The inositol 1,4,5-trisphosphate receptor (InsP$_3$R) is an intracellular Ca$^{2+}$ release channel that mediates the rise in cytoplasmic calcium in response to receptor-activated production of InsP$_3$. The InsP$_3$R-mediated signaling pathway appears to be ubiquitous and is involved in many cellular processes including cell division, smooth muscle contraction, and neuronal signaling. Different regions of the heart also express InsP$_3$$_2$ receptors. We report here that acutely dissociated ventricular myocytes from ferret and rat hearts express significant levels of InsP$_3$R as indicated by immunoblotting with a receptor consensus antibody. InsP$_3$ binding experiments ($K_D = 23.6$ nm and $B_{max} = 0.46$ pmol/mg) suggest the myocytes contain the high affinity type 2 InsP$_3$$_2$ receptor. Exhaustive mRNA screening by polymerase chain reaction, RNase protection, and subsequent DNA sequencing positively identify the InsP$_3$R as type 2. The type 2 receptor from ferret heart was then incorporated into planar lipid bilayers and formed Ca$^{2+}$-selective, InsP$_3$$_2$-activated, heparin-blocked ion channels. We conclude that the predominant InsP$_3$$_2$ receptor isoform expressed in cardiac myocytes is type 2 and that it forms a functional InsP$_3$$_2$-gated Ca$^{2+}$ channel when reconstituted in planar lipid bilayers.

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The abbreviations used are: InsP$_3$, D-myoinositol 1,4,5-trisphosphate; InsP$_3$R, D-myoinositol 1,4,5-trisphosphate receptor; CHAPS, 3-[N-(cholamidopropyl)dimethylammonio] propane sulfonate; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; RyR, ryanodine receptor.

Identification and Functional Reconstitution of the Type 2 Inositol 1,4,5-Trisphosphate Receptor from Ventricular Cardiac Myocytes*

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Inositol 1,4,5-trisphosphate (InsP$_3$)$^*$ is a well known second messenger mediating the regulated release of intracellular calcium and is produced through the action of phospholipase C. Phospholipase C is activated in response to the stimulation of cell surface receptors coupled to heterotrimeric G-proteins and tyrosine kinases resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to liberate InsP$_3$ and diacylglycerol. InsP$_3$ is a readily diffusible compound that binds to specific (InsP$_3$R) receptors localized to the endoplasmic reticulum and results in the release of calcium from intracellular stores (see reviews Refs. 1–3).

The InsP$_3$ receptor consists of a family of 3–4 highly homologous members. The primary structure of these InsP$_3$R$_n$ has been determined by cDNA cloning and sequencing (4–7). The type 1 receptor is expressed at very high levels in cerebellar Purkinje cells and has been extensively characterized (4, 6, 8, 9). When compared with the type 1 receptor, the type 2 and type 3 isoforms have an overall amino acid sequence identity of 69 and 64%, respectively (6, 7). Even though the three principal types of receptor are very similar, they exhibit significantly different affinities for InsP$_3$ binding (designated T-2 > T-1 > T-3), suggesting that the different receptor homologues have distinct functions within a cell (6, 10).

In cardiac muscle, the ryanodine receptor serves as the primary calcium release channel of the sarcoplasmic reticulum in excitation-contraction coupling (11, 12). The role of InsP$_3$-induced calcium release in cardiac cells is not well understood. Phosphatidylinositol 4,5-bisphosphate turnover coupled to α-adrenergic and muscarinic plasma membrane receptors has been shown to increase InsP$_3$ levels in cardiac muscle (13, 14). Initial studies revealed that InsP$_3$ was capable of inducing a slow release of calcium from vesicular preparations as well as activate contraction in skinned ventricular rat muscle and chick heart preparations (15). Borgatta et al. (16) identified a low conductance, InsP$_3$-sensitive, calcium release channel in sarcoplasmic reticulum vesicle preparations from canine heart. These channels were concentrated in the sarcoplasmic reticulum isolated from the ventricle septum that contains cells of the conducting system. Recently, biochemical and immunological approaches have been applied to identify the InsP$_3$ receptor in cardiac tissues. Moschella and Marks (17) observed InsP$_3$$_2$ in rat heart using in situ hybridizations and immunocytochemical analysis and concluded that the receptor expressed in cardiac myocytes is structurally similar to the type 1 receptor. The expression level was approximately 50-fold lower than that of the cardiac ryanodine receptor. Gorza et al. (18, 19) observed the highest levels of expression in Purkinje myocytes of the conduction system using type 1 (cerebellar) peptide antibodies and cRNA probes. The InsP$_3$$_2$ receptor was localized to the intercalated disc of cardiac myocytes using immunogold electron microscopy (20). These studies suggested that the receptor may be involved in cell-cell signaling or potentially calcium influx.

The biological role of this receptor family in cardiac tissues is unclear. Although the InsP$_3$ receptor is not the primary calcium release channel in cardiac tissue, InsP$_3$ may be involved in the regulation of Ca$^{2+}$-induced Ca$^{2+}$ release. Increased InsP$_3$$_2$ and the resulting increase in cytoplasmic free calcium may be an important mechanism for controlling cardiac contractile force in response to hormones and pharmacological factors as well as in the diseased state. A greater than 2-fold increase in steady state InsP$_3$$_2$ receptor mRNA was observed during end-stage heart failure in human (21). This increase was accompanied by a 31% decrease in the ryanodine receptor.
mRNA suggesting that the InsP$_3$ receptor may participate in a compensatory role in cardiac intracellular calcium signaling dynamics.

