-1 with BamHI and using T7 RNA polymerase. Tissue samples from adult rat heart were prepared for in situ hybridization as described elsewhere with several modifications (Cox et al., 1984; Kinter and Melton, 1987). Specifically, whole hearts were removed under anesthesia and fixed for 30 min in 4% paraformaldehyde in 0.1 M PBS. The hearts were subsequently cut along the sagittal plane and further fixed in 4% paraformaldehyde in 0.1 M PBS overnight. Tissue was cryoprotected in 0.5 M sucrose in 0.1 M PBS for 30 min and further cryoprotected in 1 M sucrose and 0.1 M PBS for 30 to 45 min. Tissue was frozen in Cryo-Embed compound in liquid nitrogen and stored at −80°C until use. Sections, 6–8-μm thick, were obtained by using an IBHI (Hacher) cryostat and collected onto coated slides (Superfrost plus; Fisher Scientific Co., Pittsburgh, PA). Sections were refixed with 4% paraformaldehyde for 20 min, rinsed in 2× SSPE, incubated for 30 min with proteinase K (3 mg/ml in 0.1 M Tris, pH 7.5, 0.01 M EDTA), rinsed, incubated in 0.2 N HCl, rinsed, treated with acetic anhydride (0.25% in 0.1 M triethanolamine buffer, pH 8.0) for 10 min, rinsed, and hybridized overnight.

Hybridization was performed in 50% formamide, 0.3 M NaCl, 10% dextran sulfate, 2 mM EDTA, 1× Denhardt's, 0.01 M Tris HCl, pH 8, 0.05 M DTT. Approximately $5–6 \times 10^6$ cpm of each probe was applied to individual slides. Slides were rinsed in 4× SSPE and 10 mM DTT, washed in 2× SSPE and 10 mM DTT for 1 h at room temperature, and treated with 20 μg/ml RNAse in 4× SSPE and 10 mM DTT for 30 min at 37°C. Slides were then washed at 60°C for 1 h in 50% formamide, 2× SSPE and 10 mM DTT, transferred to 0.3 M ammonium acetate, 1% glycerol before dipping in NTB2 autoradiographic emulsion (Eastman Kodak Co., Rochester, NY). Slides were exposed for 1 wk, developed in D-19 developer (Eastman Kodak Co.) for 2.5 min, rinsed in 0.2% acetic acid for 10 s, fixed for 5 min, and rinsed in water. Slides were stained with hematoxilin to visualize the nuclei.

Specimens were photographed using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with a darkfield condenser.

Additional tissue sections were analyzed using combined in situ hybridization and immunocytochemistry. These sections were prepared as in the in situ hybridization protocol (see above) with the following modifications: sections were fixed in 4% paraformaldehyde in PBS for 20 min, rinsed once in 3× PBS and twice in PBS for 5 min, washed in 2× SSPE, and incubated with proteinase K (1.5 mg/ml) at 37°C for 15 min 1 × 10^6 cpm of probe was added to the hybridization solution. Washing was with 55% formamide at 55°C. Slides were washed twice with PBS and prepared for immunocytochemical staining as per the protocol in the Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA). Sections were pretreated with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature to quench endogenous peroxidases followed by washing with PBS × 3 for 10 min each. Sections were blocked with goat serum (Vectastain) and incubated for 30 min at room temperature. On some sections primary antibody was omitted after treatment with the goat serum. On these sections, stained with secondary antibody alone, no signal was detected (data not shown), indicating the absence of endogenous biotin and avidin which would have yielded a positive signal if present. Sections were incubated with either the anti-IP3R antibody (αIP3R-1), control pre-immune serum or pre-absorbed serum (see below) at 1:50 dilution overnight at 4°C. After a 1-h incubation, the antigen–antibody complex was detected using biotinylated goat anti-rabbit IgG at a 1:300 dilution and ABC reagents (Vector Laboratories Inc.). Peroxidase stain was developed with diaminobenzidene tetrahydrochloride and the sections were then dipped in autoradiographic emulsion following dehydration in graded alcohols containing 0.3 M NH_4AOc.

To demonstrate that αIP3R-1 was sequence-specific, the antibody was pre-absorbed with an excess of the antigenic peptide (~1 mg of IP3R peptide in 250 μl of a 1/50 dilution of antiserum) by incubation at room temperature for 2 h. (αIP3R-1 antiserum incubated without antigenic peptide at room temperature for 2 h retained immunoreactivity as determined by immunostaining of heart tissue sections; see Fig. 7 *A*). Pre-absorbed antibody was tested against the antigenic peptide on dot blots and against the IP3R protein on immunoblots, demonstrating no reactivity. Pre-absorbed antibody was used as a negative control for immunocytochemistry following the protocol described above.

Separately, an antibody which recognizes sarcomeric myosin heavy chain was used to identify cardiac myocytes in adjacent sections to those used for IP3R detection. This antibody, MF20, was provided by Dr. Donald Fischman (Cornell University Medical College, New York, NY). Sections from the rat heart were examined for endogenous avidin and biotin which might result in higher background using the ABC–immunoperoxidase reagents and no significant background signal was detected (data not shown).

