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.
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 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., Columbus, MD). Sequences were edited and compared to the Genbank database using MacVector software (v5.5.3). 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-mixer (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 0.2x 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.

RNA protection was performed using the same cDNA probes (sense and antisense) as those used for in situ hybridization (see below) except that the radiolabeling was performed with [3P]dCTP instead of [32P]dCTP. Total RNA samples from adult rat were hybridized overnight at 45°C with 8 x 106 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% denatured formamide, heated at 95°C and electrohoresed 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) and electrophoresed in 6-8% denaturing polyacrylamide gels. Lys-Leu, with an amino terminal cysteine added) of the murine brain IP3R sequence using laser densitometry and the Image 1.36 software for data analysis with random primers using Klenow and if_p32 dCTP to a specific activity containing the entire coding region and part of the 3'-untranslated region (GAPDH) probe was a 1.3-kb PstI fragment from clone pUC-GAPDH13 (Brilantes et al., 1992); (c) the glyceraldehyde-3-phosphate-dehydrogenase antisense (as those used for in situ hybridization (see below) except that diethyl pyrocarbonate was used to deproteinize the eDNA probe corresponding to the sequence of the cRNA probe used 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 [32P]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 rabbit 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 overnight. Tissue was cryoprotected in 0.5 M sucrose in 0.1 M PBS for 30 to 45 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 IBBI (Hacher) cryostat and collected onto coated slides (Superfrost plus; Fisher Scientific Co., Pittsburgh, PA). Sections were fixed with 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 using an IBBI (Hacher) cryostat and collected onto coated slides (Superfrost plus; Fisher Scientific Co., Pittsburgh, PA). Sections were fixed with 4% paraformaldehyde for 20 min, rinsed in 2 x SSPE, incubated for 20 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 acid/water (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, 1x Denhardt's, 0.01% Triton X-100, pH 8.8, 0.05 M DTT. Approximately 5 x 106 cpm of each probe was applied to individual slides. Slides were rinsed in 4 x SSPE and 10 mM EDTA, washed in 2 x SSPE and 10 mM EDTA for 1 h at room temperature, and treated with 20 µg/ml RNase in 4 x SSPE and 10 mM EDTA for 30 min at 37°C. Slides were then washed at 60°C for 1 h in 50% formamide, 2 x SSPE and 10 mM EDTA, 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 hematoxylin to visualize the nuclei.

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 [32P]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 rabbit 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 IBBI (Hacher) cryostat and collected onto coated slides (Superfrost plus; Fisher Scientific Co., Pittsburgh, PA). Sections were fixed with 4% paraformaldehyde for 20 min, rinsed in 2 x SSPE, incubated for 20 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 acid/water (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 x Denhardt's, 0.01% Triton X-100, pH 8.8, 0.05 M DTT. Approximately 5 x 106 cpm of each probe was applied to individual slides. Slides were rinsed in 4 x SSPE and 10 mM EDTA, washed in 2 x SSPE and 10 mM EDTA for 1 h at room temperature, and treated with 20 µg/ml RNase in 4 x SSPE and 10 mM EDTA for 30 min at 37°C. Slides were then washed at 60°C for 1 h in 50% formamide, 2 x SSPE and 10 mM EDTA, 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 hematoxylin to visualize the nuclei.
As a negative control no hybridization was seen with yeast RNA added to either antisense or sense riboprobe (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 (oIP3R-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 diaminobenzidine tetrahydrochloride and the sections were then dipped in autoradiographic emulsion following dehydration in graded alcohols containing 0.3 M NH₄OAc.

To demonstrate that oIP3R-1 was sequence-specific, the antibody was pre-absorbed with an excess of the antigenic peptide (∼1 µg of IP3R peptide in 250 µl of a 1/50 dilution of antiserum) by incubation at room temperature for 2 h. oIP3R-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 (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).

**Results**

**Specificity of the IP3R Riboprobe**

IP3R riboprobe 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.

**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,
The present study demonstrates that 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 5. In situ hybridization showing IP3R mRNA in regions of the rat heart. Antisense and sense ³²P-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 35S-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.
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 35S-labeled IP3R RNA probe was prepared as described in Materials and Methods. Bar, 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.