In this study, we have examined the expression of InsP$_3$ receptor in rat and ferret ventricular cardiac myocytes using immunological, molecular, and electrophysiological techniques. We show that in both rat and ferret myocytes the principal isoform of the InsP$_3$ receptor expressed is the type 2 homologue. Functional reconstitution of this receptor isoform into planar lipid bilayers resulted in the identification of a Ca$^{2+}$ release channel responsive to low levels of InsP$_3$ and inhibited by heparin.

**EXPERIMENTAL PROCEDURES**

**Materials**—[H]InsP$_3$ (21 Ci/mmol) was obtained from NEN Life Science Products. [γ-32P]dATP (3000 Ci/mmol) and [α-32P]UTP (800 Ci/mmol) were obtained from Amersham Corp. Unlabeled inositol 1,4,5-trisphosphate was purchased from LC Laboratories. Heparin was purchased from Fluka. CHAPS was from Boehringer Mannheim. N-α-Phosphatidylcholine, L-α-phosphatidylethanolamine, and L-α-phosphatidylserine were obtained from Avanti Polar Lipids Inc., Pelham, AL.

**Membrane Preparation**—Rat and ferret ventricular cardiac myocytes were used as described by Bassani et al. (22). Acutely dissociated ventricular myocytes were homogenized in 50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 1 mM PMSF, and 1 mM Na$_3$VO$_4$. The microsomes were pelleted with a 10-min 135,240 g centrifugation, resuspended, and stored at –80 °C until use.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**—SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described (23, 24) using 5% SDS-polyacrylamide gels. Antibodies were directed against the 19 carboxy-terminal (type 1-specific) (24) amino acid residues 2463–2476 (type 1 loop) (25) and the bacterially expressed C-terminal 324 amino acids (receptor consensus) of the type 1 receptor (26). Peptide competition experiments for the type 1 carboxy-terminal and type 1 loop antisera were performed by preincubation in specific peptide (1 mg/ml) for 1 h prior to incubation with the immunoblot.

**InsP$_3$ Saturation Binding Measurement**—InsP$_3$ binding to ferret myocyte membrane preparations was performed in the presence of increasing concentrations of [H]InsP$_3$ ranging from 0.42 to 50.5 nM. Each 100 μl assay contained 100 μg of protein in 50 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 8.3, using the centrifugation binding assay as described previously (26). The binding assays were incubated on ice for 10 min in the presence of [H]InsP$_3$ and then microsomes were pelleted with a 10-min 29,000 × g$_{av}$ centrifugation, resuspended, and washed in 50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 1 mM PMSF, 1 mM β-mercaptoethanol three times. Washed microsomes were resuspended in approximately 5–10 μg/ml protein and either used immediately or stored at –80 °C until use.

**Reverse Transcription/PCR Analysis**—The procedures for reverse transcription of cDNA and PCR analysis are essentially as described by Newton et al. (10). Total RNA was isolated from rat and ferret ventricular myocytes, as well as rat left ventricular myocardium, whole heart, and cerebellum as described previously by Perin et al. (27). Five μg of total RNA from rat and ferret samples were used as template for first strand cDNA synthesis. Each 50-μl reaction contained 500 μM dNTPs (Pharmacia Biotech Inc.), 20 units of RNase inhibitor (Promega), 1 μg of pdN1, and 0.25 μg of hexamer (Pharmacia) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl$_2$, 10 mM diithiothreitol plus 200–400 units Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Reactions were incubated at 37 °C for 60 min and heat-inactivated for 5 min at 95 °C.

The rat and ferret cDNAs were used as templates in 100-μl PCR assays containing 10 μl of cDNA, 200 μM dNTPs, 1 μM each sense and antisense oligonucleotides, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM MgCl$_2$, and 5 units of AmpliTaq® DNA polymerase (Perkin-Elmer). Each pcDNA receptor type-specific control templates were derived from pl7, pl15, and pl924-2 for the types 1, 2, and 3 receptors, respectively (5, 10). The oligonucleotide primers common to all receptor types used in the PCR reactions were ACGAGCTGCTCGCT/CCTGCTTCTC/TCTCCAAGGCA/G/ACAG-ATGAAGCA. Reaction conditions consisted of two cycles at 94 °C for 5 min, 65 °C for 2 min, and 72 °C for 10 min and 35 cycles of 94 °C for 1 min, 65 °C for 2 min, and 72 °C for 4 min.

**Southern Blotting**—PCR products derived from rat and ferret total RNA primed with the InsP$_3$ receptor consensus oligonucleotides were resolved on triplicate 1% agarose gels and blotted to individual nitrocellulose membranes as described by Sambrook et al. (28). Receptor type-specific control PCR products were amplified using the same common-primer pair and were generated from cDNA templates pl7, pl15, and pl924-2 corresponding to the type 1, 2, and 3 InsP$_3$ receptor, respectively (5, 6, 10). Blots were hybridized to receptor type-specific [32P]-labeled oligonucleotides and washed at high stringency. The receptor-specific oligonucleotides were purified into sequences within the PCR product as follows: type 1, CCGATCGA-TGTTCTGTGTGGCTCTCTGGTTTACCTGTTCCTC; type 2, CTCCTCTCA-CGGGCTGCAATCGAAGA; and type 3, AGGGCCTGCTTAGAATGCG.