Results

Specificity of the IP3R Riboprobe

IP3R riboprobes were synthesized from a cDNA template encoding a portion of the rat aortic smooth muscle IP3R (R-IP3R-1). This cDNA corresponds to nucleotides 987-2705 of the mouse aortic smooth muscle IP3R (Marks et al., 1990) and is >90% identical to the mouse cDNA sequence. Fig. 1 shows the specificity of the antisense and sense IP3R riboprobes used for in situ hybridizations. As demonstrated in Fig. 1 the antisense IP3R riboprobe identifies a fully protected fragment in total RNA from rat heart (Fig. 1, lane *1*), rat aortic smooth muscle cells (Fig. 1, lane *2*), and rat brain (Fig. 1, lane *3*). No hybridization is seen with the sense IP3R riboprobe corresponding to the same sequence as the antisense (Fig. 1, lane *4*). The undigested probe (Fig. 1, lane *5*) contains additional sequence from pBluescript vector and migrates slightly slower than the fully protected fragment. As a negative control no hybridization was seen with yeast RNA added to either antisense or sense riboprobe (data not shown).

Fig. 2 shows a Northern blot analysis of rat aortic smooth muscle total RNA demonstrating that a cDNA probe corresponding to the sequence of the riboprobe used for in situ hybridization binds to a single ~10-kB mRNA. A long exposure (10 d) is shown to demonstrate that no hybridization to minor species is seen.

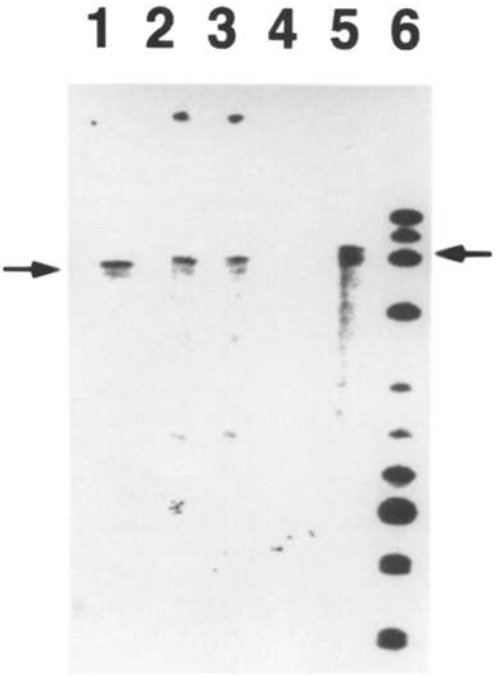


Figure 1. RNAse protection showing specificity of the IP3R riboprobes. Using the antisense-strand cRNA probe and total RNA (10 μg each sample) fully protected bands (870 nt, indicated by arrow at left) are seen in: rat heart (lane *1*); rat aortic smooth muscle (lane *2*), and rat brain (lane *3*). No protection is seen using the sense-strand probe with mouse heart RNA (lane *4*). Lane *5* shows the full-length probe (1,010 nt, indicated by arrow at right). Molecular weight markers are a labeled ϕX174 HaeIII digest (New England Biolabs).

Specificity of the IP3R Antibody

Anti-IP3R antibody was raised in rabbits against a synthetic IP3R peptide. After affinity purification this antibody reacted with a single ~260-kD band in a crude rat heart homogenate (Fig. 3, lane *1*) and rat brain homogenate (Fig. 3,

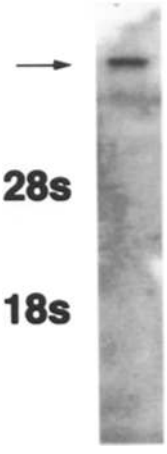


Figure 2. Northern blot analysis demonstrating specificity of the IP3R probe. The IP3R mRNA was detected as a single ~10-kb band in rat heart total RNA (30 μg) using an IP3R cDNA as probe corresponding to the sequence of the riboprobe used for in situ hybridization. The positions of the 28s and 18s ribosomal RNAs are depicted at left.

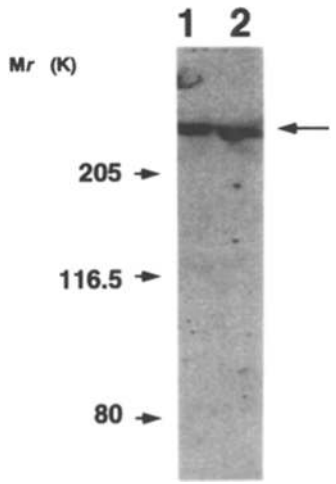


Figure 3. Western blot analysis demonstrating specificity of the rabbit anti-IP3R antibody. Rat heart and brain homogenates (30 μg total protein) were size fractionated on a 6% SDS-polyacrylamide gel, blotted to a polyvinylidene difluoride membrane, and probed with affinity-purified anti-IP3R antibodies. Lane *1*, rat heart; lane *2*, rat brain. A single band migrating at ~260,000 D is seen in both heart and brain, no additional non-specific or specific binding was seen. Antibody was used at a 1:50 dilution. Position of molecular weight markers are indicated: myosin (205 kD), β-galactosidase (116.5 kD), BSA (prestained, migrates at 80 kD).

lane *2*). A peptide blocking experiment was performed to further demonstrate the specificity of the α-IP3R-1 antibody. α-IP3R-1 antibody, pre-absorbed with buffer alone (PBS) retained immunoreactivity (see Fig. 7 *A*), however α-IP3R-1 antibody, pre-absorbed with an excess of the antigenic synthetic IP3R peptide demonstrated no immunoreactivity (Fig. 7 *B*). This experiment indicates the specificity of the α-IP3R-1 antibody used for immunolocalization of the IP3R in cardiac myocytes.