A prelude to determining the significance of IP3-induced Ca2+ release in cardiac muscle is to establish that cardiac myocytes express an IP3R in addition to the cardiac RYR which mediates Ca2+-induced Ca2+ 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 Ca2+ 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 concent-

Figure 8. 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 than 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.

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 than 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.
tations 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 kDa based on the deduced amino acid sequence from cDNA cloning (Furuichi et al., 1989; Mignery et al., 1990) and an \(\sim 260,000\) Mr, 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 \(\sim 1,000\) kDa (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 \(\sim 260,000\) Mr 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 triitated 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 Synx Scholar.

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

References

Berridge, M. J., and R. F. Irvine. 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature (Lond.). 312:315–321.

Berridge, M. J., and R. F. Irvine. 1989. Inositol phosphates and cell signaling. Nature (Lond.). 341:197–205.

Bers, D. M. 1991. Excitation-Contraction Coupling and Cardiac Contractile Force. Kluwer Academic Publishers, Boston, 258 pp.

Brillanter, A., P. Allen, T. Takahasi, S. Izumo, and A. R. Marks. 1992. Differences in cardiac calcium release channel (ryanodine receptor) expression in myocardium from patients with end-stage heart failure caused by ischemic versus dilated cardiomyopathy. Circ. Res. 71:18–26.

Catterall, W. A. 1991. Excitation-contraction coupling in vertebrate skeletal muscle: a tale of two calcium channels. Cell. 64:871–874.

Chadwick, C. C., A. Saito, and S. Fleischer. 1990. Isolation and characterization of the inositol trisphosphate receptor from smooth muscle. Proc. Natl. Acad. Sci. USA. 87:2132–2136.

Cox, K., D. DeLeon, L. Angerer, and R. Angerer. 1984. Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. Dev. Biol. 101:485–502.

Danoff, S., C. Ferris, C. Donath, G. Fischer, S. Munemitsu, A. Ulrich, S. Snyder, and C. Ross. 1991. Inositol 1,4,5-trisphosphate receptors: distinct neuronal and nonneuronal forms derived by alternative splicing differ in phosphorylation. Proc. Natl. Acad. Sci. USA. 88:2951–2955.

Fabio, A. 1990. Comparison and relation between inositol (1,4,5)-trisphosphate induced release and calcium induced release from the sarcoplasmic reticulum. Recent Advances in Calcium Channels and Calcium Antagonists. Pergamon Press, Elmsford, NY. pp. 35–39.

Fabio, A., and F. Fabio. 1984. Calcium and cardiac excitation-contraction coupling. Annu. Rev. Physiol. 46:473–484.

Ferris, C. D., R. L. Huganir, S. Suppatapone, and S. H. Snyder. 1989. Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles. Nature (Lond.). 342:87–89.

Ferris, C. D., R. L. Huganir, D. S. Brent, A. M. Cameron, and S. H. Snyder. 1991. Inositol trisphosphate receptor: phosphorylation by protein kinase C and calcium-calmodulin-dependent protein kinases in reconstituted vesicles. Proc. Natl. Acad. Sci. USA. 88:2232–2235.

Fleischer, S., and M. Iaini. 1989. Biochemistry and biophysics of excitation-contraction coupling. Annu. Rev. Biophys. Biophys. Chem. 18:333–364.

Fort, P. L., M. Marty, M. Piechaczyk, S. Sabrouty, C. Dani, P. Jaenteur, and J. Lovenberg. 1990. Endothelin: a review of its effects and possible mechanisms of action. Neurochem. Res. 15:407–417.

Ginard, B. F. 1990. Smooth muscle and brain inositol 1,4,5-trisphosphate receptors are structurally and functionally similar. J. Biol. Chem. 265:20719–20722.

Marks, A. R., P. Tempst, K. S. Hwang, M. B. Taubman, M. Inui, C. Chadwick, S. Fleischer, and B. Nadal-Ginard. 1989. Molecular cloning and characterization of the ryanodine receptor/junctional channel complex from mouse skeletal muscle sarcoplasmic reticulum. Proc. Natl. Acad. Sci. USA. 86:9083–9087.

Marks, A. R., S. Fleischer, and P. Tempst. 1990. Surface topography analysis of the ryanodine receptor/junctional channel complex based on proteolysis sensitivity mapping. J. Biol. Chem. 265:13143–13149.