**RNase Protection Assays**—Ribonuclease protection assays were performed using a HybSPEED RPA kit (Ambion) as described by the manufacturer. Receptor type-specific transcription products were constructed by PCR amplification of nucleotides 7664–8186, 7448–7959, and 7254–7772 of the rat type 1, 2, and 3 isoforms, respectively, and insertion into EcoRI and BamHI digested pGEM-3Z (Promega). The oligonucleotides primers comprised of CGGGATCGCTGCTTTCCTTGGTTTACCTGTTCCTC and CGAATTCGTCGTCTTCCTCTTCTCCCTCCACCCACCAGAAAACAACCAGAAGCA for the type 1, CGGGAATCCTGCTTTCCTTCCATTATCCTGCTTTA and CGGAATTCGTCGTCTTCCTCCCTTCCACCCACCAGAAAACAACCAGAAGCA for the type 2, and CGGGAATCCTGCTTTCCTTCCATTATCCTGCTTTA and CGGAATTCGTCGTCTTCCTCCCTTCCACCCACCAGAAAACAACCAGAAGCA for the type 3 receptor. InsP$_3$-specific antisense probes were generated from these plasmids, linearized with BamHI, using 77 phage DNA polymerase and the MAXIscript (Ambion) in vitro transcription kit in the presence of [35S]UTP. The expected run-off transcription product sizes were 531, 526, and 528 nucleotides for types 1, 2, and 3 receptors, respectively. Transcription products were purified on 5% acrylamide, 8M urea denaturing gels. Typical specific activities of the probes were greater than 1.3 × 10$^6$ dpm/μg and varied less than 5% between isoform-specific probes. The β-actin control antisense probe was derived from linearized pTMR-β-actin-mouse plasmid DNA. Ribonuclide protection assays were performed in the presence of 80,000 cpm each InsP$_3$-specific and mouse β-actin antisense probes using 10 μg of total RNA for all cardiac samples and with 2.5 μg of total RNA from cerebellum. Products from the RNase protection reactions were resolved on 5% acrylamide, 8% urea denaturing gels, dried, and autoradiographed or quantitated using a Packard Instant Image running Packard Imager for Windows V2.03.

**PCR Product Cloning and Sequencing**—PCR products from rat and ferret RNA were digested with HindIII and ligated into similarly cut pBluescript SK. DNA sequencing was performed on double-stranded templates using [35S]-dATP and Sequenase version 2.0 sequencing reagents (U. S. Biochemical Corp.).

**Reconstitution of InsP$_3$ Receptor into Liposomes**—Microsomes from ferret ventricular cardiac myocytes were solubilized in 1% CHAPS and fractionated on 5–20% sucrose gradients as described previously (5, 8). Gradient fractions containing the highest levels of receptor protein were identified and the resulting microsomal proteoliposomes essentially as described by Ferris et al. (29).
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FIG. 1. Analysis of the InsP₃ receptor protein in ventricular cardiac myocytes by SDS-PAGE and immunoblotting. 10 µg of protein from microsomal membrane fractions of bovine cerebellum and 100 µg of protein from ferret cardiac myocyte microsomes were probed after separation on a 5% SDS-PAGE gel with antibodies directed against the the InsP₃R type 1 carboxy-terminal 19 amino acids in the absence or presence of 1 mg/ml immunogenic peptide (1st and 2nd panels), amino acid residues 2463–2476 (type 1 loop) in the absence or presence of 1 mg/ml immunogenic peptide (3rd and 4th panels), and the consensus antibody directed against the 324 NH₂-terminal amino acids of the conserved ligand binding domain (5th panel). Migration of molecular weight standards is shown on the right. Note the significant immunoreactive band detected in the ferret myocytes lane in the 5th panel.

digitized at 5–10 kHz and filtered at 1 kHz. Channel sidedness was determined by InsP₃ sensitivity. The orientation of the channels studied was such that the InsP₃-sensitive side (i.e. cytoplasmic side) was in the cis compartment.

The ion selectivity of previously reconstituted single InsP₃R channels was nearly identical to that of single ryanodine receptor channels (30). Thus, a simple reconstitution strategy analogous to that successfully applied to study ryanodine receptor channels (31) was used here to examine single InsP₃R channels. This strategy utilized a monovalent cation (Ca²⁺) instead of a divalent cation as charge carrier. The use of a monovalent charge carrier improves experimental success rate and increases the single-to-noise ratio.

RESULTS

Expression of InsP₃ Receptor in Cardiac Ventricular Myocytes—To define the expression of InsP₃ receptor(s) in ventricular cardiac myocytes, microsomal protein from both rat and ferret were electrophoresed on 5% SDS-polyacrylamide gels along with bovine cerebellar microsomal proteins as a control. Following electrophoretic transfer to nitrocellulose membranes, the blots were either incubated with antibodies directed against the carboxy-terminal 19 amino acids (24), amino acid residues 2463–2476 (type 1 loop) (25), or the amino-terminal 324 amino acids of the type 1 InsP₃R. The antisera derived from the amino-terminal 324 amino acids is directed against the highly conserved ligand binding domain and reacts with all receptor types tested (10, 26).