IP3R mRNA and Protein Are Expressed in Cardiac Myocytes

To determine whether the IP3R is expressed in cardiac myocytes or in non-muscle cells in the heart, combined in situ hybridization and immunocytochemistry was performed on whole heart sections from adult rat. IP3R protein was detected throughout the heart (Fig. 4, *A*, *B*, *D*, *E*, and *F*) and in smooth muscle cells of the aorta (positive control, Fig. 4 *C*). At higher magnification, IP3R protein was evident within the cardiac myocytes (Fig. 4, *E* and *F*). The identity of cells in which IP3R was detected was further established by detection of sarcomeric myosin heavy chain using an anti-myosin antibody (MF20) on sections adjacent to those used for the IP3R detection (data not shown). IP3R mRNA is detected in the wall of left ventricle and in papillary muscle (Fig. 5 *A*), in the structures surrounding the left ventricular outflow track (Fig. 5 *B*), the wall of the left atrium (Fig. 5 *C*), and at the apex of the left ventricle (Fig. 5 *E*). No significant signal above background is seen in a representative section using the sense-strand riboprobe (Fig. 5 *D*). These sections are representative of sections throughout the heart, all of which showed signals for IP3R mRNA and protein within the cardiac myocytes. In regions of the left ventricle which contained both cardiac myocytes and non-muscle cells (fibroblasts) no IP3R mRNA or protein was detected in the non-muscle cells (data not shown).

The signal for antisense IP3R riboprobe (Fig. 6 *A*) was compared to that for the sense riboprobe (Fig. 6 *B*) in ascending aorta. No significant signal above background is seen in a representative section of the ascending aorta using the sense riboprobe, while substantial signal demonstrating IP3R mRNA is detected using the antisense riboprobe for IP3R. To demonstrate the specificity of the IP3R antibody used for immunocytochemistry (α-IP3R-1), antibody staining of left ventricular myocardium was compared to staining with control serum (Fig. 6, *C* and *D*). Staining a section of left ventricular myocardium with α-IP3R-1 demonstrated the presence of IP3R in cardiac myocytes (Fig. 6 *C*), in contrast no signal was seen after staining with control serum (Fig. 6 *D*). IP3R mRNA was detected by in situ hybridization in the vascular smooth muscle of an artery in the left ventricular myocardium (Fig. 8).

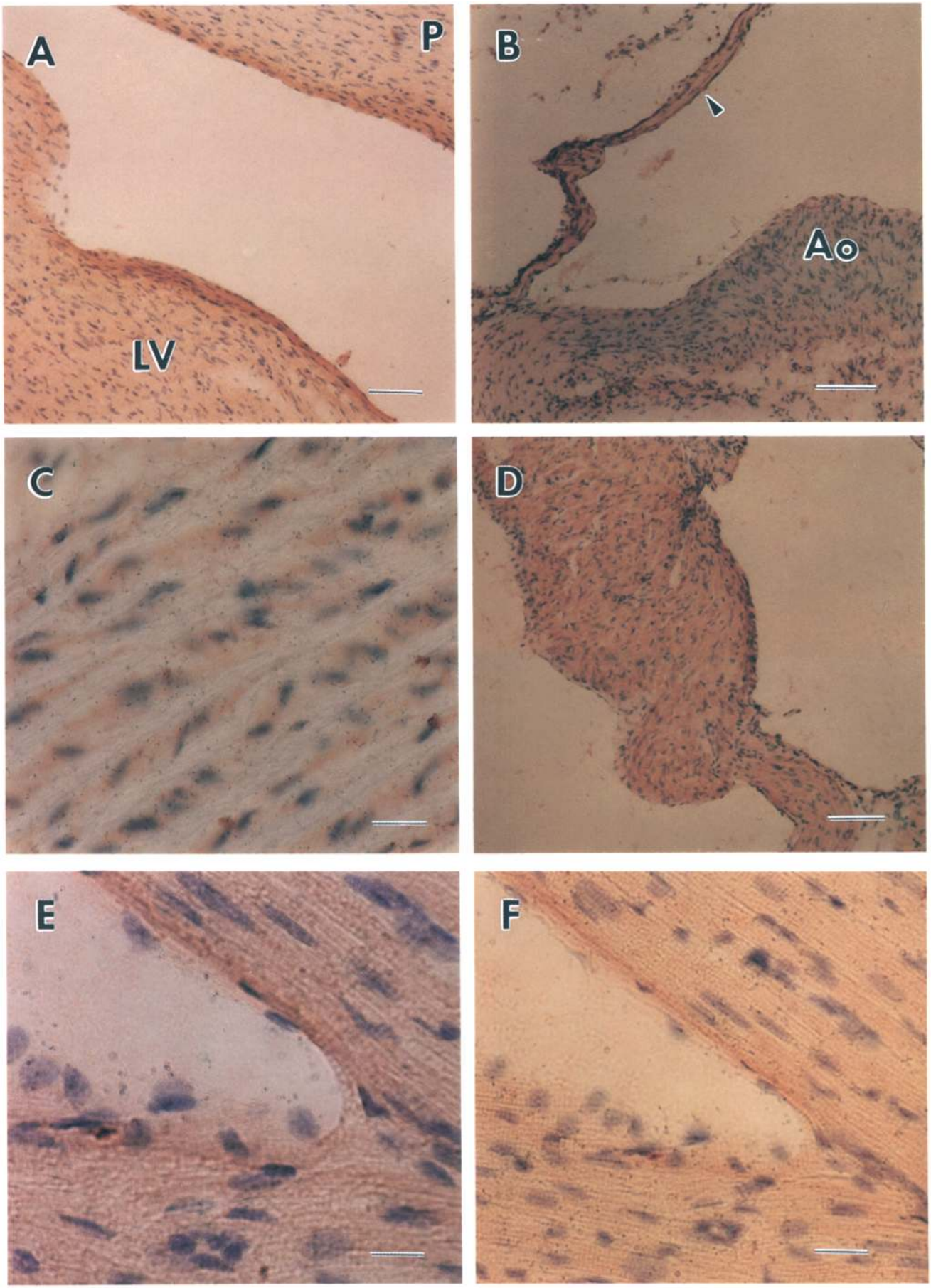
Relative Amounts of IP3R and RYR mRNAs in Cardiac Myocytes