Mayr-Leitner, M., C. Chadwick, A. Timmerman, S. Fleischer, and S. Schindler. 1991. Purified IP3-receptor from smooth muscle forms an IP3 gated and heparin sensitive calcium channel in planar bilayers. Cell Calcium. 12:505–514.

Mignery, G. A., and T. C. Sudhof. 1990. The ligand binding site and transduction mechanism in the inositol 1,4,5-trisphosphate receptor. EMBO (Eur. Mol. Biol. Organ.) J. 9:3893–3898.

Mignery, G., T. Sudhof, K. Takei, and P. Camilli. 1989. Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor. Nature (Lond.). 342:192–195.

Mignery, G. C., B. Newton, A. Verma, and T. Sudhof. 1990. Structure and expression of the rat inositol-1,4,5-trisphosphate receptor. J. Biol. Chem. 265:12679–12685.

Neubauer, R. J., A. Verma, S. Suppatapone, and S. H. Snyder. 1990. Purification and characterization of the inositol 1,4,5-trisphosphate receptor protein from rat vas deferens. Biochem. 27:383–389.

Moyesian, M., A. Thomas, M. Selak, and J. Williamson. 1985. Inositol trisphosphate does not release Ca2+ from permeabilized cardiac myocytes and sarcoplasmic reticulum. FEBs (Fed. Eur. Biochem. Soc.) Lett. 185:328–332.

Nakagawa, T., H. Okano, T. Furushih, J. Aruga, and K. Mikoshiba. 1991. The subtypes of the mouse inositol 1,4,5-trisphosphate receptor are expressed in a tissue-specific and developmentally specific manner. Proc. Natl. Acad. Sci. USA. 88:6244–6248.

Nosek, T., M. Williams, S. Ziegler, and R. Godt. 1986. Inositol trisphosphate enhances calcium release in skinned cardiac and skeletal muscle. Am. J. Physiol. 250:C807–C811.

Otani, H., R. Otani, and D. Das. 1988. α-Adrenoreceptor-mediated phospholipase C breakdown and inositol response in rat left ventricular papillary muscles. Circ. Res. 62:8–17.

Osu, K., H. F. Willard, V. K. Khanna, F. Zorzato, N. M. Green, and D. H. MacLennan. 1990. Molecular cloning of cDNA encoding the Ca2+ release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J. Biol. Chem. 265:13472–13483.

Poggioli, J., J. Sulpic, and G. Vassort. 1986. Inositol phosphate production following α-adrenergic, muscarinic, or electrical stimulation in isolated rat left ventricular papillary muscles. Circ. Res. 58:18–27.

Sambrook, J., E. F. Frisch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sharp, A., S. Snyder, and S. Nigam. 1992. Inositol 1,4,5-trisphosphate receptors: localization in epithelial tissue. J. Biol. Chem. 267:7444–7449.

Somlo, A. 1985. Excitation-contraction coupling and the ultrastructure of smooth muscle. Circ. Res. 57:497–507.

Sudhof, T. C., B. Newton, A. Verma, V. Usukharyev, and G. Mignery. 1991. Structure of a novel InsP3 receptor. EMBO (Eur. Mol. Biol. Organ.) J. 11:3199–3206.

Suppatapone, S., P. F. Worley, J. M. Baraban, and S. Snyder. 1988. Solubilization, purification, and characterization of an inositol trisphosphate receptor. J. Biol. Chem. 263:1530–1534.

Takeshima, H., S. Nishimura, T. Matsumoto, H. Ishida, K. Kangawa, N. Minamino, H. Massou, M. Ueda, H. Nakaoka, and T. Hirose. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. Nature (Lond.). 339:439–445.

Taubman, M. B., S. Izumo, T. Tsuda, R. Alexander, and B. Nadal-Ginard. 1989. Angiotensin II induces c-fos mRNA in aortic smooth muscle. J. Biol. Chem. 264:526–530.

Verma, A., D. Hirsch, and S. Snyder. 1992. Calcium pools mobilized by calcium or inositol 1,4,5-trisphosphate are differentially localized in rat heart and brain. Mol. Biol. Cell. 3:621–631.