The type 1-specific carboxy-terminal antisera elicited a strong signal in proteins from bovine cerebellar microsomes (Fig. 1, 1st lane) but failed to detect the presence of any immunologically cross-reactive species of similar size in the myocyte preparations of ferret (Fig. 1, 1st lane, 2nd lane). The signal observed in the cerebellar sample was generated from one-tenth (10 µg) the amount of total membrane protein applied to the myocyte sample wells. Microsomes from rat and ferret cerebella were also immunoreactive (not shown). An immunoreactive signal was observed at Mᵣ = 97,700 in ferret myocytes which was eliminated by pre-absorbing the carboxy-terminal antibody with its corresponding peptide (Fig. 1, 2nd panel). To investigate further whether the immunoreactive signal (Mᵣ = 97,700) observed in the myocyte preparation with the carboxy-terminal antisera represents a proteolytic fragment of the type 1 receptor, a second type 1-specific antibody directed against amino acid residues 2463–2476 (type 1 loop) was used for immunoblotting (Fig. 1, 3rd panel). In this panel, the type 1 receptor of cerebellum is strongly immunoreactive with no apparent signal of similar mobility as observed in the myocyte sample. As was seen with the type 1 carboxy-terminal antisera, a different band of lower apparent Mᵣ (~148,000) is seen which could be eliminated in peptide competition experiments (Fig. 1, 4th panel). The band of Mᵣ = 97,700 observed using the carboxy-terminal antisera is not detected with this antibody and suggests that the cross-reacting species is not the type 1 receptor. This conclusion is based on the observation that the carboxy-terminal antibody is reacting with a protein species of approximately 97.7 kDa, and if this was a proteolytic fragment of the type 1 receptor it would cross-react with the type 1 loop antibody which is directed against residues 2463–2476 only 267 amino acids NH₂-terminal to the COOH-terminal antibody and thus should reside on the same proteolytic fragment. The identity of the protein species which was faintly detected by the type 1 “loop” antibody remains uncertain. The weak signals observed with this and the COOH-terminal antisera may be a consequence of the large amounts of myocyte membrane proteins loaded onto the SDS-PAGE gels.

When the Western blots were incubated with the consensus antisera derived from the receptor amino-terminal 324 amino acids, there was a significant signal observed at the expected apparent molecular weight (Mᵣ = 260 × 10⁶) for the InsP₃ receptor in both cerebellum and ferret cardiac myocytes (Fig. 1, 5th panel). As judged by the signal intensity between the myocytes and cerebellar protein samples, these data suggest that there is a significant amount of InsP₃ receptor protein present in cardiac myocytes and that the predominant isoform expressed is not type 1. It is unlikely that this signal represents the type 3 InsP₃R due to its similar mobility to the cerebellar type 1 receptor on SDS-PAGE, since the type 3 receptor has a significantly smaller apparent Mᵣ (10).

Insolit 1,4,5-Triphosphosphate Binding to Cardiac Ventricular Myocyte Membrane Preparations—The ligand binding properties of the isoform expressed in cardiac myocytes was investigated using saturation binding measurements. Even though the various InsP₃ receptor family members exhibit a remarkable degree of sequence similarity, they have markedly distinct affinities for InsP₃. The type 2 InsP₃R receptor has the highest InsP₃ affinity (Kₛ = 27 nM) of the three characterized isoforms with a relative order of, type 2 > type 1 > type 3 (6, 10). In Fig. 2, [³²P]InsP₃ binding to ferret myocyte membrane preparations was measured in the presence of increasing concentrations of [³²P]InsP₃ ranging from 0.42 to 50.5 nM in 50 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 8.3, using the centrifugation binding assay as described previously (26). All assays were performed in triplicate and nonspecific [³²P]InsP₃ binding for each concentration was determined in the presence of 10 µM unlabeled InsP₃. Similar results were obtained from three independent determinations.

Analysis of the binding data using the Scatchard transformation results in a binding isotherm with a calculated Kₛ equal to 23.6 nM and a B_max = 0.46 pmol/mg (Fig. 2). These values are very similar to values previously reported (21 nM, 0.66 pmol/mg) with canine cardiac microsomes (32) and that of the cloned and expressed type 2 InsP₃ receptor ligand binding domain (27 nM) (6). Although significant variability exists for the reported Kₛ values of the InsP₃ receptors due to differences in purity and experimental conditions, the values obtained in this study are consistent with those of previous studies (6) where membrane preparations and assays were performed us-
recognize all three principal types of InsP3 receptor cDNA. The total RNA was reverse-transcribed and used as templates for types. The distribution may be limited as compared with the other receptor isoforms. Newton and others (31) have characterized the expression of the InsP3 receptor in various tissues using similar methodologies. These results (Fig. 2), together with the Western blotting data (Fig. 1), suggest that the predominant species of InsP3R in cardiac ventricular myocytes is not the type 1 or type 3 InsP3 receptor and is consistent with the hypothesis that it may represent the type 2 isoform.

Polymerase Chain Reaction Analysis of InsP3 Receptor Expression—To establish the identity of the InsP3R isoforms expressed in heart, polymerase chain reaction (PCR) screening strategies were performed. Several studies (10, 33) have characterized the expression of the InsP3 receptor in various tissues and cell lines using PCR strategies. Multiple homologues of InsP3 receptor were detected in all tissues and cell types examined. Within a particular cell type, the receptors detected are usually the type 1 and type 3 isoforms. Newton et al. (10) observed that in many cultured cell lines, the type 2 receptor is not expressed at any significant level and proposed that its hypothesis that it may represent the type 2 isoform.