We also examined the relative abundance of IP3R and RYR transcripts in heart, aortic smooth muscle, and brain from rabbit using densitometric analyses of Northern blots of total RNA (Fig. 9). The level of RYR mRNA in the heart was ~50-fold higher than that of the IP3R. In contrast, in total RNA from aortic smooth muscle and brain, the IP3R mRNA was more abundant (approximately fivefold) than the RYR mRNA. These Northern blots are representative of multiple (≥3 for each tissue and probe) experiments performed on total RNA isolated from several animals (≥6 for each tissue). GAPDH mRNA levels and 28s and 18s ribosomal RNA levels (by ethidium bromide staining) were used to control for the amounts of RNA loaded in each lane. Similar results have been obtained using total RNA isolated from mouse tissues (data not shown).

Discussion

The present study demonstrates that an IP3R mRNA and protein are expressed in cardiac myocytes. While the dominant mechanism of E-C coupling in cardiac muscle is Ca^{2+}-induced Ca^{2+} release, evidence has accumulated in favor of a role for IP3-induced Ca^{2+} release as well. An important

Figure 4. Combined in situ hybridization and immunocytochemical localization of IP3R in the rat heart. An antisense ^{35}S-labeled IP3R RNA probe was used for these studies (for details see Materials and Methods and Fig. 3 legend). Anti-IP3R peptide antibodies were used to stain rat heart tissue sections and were visualized using peroxidase (for details see Materials and Methods). (*A*) Immunoreactive IP3R detected in a section showing the lumen of the left ventricular cavity with the left ventricular free wall (*LV*) facing a portion of the papillary muscle (*P*). (*B*) Immunoreactive IP3R detected in the region of the left ventricular outflow tract including the wall of the ascending aorta (*Ao*) just above the anterior leaflet of the aortic valve (*arrow*). (*C*) Higher power bright field view of the wall of the ascending aorta showing both immunoreactive IP3R and IP3R mRNA within aortic smooth muscle cells. (*D*) Section of the wall of the left atrium showing immunoreactive IP3R. (*E*) High power bright field view of the apex of the left ventricular cavity showing immunoreactive IP3R in cardiac myocytes. (*F*) Lower power bright field view of the same section as in E showing both immunoreactive IP3R and IP3R mRNA. Bars: (*A*, *B*, and *D*) 80 μm; (*C* and *F*) 40 μm; (*E*) 12.7 μm.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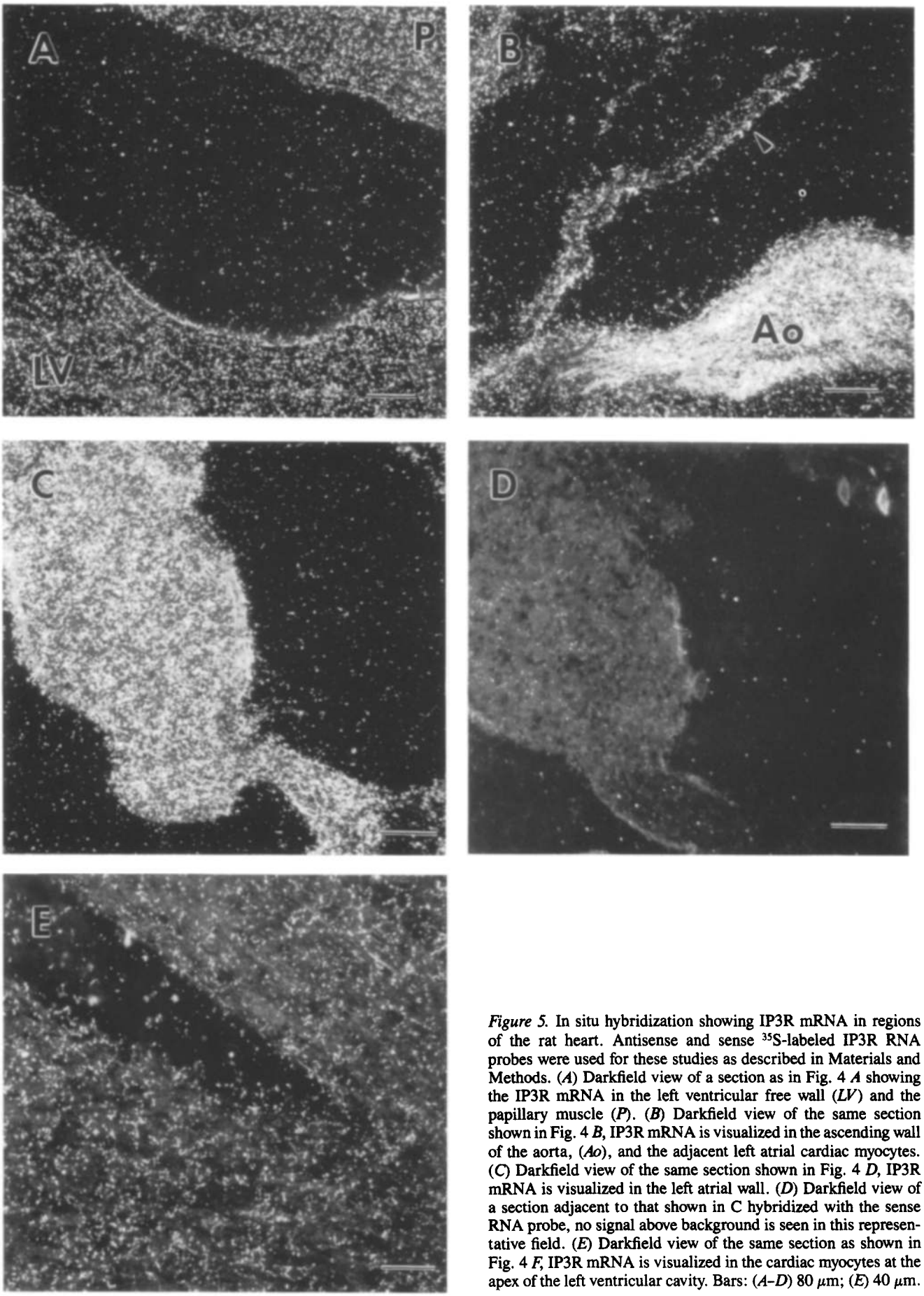

Figure 5. In situ hybridization showing IP3R mRNA in regions of the rat heart. Antisense and sense ^{35}S-labeled IP3R RNA probes were used for these studies as described in Materials and Methods. (*A*) Darkfield view of a section as in Fig. 4 *A* showing the IP3R mRNA in the left ventricular free wall (*LV*) and the papillary muscle (*P*). (*B*) Darkfield view of the same section shown in Fig. 4 *B*, IP3R mRNA is visualized in the ascending wall of the aorta, (*Ao*), and the adjacent left atrial cardiac myocytes. (*C*) Darkfield view of the same section shown in Fig. 4 *D*, IP3R mRNA is visualized in the left atrial wall. (*D*) Darkfield view of a section adjacent to that shown in C hybridized with the sense RNA probe, no signal above background is seen in this representative field. (*E*) Darkfield view of the same section as shown in Fig. 4 *F*, IP3R mRNA is visualized in the cardiac myocytes at the apex of the left ventricular cavity. Bars: (*A–D*) 80 μm; (*E*) 40 μm.

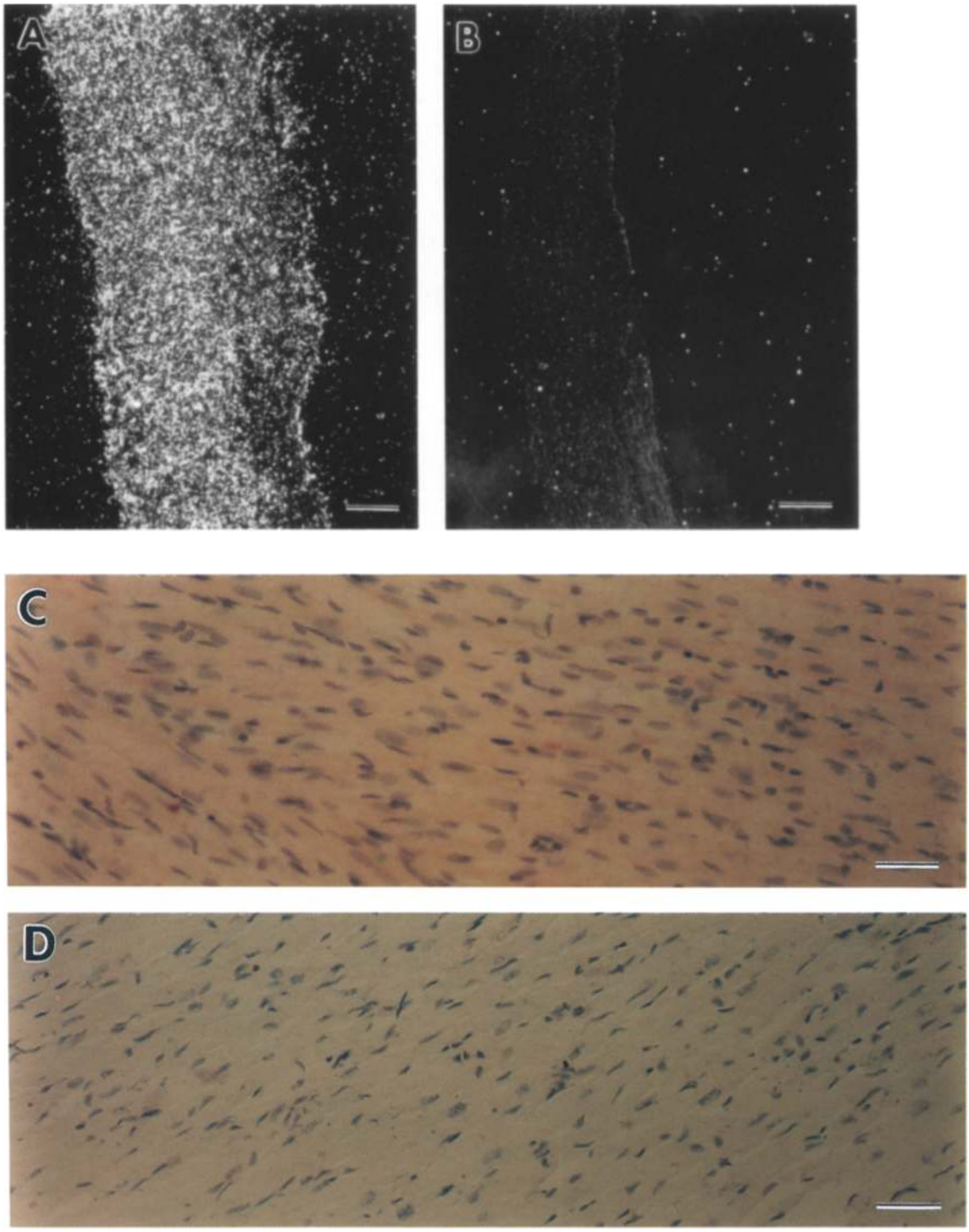

Figure 6. In situ hybridization and immunocytochemistry demonstrating specificity of the antisense-strand IP3R riboprobe in rat ascending aorta and the anti-IP3R antibody (α-IP3R-1) in left ventricular myocardium. (*A*) Darkfield view of a section of ascending aorta labeled with antisense-strand IP3R riboprobe showing the IP3R mRNA. (*B*) Darkfield view of an