Prism, version 2.0 software.

To establish the pattern of the InsP3 receptor mRNA expression in ventricular cardiomyocytes, total RNA was prepared from isolated cells essentially as described by Perin et al. (27). The total RNA was reverse-transcribed and used as templates in a PCR assay in which “consensus” oligonucleotides that recognize all three principal types of InsP3 receptor cDNA sequences were used as primers. These amplification products, along with specific receptor type products from cloned rat and ferret ventricular myocytes, the type 2 receptor isoenzymes were pelleted, and bound InsP3 was determined by scintillation counting. For each [InsP3] nonspecific binding was determined in the presence of 10 μM unlabeled InsP3. The data illustrated are the means from a single experiment performed in triplicate. Scatchard transformation of the data is shown (inset). Similar results were obtained in three separate experiments. Data analysis were performed using GraphPad Prism, version 2.0 software.

Analysis of the mRNA expression and distribution of the type 1, type 2, and type 3 InsP3 receptor in rat and ferret cardiac tissues as determined by PCR using primers that amplify all three receptor sequence types. A, PCR amplifications were performed on single-stranded cDNA prepared from rat and ferret ventricular myocyte RNA templates. B, PCR analysis of cDNA from rat ventricular myocytes, left ventricular myocardium (myocardium), whole heart, and cerebellum. PCR reaction controls consisted of a minus-RNA cDNA reaction (negative control) and cloned InsP3R cDNA type 1, type 2, and type 3 templates (amplification and hybridization controls). PCR reaction products were resolved on 1% agarose gels and Southern blotted with receptor type-specific 32P-labeled oligonucleotide probes internal to the PCR primer pair as indicated.

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Fig. 2. Saturation binding analysis of InsP3 binding to ferret cardiac myocytes microsomes. Microsomes from ferret myocytes (100 μg) were incubated in the presence of increasing [3H]InsP3 concentrations (0.42–50.5 nM) in 50 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 8.3. After a 10-min incubation, the microsomes were pelleted, and bound InsP3 was determined by scintillation counting. For each [InsP3] nonspecific binding was determined in the presence of 10 μM unlabeled InsP3. The data illustrated are the means from a single experiment performed in triplicate. Scatchard transformation of the data is shown (inset). Similar results were obtained in three separate experiments. Data analysis were performed using GraphPad Prism, version 2.0 software.

Fig. 3. Analysis of the mRNA expression and distribution of the type 1, type 2, and type 3 InsP3 receptor in rat and ferret cardiac tissues as determined by PCR using primers that amplify all three receptor sequence types. A, PCR amplifications were performed on single-stranded cDNA prepared from rat and ferret ventricular myocyte RNA templates. B, PCR analysis of cDNA from rat ventricular myocytes, left ventricular myocardium (myocardium), whole heart, and cerebellum. PCR reaction controls consisted of a minus-RNA cDNA reaction (negative control) and cloned InsP3R cDNA type 1, type 2, and type 3 templates (amplification and hybridization controls). PCR reaction products were resolved on 1% agarose gels and Southern blotted with receptor type-specific 32P-labeled oligonucleotide probes internal to the PCR primer pair as indicated.

Analysis of InsP3R Isoform Expression Levels by Ribonuclease Protection—To establish the relative levels and patterns of expression of the InsP3R gene family in myocytes and other cardiac tissues, ribonuclease protection assays were performed using InsP3R subtype-specific antisense RNA probes. InsP3 receptor subtype-specific antisense probes corresponding to nucleotides 7664–8186, 7448–7959, and 7254–7772 of the rat types 1–3 receptors, respectively, were synthesized using T7 phage RNA polymerase from linearized pGEM-3Z vector. These probes were hybridized to 10 μg of total RNA from rat whole heart, left ventricular myocardium, and cerebellum (2.5 μg of total RNA) and subjected to RNase A/T1 digestion. As illustrated in Fig. 4A, the type 1 probe strongly protects RNA from cerebellum comprising approximately 98.5% of the total InsP3R transcripts detected (see Table I). The type 2 and type 3 receptor accounts for approximately 1.3 and 0.2% of cerebellar transcripts, respectively. In both rat and ferret ventricular myocardium, the type 1 receptor predominates the receptor steady state transcript pool (~60%) with the type 2 (~27%) and type 3 (~13%) at lower levels (Fig. 4A and Table I). The discrepancy between these quantitative assays and the PCR screening (Fig. 3B) most likely reflects affinity differences between the consensus oligonucleotides and the geometric nature of the PCR amplification. In either case, however, both assays clearly demonstrate the expression of all three isoforms in whole heart and myocardium.
InsP3R probes were less than 5% and not taken into account for these comparisons.