adjacent section of ascending aorta labeled with the sense-strand riboprobe showing no significant signal above background. (*C*) Left ventricular myocardium showing immunoreactive IP3R in cardiac myocytes. (*D*) An adjacent section of left ventricular myocardium stained with control pre-immune serum showing background signal. Antisense and sense ^{35}S-labeled IP3R RNA probes were used for these studies as described in Materials and Methods. Anti-IP3R peptide antibodies were used to stain rat heart tissue sections and were visualized using peroxidase (for details see Materials and Methods). Bars: (*A* and *B*) 80 μm; (*C* and *D*) 40 μm.

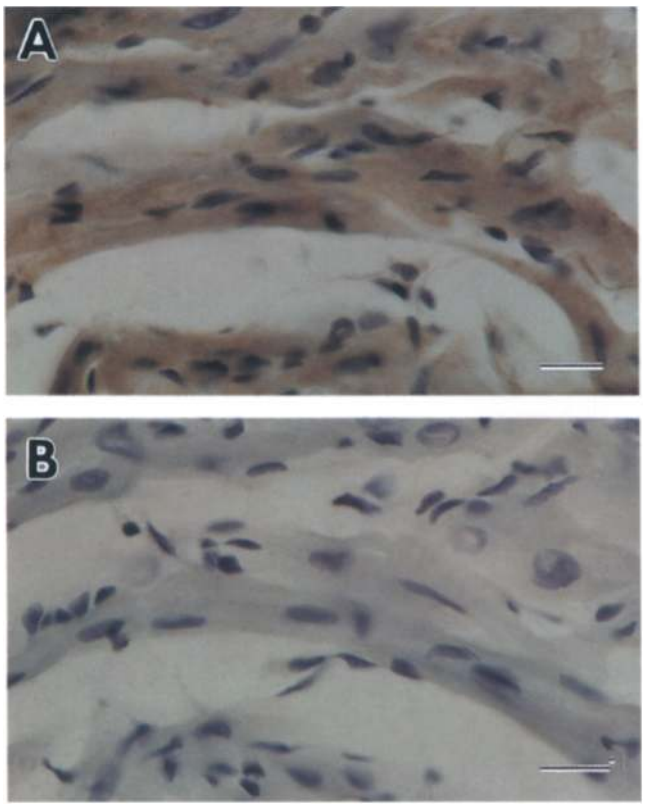

Figure 7. Immunocytochemistry demonstrating specificity of the anti-IP3R antibody. (*A*) Immunoreactive IP3R is detected in a section of the left atrium stained with anti-IP3R antibody (α-IP3R-1) pre-absorbed with buffer (PBS) alone. (*B*) An adjacent section of the left atrium stained with α-IP3R-1 antibody pre-absorbed with the antigenic peptide (anti-IP3R antibody was pre-absorbed with ~1 mg of IP3R peptide in 250 μl of a 1/50 dilution of antiserum incubated at room temperature for 2 h). No signal is detected with the pre-absorbed antibody. Anti-IP3R peptide antibodies used to stain rat heart tissue sections were visualized using peroxidase (for details see Materials and Methods). Bars, 40 μm.

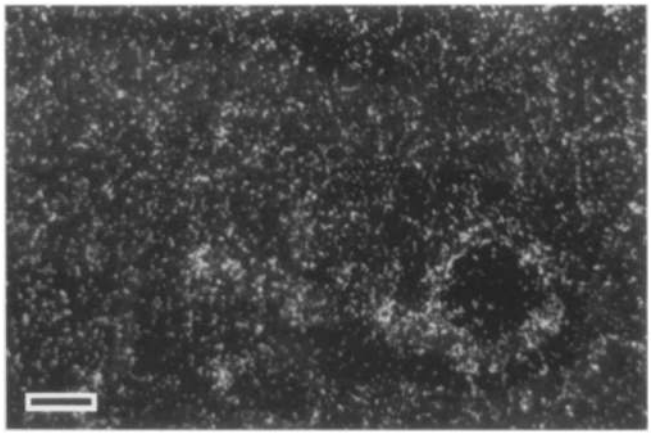

Figure 8. In situ hybridization demonstrating IP3R mRNA detected in the myocardium and in the vascular smooth muscle surrounding an artery in the left ventricular myocardium. An antisense ^{35}S-labeled IP3R RNA probe was prepared as described in Materials and Methods. Bar, 40 μm.

prelude to determining the significance of IP3-induced Ca^{2+} release in cardiac muscle is to establish that cardiac myocytes express an IP3R in addition to the cardiac RYR which mediates Ca^{2+}-induced Ca^{2+} release.

On the basis of immunoreactivity with a sequence-specific anti-IP3R antibody (Fig. 1) and RNAse protection using a smooth muscle IP3R cRNA probe (Fig. 3), we have now shown that the IP3R expressed in cardiac myocytes is structurally most similar to the type 1 IP3R expressed in vascular smooth muscle and in the cerebellum (Furuichi et al., 1989; Marks et al., 1990; Mignery et al., 1990).

IP3Rs have been identified as functional intracellular calcium release channels in reconstituted vesicles (Ferris et al., 1989) and in lipid bilayers (MayrLeitner et al., 1991). cDNAs encoding the IP3R have been cloned from mouse (Furuichi et al., 1989) and rat (Mignery et al., 1990) brain and partially cloned from mouse aortic smooth muscle (Marks et al., 1990) revealing structural similarity to the RYR/calcium release channel from the sarcoplasmic reticulum (Mignery et al., 1989). IP3Rs are suspected to be present in most tissues, but their structure has been identified only in brain and smooth muscle.

In the heart, a role for IP3-mediated pathways in excitation–contraction (E–C) coupling has been proposed based on studies suggesting IP3-induced calcium release and contraction in cardiac muscle (Fabiato and Fabiato, 1984; Fabiato, 1990). IP3-induced Ca^{2+} release from cardiac SR has been shown in skinned ventricular fibers from rat (Kentish et al., 1990) and cardiac SR vesicles (Hirata et al., 1984). IP3 has also been shown to potentiate the effects of caffeine-induced calcium release in skinned guinea pig papillary muscle (Nosek et al., 1986). Moreover, IP3-induced intracellular calcium release has been reported with high concen-

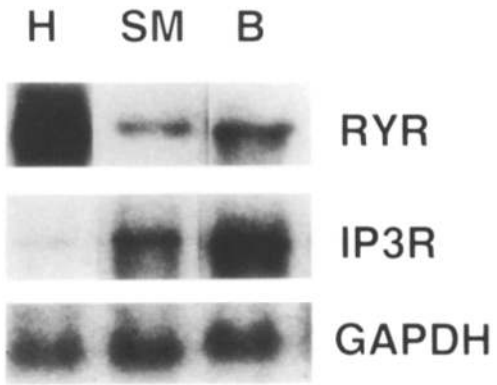

Figure 9. Northern blot analyses demonstrating relative levels of IP3R and RYR mRNAs in heart and smooth muscle. 20 μg of total RNA was isolated from the following rabbit tissues: *H*, heart; *SM*, aortic smooth muscle; and *B*, brain. Northern blot analysis was performed using cDNA probes for the cardiac RYR, aortic smooth muscle IP3R, and GAPDH (see Materials and Methods for details). In the heart, RYR mRNA is considerably more abundant that IP3R, whereas there is more IP3R mRNA in brain and smooth muscle. Hybridization of GAPDH is used to control for the amount of RNA loaded in each lane. Only the relevant portions of the blots are shown. Only specific bands corresponding to the indicated mRNAs were seen.

trations of α-adrenergic agonists in rat left ventricular muscle (Poggioli et al., 1986; Otani et al., 1988).

In contrast, it has been reported that IP3 had no effect on isolated cardiac SR or in permeabilized myocytes (Movesian et al., 1985). In general the rate and degree of IP3-induced Ca^{2+} release in the heart has been significantly lower than that observed for Ca^{2+}-induced Ca^{2+} release. Endothelin, a potent inducer of contraction in the heart, has also been implicated as an activator of IP3 response in cardiac myocytes (Lovenberg and Miller, 1990). However, it has not been conclusively demonstrated that endothelin activates cardiac contractility via an IP3 pathway and subsequent release of intracellular Ca^{2+}, in part because investigators have not previously known whether an IP3R is present in cardiac myocytes.

Northern blot and RNAse protection analyses of total heart RNA have demonstrated a signal corresponding to IP3R mRNA (Marks et al., 1990; Nakagawa et al., 1991). Taken together, these studies, albeit incomplete, have raised the possibility that the IP3R may be expressed in cardiac myocytes and therefore could be involved in physiologic modulation of cardiac contractility in response to pharmacologic agents and hormones. However, until the present study, it has not been possible to ascertain whether the IP3Rs detected in these earlier studies were expressed in cardiac myocytes or in non-muscle cells in the heart.