In acutely dissociated myocytes, the predominant protected receptor species corresponds to the type 2 receptor (85%), with only minor contributions from the type 1 and type 3 receptor (1.9 and 13%, respectively) (Fig. 4A and Table I). The high levels of the type 2 receptor in myocytes is consistent with the immunoblotting as well as the InsP3 binding results and suggests that the other receptor isoforms are expressed at significant levels in other cell types of the heart such as smooth muscle and the conducting myocytes such as Purkinje cells (18, 19). Fig. 4B illustrates RNase protection analysis at several stages of myocyte preparation. The experiments were intended to examine the fractionation of InsP3R subtypes during myocyte isolation. With incompletely dissociated myocardium the receptor distribution is essentially as that observed for whole heart and intact ventricular myocardium (Fig. 4A), with the type 1 receptor predominating at approximately 56% of the total receptor population. However, in unwashed myocytes (~4, 1 × g sedimentations/platings in normal Tyrode buffer), the relative levels of receptor isoforms are dramatically shifted with the type 2 isoform predominating at greater than twice that observed in whole heart and myocardium (Fig. 4A). In washed myocytes (Fig. 4A, B, and Table I) the type 2 receptor levels are further enriched and represent approximately 85% of the total receptor population with the type 1 receptor at almost undetectable levels and the type 3 receptor at 13%. It is possible that some RNA degradation occurs during the washing process; however, based on the integrity of the protected band and the β-actin signal, intensity between washed and unwashed myocytes in non-saturating exposures suggests that it is minimal. In addition, the levels of the type 3 receptor RNA remain almost constant throughout the isolation.

Sequence Analysis of PCR Products—To definitively establish that the type 2 receptor is the predominant isoform expressed in rat and ferret ventricular myocytes, products from the PCR reactions designed to amplify all InsP3 receptor subtype mRNA were cloned and subjected to DNA sequence analysis.

The nucleotide sequences derived from four independent clones of the rat ventricular myocyte PCR products are identical to one another and reveal a very high degree of similarity (99.4%) with the previously reported type 2 InsP3 receptor from rat brain (6) (Fig. 5). The three nucleotide changes observed between the published full-length rat type 2 sequence and those from rat cardiac myocytes are all silent changes and encode identical amino acid sequences.

A total of six ferret clones were sequenced and found to be very homologous to those of the rat type 2 sequences from brain and cardiac myocytes (Fig. 5). These clones exhibited an average similarity to the rat brain type 2 sequence of 99.2%. Only two of the ferret clones sequenced (F7 and F20) were identical and were 99% similar to the published rat sequence. The one notable difference between these two isolates and those of the other ferret or rat sequences is the presence of a G at nucleotide 548 (nt 7893 in X61677 ITPR2 (Genbank) Ref. 6) encoding an arginine. The other four ferret sequences characterized are very homologous to each other as well as the rat sequences and exhibit minimal sequence heterogeneity at the same nucleotide residues in the full-length rat type 2 sequences.

Taken together, the rat and ferret sequences obtained from ventricular cardiac myocytes encode the type 2 InsP3 receptor and have a high degree of similarity with the published rat type 2 receptor. The micro-heterogeneity at similar nucleotide positions observed in the ferret clones suggests that the type 2 receptor may be encoded by a small gene family of type 2 receptors or indicate that the ferret population tested was not as genetically homogeneous as that of rat.

Functional Reconstitution of Type 2 InsP3 Receptors from Ferret Ventricular Myocytes—To examine the functional features of the ferret InsP3 receptor channels, its large size was
exploited to enrich for InsP₃ receptor protein for reconstitution in planar lipid bilayers. Microsomes from ferret ventricular myocytes were solubilized in CHAPS detergent and subjected to sedimentation over 5–20% sucrose gradients. Following fractionation, the position of the receptor was established by immunoblotting using the NH₂-terminal consensus antibody (not shown). Fractions with the greatest amounts of receptor protein were reconstituted into proteoliposomes using a method similar to Ferris et al. (29). The resulting liposomes were fused into planar lipid bilayers.

The conductance of Cs⁺ through single ryanodine receptor (RyR) channels in planar bilayers is quite large (500–600 picoSiemens; Ref. 12). The homology between RyR and InsP₃R transmembrane segments (24) and the similar selectivity of single InsP₃R channels in bilayers (30) led to the assumption that Cs⁺ conduction through single InsP₃R channels may also be quite large. The function of individual type 2 InsP₃R channels was defined by fusing proteoliposomes into planar lipid bilayers with Cs⁺ as the main cationic charge carrier. The free [Ca²⁺] in the bathing solutions on both sides of the bilayer was buffered (1 mM EGTA) at 200 nM. The RyR channel, which is abundant in cardiac ventricular myocytes, will be closed at this relatively low [Ca²⁺] (12). However, single InsP₃R channels can be activated by InsP₃ at such low free [Ca²⁺] (34).

Cationic single channels appeared in the bilayer following the incorporation of InsP₃R-enriched proteoliposomes. Spontaneous infrequent single channel events were observed in the absence of added exogenous InsP₃ (Fig. 6 A). Open events were brief with few events lasting longer than 20 ms and there were long periods (10–20 s) during which no events were observed. The overall open probability (Pₒ) in the absence of added InsP₃ was 0.02 ± 0.01 (n = 4). Addition of 60 nM InsP₃ to the cis solution increased the Pₒ to 0.23 ± 0.14 (n = 4; Fig. 6B). Thus, the Cs⁺-conducting channel reconstituted from the type 2 InsP₃R-enriched proteoliposomes was activated by InsP₃.