The IP3R has a molecular weight of 313 kD based on the deduced amino acid sequence from cDNA cloning (Furuichi et al., 1989; Mignery et al., 1990) and an ~260,000 M_r, based on protein purification (Supattapone et al., 1988; Chadwick et al., 1990; Mourey et al., 1990). The molecular mass of the native IP3Rs from brain and vas deferens, as determined by gel filtration, was ~1,000 kD (Mourey et al., 1990) and ultrastructural analysis of the purified bovine aortic smooth muscle IP3R demonstrates fourfold symmetry (Chadwick et al., 1990). Thus, the functional IP3R/calcium release channel is a homotetramer comprised of four ~260,000 M_r subunits. Analysis of hydropathy plots of the deduced amino acid sequence led to the prediction that the IP3R contains multiple (eight or nine) transmembrane regions located near the carboxy terminus (Furuichi et al., 1989; Mignery et al., 1990) and a large cytoplasmic region to which IP3 binds (Mignery and Sudhof, 1990).

Three domains have been proposed for the IP3Rs, a ligand binding domain at the amino-terminal end, a coupling domain presumably linking IP3 binding to calcium release channel activation, and a carboxy-terminal channel region (Mignery et al., 1990; Ferris et al., 1991). The basic structure is similar to that of the calcium release channels/RYRs of the sarcoplasmic reticulum. Two of these related calcium release channels, the skeletal and cardiac RYRs, have been well characterized (Marks et al., 1989; Mignery et al., 1989; Takeshima et al., 1989; Marks, 1990; Otsu et al., 1990). The RYRs are also large, symmetrical intracellular calcium release channels comprised of four identical subunits. Structural similarities between RYRs and IP3Rs suggest that the two forms of intracellular calcium release channels are members of a gene super family.

Alternative splicing of the IP3R transcript has been characterized (Mignery et al., 1990; Nakagawa et al., 1991) defining neuronal and non-neuronal forms (Danoff et al., 1991). A second and third form of the IP3R have been cloned from rat cerebellum (IP3R types 2 and 3) (Sudhof et al., 1991). An IP3R has also recently been characterized in human T lymphocytes (Khan et al., 1992).

The level of IP3R mRNA in the heart is considerably lower than that encoding the cardiac RYR, suggesting that the amount of IP3R in the heart may be significantly less than that of the RYR. This contrasts markedly with the brain and aortic smooth muscle, both of which express more IP3R mRNA than RYR mRNA. Some of the IP3R mRNA signal detected in the total heart RNA is likely secondary to vascular smooth muscle in the walls of arteries in the heart, thus the signal on Northern blot analysis probably overestimates the amount of IP3R mRNA in the cardiac myocytes. The distribution of the IP3R in the heart suggests that it may have a role in regulating cytoplasmic calcium concentration in all regions of the heart including both ventricles and the atria. No striking regional differences in the level of IP3R mRNA or protein expression in cardiac myocytes from different regions of the heart were seen.

In the present study we demonstrated that the cDNA probe used for in situ hybridization is specific for IP3R mRNA (Fig. 1) and that the antibody used for immunocytochemistry is specific for IP3R protein (Fig. 3). Using conditions similar to those used for the combined in situ hybridization and immunocytochemistry, the cRNA probe and anti-IP3R antibody detected single, specific IP3R mRNA and protein signals, respectively. The subcellular localization of the IP3R in cardiac myocytes was not addressed.

Recently, an IP3R was detected in both the intracellular and plasma membrane fractions in canine pancreatic homogenates (Sharp et al., 1992) and on the plasma membrane of T cells (Khan et al., 1992). These findings raise the possibility that the IP3R detected in the heart may be located on multiple distinct membranes. Differential localization of tritiated ryanodine and IP3 binding has been reported in brain and heart (Verma et al., 1992). IP3 binding was most prominent in the vascular smooth muscle surrounding arteries but was detected at lower levels throughout the rat heart in agreement with the findings of the present study. While IP3 is the major signal for contraction in smooth muscle (Somlyo, 1985), it appears to subserve a different, yet possible physiologically significant role in cardiac muscle. The IP3R may determine calcium release from either an anatomically distinct pool of intracellular calcium in cardiac myocytes or directly from the SR. Demonstration that both forms of calcium release channels are expressed in cardiac myocytes raises the possibility that a complex feedback may exist involving interactions between the IP3R and the RYR calcium pools. IP3-sensitive pathways may play a role in regulating cytoplasmic calcium concentration in cardiac muscle, this in turn may affect contractility. Alternative pathways for regulating cytoplasmic calcium concentration may become more significant physiologically in disease states such as end-stage heart failure where we have recently shown that the cardiac RYR mRNA is down regulated (Brillantes et al., 1992).

We thank B. Ehrlich for comments on the manuscript and the following members of the Marks laboratory for their encouragement and help with various aspects of this project: Yong-Sheng Ma, Anne-Marie Brillantes, T. Jayaraman, Tania Nanevicz, and Ding-Ming Yang.

Supported in part by grants from the National Institutes of Health and American Heart Association (to A. R. Marks). A. R. Marks is an Estab-

lished Investigator of the American Heart Association and a Syntex Scholar.

Received for publication 30 May 1992 and in revised form 1 December 1992.

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