The action of heparin on the InsP₃-activated Cs⁺-conducting channel was defined. Control channel activity was monitored in the presence of 60 nM InsP₃ in the cis solution (Fig. 7A). Hep-
channels pretreated with ryanodine (7 mM) did not open. After addition of exogenous 60 nM InsP3, the frequency of single channel events dramatically increased. Open probability was greater than 0.20 and remained relatively constant.

A reliable method to define the reversal potential in multiple channel experiments was blocked by heparin. Channel experiments were performed in the presence of 200 mM free Ca2+ to minimize the activity of RyR channels that may also be present in the proteoliposomes. The plant alkaloid ryanodine binds to the RyR protein with nanomolar affinity. Ryanodine binding dramatically alters single RyR channel activity in bilayers (11). To demonstrate that the Ca2+-conducting channel examined in this study was not a RyR, ryanodine (7 μM) was added to the cis solution and vigorously stirred for 1 min (Fig. 8A). The addition of ryanodine did not change the P_o.

The conductance of the Ca2+-conducting channel was determined by plotting the amplitude of single channel events as a function of membrane potential (Fig. 8B). This analysis, however, was complicated by the existence of multiple conduction levels (see Figs. 7A and 8A). Data pooled from eight different single channel experiments are shown in Fig. 8B (filled circles). Data points clustered around two conductance levels (388 and 274 picosiemens). The fitted lines are linear regressions. The same two conduction levels were also commonly observed in channels pretreated with ryanodine (7 μM; open circles) indicating that single channel current, like P_o, was also unaffected by the presence of ryanodine.

A reliable method to define the reversal potential in multiple or subconducting channel experiments is to apply membrane voltage ramps. Voltage ramps were applied to the Ca2+-conducting channels to define reversal potential in the presence of 220 mM cis and 20 mM trans-CaCl2SO4 (Fig. 8C, top record). Note that all conduction levels appear to reverse at the same point. In these solutions, the reversal potential of an ideal cationic selective channel would be near −60 mV. The measured reversal potential (−53 mV, see arrow) indicates that the InsP3-sensitive channel was nearly a perfect cationic selective channel. To determine if the channel was Ca2+-selective, 100 mM CaCl2 was added to the trans solution (Fig. 8C, bottom record). A reversal potential shift would indicate that the channel is permeable to the added ion. In the presence of 100 mM CaCl2, the reversal potential shifted (−53 versus −15 mV, see arrows) toward the theoretical Ca2+ equilibrium potential. These data indicate that this channel was indeed a Ca2+-selective channel. This channel is Ca2+-selective, InsP3-activated, inhibited by heparin, and thus is likely to be the InsP3 channel from cardiac ventricular myocytes. Since the identity of the InsP3 channel was established as type 2, this study is the first to examine the functional attributes of the type 2 InsP3-gated channel.

**DISCUSSION**

Identification of the Type 2 Receptor—The present study identifies the type 2 InsP3 receptor as the predominant isoform of the InsP3 receptor family expressed in rat and ferret ventricular myocytes. In addition, this study demonstrates that the isoform identified as a type 2 receptor encodes a functional calcium release channel activated by low levels of InsP3 and inhibited by heparin.

In whole heart there is significant evidence that the type 1 receptor is expressed at high levels in the conduction system,
In a similar study, Moschella and Marks (17) reported that the shares regions of sequence homology with the type 2 receptor. From cross-hybridization of type 2 mRNA, since the antisense were only weakly reactive (18). These weak signals may result kinje bundles. Working ventricular myocytes on the other hand conduction system myocytes probably corresponding to Purkinje.

Analysis at the RNA level revealed intense hybridization to via boxyl-terminal amino acids of the type 1 receptor isoform and achieved using a peptide antisera directed against the 19 car boxyl-terminal amino acids of the conserved ligand binding domain (Ref. 5, Fig. 1). The InsP3-sensitive channel was defined by applying voltage ramps. In the standard solution (220/20 Cs+), single channel current reversed at −52 mV (top record, arrow). Addition of 100 mM CaCl2 to one side shifted the reversal potential (bottom record, arrow) and attenuated single unit current. Fitted lines were fit to randomly selected regions of both records to confirm reversal potential. Horizontal lines represent the zero current level for each record.

particularly Purkinje myocytes (18, 19). Initially, the expression of the type 1 InsP3 receptor was reported in whole rat heart on Western blots using a type 1-specific antibody (5). However, interpretation of that study is complicated because it was uncertain as to whether or not the signal was derived from cardiac muscle, the associated vascular smooth muscle, or neural tissue. Recently, it has been reported by at least two groups and not altered by ryanodine. A, representative control single recordings (60 nM InsP3) are shown at top. Ryanodine (7 μM) was added to the solutions on both sides of the bilayer. Zero current levels are marked, and single channel openings are shown as upward deflections. B, relationship between single channel current (I) and membrane voltage (V). The InsP3-sensitive channels were ohmic (i.e., linear I–V relation). Data points (filled circles) collected from different channel experiments are summarized in the plot. The data were best described by two lines with slopes (i.e., conductances, IV) of 388 and 274 picosiemens. Data collected in the presence of ryanodine (7 μM) are also shown (B, open circles). The reversal potential of the multi- or substat ing InsP3-sensitive channel was defined by applying voltage ramps. In the standard solution (220/20 Cs+), single channel current reversed at −52 mV (top record, arrow). Addition of 100 mM CaCl2 to one side shifted the reversal potential (bottom record, arrow) and attenuated single unit current. Fitted lines were fit to randomly selected regions of both records to confirm reversal potential. Horizontal lines represent the zero current level for each record.

To investigate further the properties of the receptor expressed in cardiac myocytes, total RNA was prepared and polymerase chain reaction amplifications were performed using oligonucleotide primers that anneal to all three receptor subtypes. Southern hybridizations of the PCR products from both rat and ferret RNA using receptor type-specific probes indicated that the type 2 receptor is the primary species amplified from ventricular myocytes. In whole heart and left ventricular myocardium, however, all three of the receptor isoforms are detected.

The pattern and levels of expression for the individual InsP3R isoforms were examined using RNase protection with receptor subtype-specific antisense probes. The type 1 receptor was found to predominate in RNA derived from whole heart and left ventricular myocardium, whereas in myocytes the type 2 receptor was highly enriched comprising nearly 85% of the detectable signal. These results confirm the PCR screening results and suggest that in cardiac tissues all receptor isoforms are expressed, but their distribution and levels of expression are heterogeneous, possibly reflecting specialized roles for the InsP3R family members in various regions or cell types of the heart.

Sequence analysis of the PCR products of rat and ferret cardiac myocytes confirmed that the type 2 receptor sequence is present and highly conserved between rat and ferret. The ferret sequences show some micro-heterogeneity suggesting that the type 2 receptor may itself be encoded by a small highly homologous gene family of several distinct members.

Different homologues of the InsP3R receptor are found in different cell types associated with the heart. The physiological role for the differential expression of distinct InsP3R isoforms in heart is unknown. However, differences between the patterns of expression for other calcium handling proteins, for example the sarcoplasmic reticulum-Ca2+ channel, and Ca2+-pump mRNA in conduction and working myocytes have been.
noted (19). Conduction system myocytes exhibit preferential expression of the type 1 InsP₃ and type 3 ryanodine receptors, whereas the working myocytes show enhanced levels of SERCA2a as well as the type 2 ryanodine and InsP₃ receptors. It has been suggested that different InsP₃ receptor subtypes may have distinct biological roles within a cell (10). This hypothesis remains to be tested, but it is noteworthy that in whole heart, multiple types of InsP₃ receptors are expressed with the type 2 at significant levels in working myocytes and the type 1 predominating in conduction tissues.

Functional Reconstitution—Microsomal proteins from ferret ventricular myocytes were solubilized and sediments on sucrose gradients to enrich for the InsP₃ receptor. These fractions were subsequently reconstituted into proteoliposomes and used to examine whether the type 2 receptors tetramers are functional Ca²⁺ channels.

Cationic single channels appeared in the bilayer following the incorporation of proteoliposomes. These channels were Ca²⁺-selective, InsP₃-activated, and blocked by heparin. Spon-
taneous infrequent single channel events were observed in the absence of added exogenous InsP₃ (Fig. 6). Spontaneous open-
ings of an InsP₃-activated channel in the absence of InsP₃ were also reported by Borgatta et al. (16). It is possible that these openings are in response to contaminant InsP₃. Alternatively, the presence of multiple channels may lead to an over-estima-
tion of the single channel Pₒ in the absence of InsP₃. Addition of 60 nM InsP₃ to the cis solution increased the Pₒ dramatically. Despite the basal activity of the channel in the absence of InsP₃, the channel was activated by a relatively low [InsP₃].

We conclude that the single channel activity represents the functional attributes of the type 2 InsP₃R channel. This con-
clusion is supported by several lines of evidence. 1) The chan-
els are derived from preparations that are significantly en-
riched for the type 2 receptor isoform. 2) The activation of channel openings at relatively low concentrations of InsP₃ (60 nM) (Fig. 6) suggests that the affinity for InsP₃ of this channel is higher than reported in previous studies. The IC₅₀ for max-
imal activation is approximately 57 nM as compared with 200 nM for the InsP₃R of cerebellum (34). This difference may reflect the 3–4-fold higher InsP₃ binding affinity for InsP₃ of the type 2 receptors as compared with the type 1 receptor that predominates in cerebellar Purkinje cells. 3) The channel open-
ings are inhibited by heparin, which is known to inhibit ligand binding to the InsP₃ receptor. 4) Single channel activity and current amplitude were not altered by ryanodine. 5) The chan-
el is calcium-selective and permeable to monovalent cations, similar to the type 1 receptor of cerebellum (30).

Even though the type 2 InsP₃ receptor channels examined here significantly lower IC₅₀ for maximal open probability compared with those studied from cerebellum, where the type 1 receptors predominate, similarities between the two channels are conspicuous. The similarities include multiple conductance states, Ca²⁺ selectivity, permeability to monovalent cations, blockade by heparin, insensitivity to ryan-
odine, and activation by InsP₃ at low [Ca²⁺]. The only clear difference appears to be the InsP₃ regulation of the channel. Currently, the biological significance of the similarities and differences is not clear. It is also unclear whether channel dysfunction due to experimental manipulations such as mem-
brane isolation, solubilization, and reconstitution alters func-
tion. A detailed systematic characterization of type 1 and type 2 InsP₃R channel function is currently being done.²

In conclusion, this study demonstrates that the type 2 recep-

² J. Ramos-Franco, G. Mignery, and M. Fill, manuscript in preparation.

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