Inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) are ubiquitous intracellular Ca$^{2+}$ release channels whose functional characterization by transfection has proved difficult due to the background contribution of endogenous channels. In order to develop a functional assay to measure recombinant channels, we transiently transfected the rat type I IP$_3$R into COS-7 cells. Saponin-permeabilized COS cells transfected with type I IP$_3$R showed a 50% increase in inositol 1,4,5-trisphosphate (IP$_3$)-mediated Ca$^{2+}$ release at saturating [IP$_3$] (10 μM) but no enhancement at subsaturating [IP$_3$] (300 nM).

However, cotransfection of the IP$_3$R and human sarco/endoplasmic reticulum ATPase (SERCA)-2b ATPase cdNA resulted in 60 and 110% increases in Ca$^{2+}$ release at subsaturating and saturating doses of IP$_3$, respectively. IP$_3$ or adenophostin A failed to release 46Ca$^{2+}$ from microsomal vesicles prepared from cells expressing either type I IP$_3$R or SERCA cDNAs alone. However, microsomal vesicles prepared from cells doubly transfected with IP$_3$R and SERCA cDNAs released 33.0 ± 0.04% of the A23187-sensitive pool within 30 s of 1 μM adenosphin A addition. Similarly, the initial rate of 46Ca$^{2+}$ influx into oxalate-loaded microsomal vesicles was inhibited by IP$_3$ only when the microsomes were prepared from COS cells doubly transfected with SERCA-2b and IP$_3$R DNA. The absence of a functional contribution from endogenous IP$_3$Rs has enabled the use of this assay to measure the Ca$^{2+}$ sensitivities of IP$_3$-mediated 46Ca$^{2+}$ fluxes through recombinant neuronal type I (SII(+)), peripheral type I (SII(−)), and type III IP$_3$Rs. All three channels displayed a biphasic dependence upon [Ca$^{2+}$]$_{cyt}$. Introduction of mutations D2550A and D2550N in the putative pore-forming region of the type I IP$_3$R inhibited IP$_3$-mediated 46Ca$^{2+}$ fluxes, whereas the conservative substitution D2550E was without effect. This assay therefore provides a useful tool for studying the regulatory properties of individual IP$_3$R isoforms as well as for screening pore mutations prior to more detailed electrophysiological analyses.
tivity while minimizing the functional contribution of endogenous channels.

In the present study, we show that this can be achieved by co-expressing the sarco/endoplasmic reticulum ATPase (SERCA) together with recombinant IP_3 Rs in COS-7 cells. Microsomes prepared from cells expressing only IP_3 Rs or SERCA pumps exclusively were not sensitive to IP_3, whereas those co-expressing both proteins were sensitive to IP_3 in a 45Ca^2+ flux assay. This suggests that endogenous IP_3 Rs and SERCA pumps segregate to different vesicle populations during microsome preparation, and coexpressing both recombinant proteins allows co-segregation into the same vesicle pool, enabling Ca^2+ accumulation in these stores to be released by IP_3. Using this assay, we have measured the Ca^2+ sensitivities of recombinant type I (SII(+)), type I (SII(-)), and the type III IP_3 Rs in the absence of a functional contribution from the endogenous channel population. We also show that a point mutation in the putative pore-forming region eliminates channel function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Taq DNA polymerase, EXPOND long template DNA polymerase mixture, shrimp alkaline phosphatase, T4 DNA ligase, ATP, dNTPs, and protease inhibitor mixture were from Roche Molecular Biochemicals. Koz-III cDNA was purchased from Stratagene (Madison, WI). Oligonucleotides were synthesized by Life Technologies, Inc. (Grand Island, NY). Sequences were confirmed by automated dye terminator cycle sequencing. This plasmid is referred to as Koz-I/SII(+) and we refer to cloning junctions and the absence of the SII region were confirmed by automated sequencing. This plasmid is referred to as Koz-I/SII(+), and we refer to Koz-I/SII(-), and the type III IP_3 Rs in the absence of a functional contribution from the endogenous channel population. We also show that a point mutation in the putative pore-forming region eliminates channel function.

**Expression Constructs**

Koz-I/SII(+)—The cDNA encoding the rat IP_3 R type I SII(+), SII(+), SII(+) splice variant in pCMV3 was the kind gift of Dr. Thomas Sudhof (University of Texas Southwestern Medical Center). To ensure high levels of expression, the 5′-untranslated region was removed, and a Kozak sequence (24) was engineered immediately preceding the start codon using polymerase chain reaction as described previously (17). Sequences were confirmed by automated dye terminator cycle sequencing (Applied Biosystems model 377, Nucleic Acid Facility, Thomas Jefferson University). This plasmid is referred to as Koz-I/SII(+), and we refer to the protein expressed from this plasmid as type I SII(+).

Koz-I/SII(−)—The generation of the type I construct lacking the sequence corresponding to amino acids 1693–1732 in pCMV3 has been described elsewhere (14). This plasmid was cut with BamHI, which cleaves the type I C DNA at bases 4126 and 6158, which flank the SII splice region. The 1912-base pair cassette was then ligated into the plasmid containing the 7082-base pair 3′ IP_3-R-III fragment. The resulting plasmid was sequenced over the entire amplified portion and is referred to as Koz-III.

D2550EF,D2550N,D2550A—Aspartic acid 2550 (rat) was mutated to glutamic acid, aspartate, and alanine using the QuickChange point mutation kit (Stratagene, La Jolla, CA). Briefly, a cassette encompassing base pairs 7001–9466 in the C-terminal portion of the receptor was excised from Koz-III(+) using BstBI and XhoI and subcloned into pBLUEScript (Stratagene). Forward primers were designed following manufacturer’s recommendations as follows: D2550EF, 5′-GGCGGAGGTGAGTGTCCAGAGG-3′; D2550N, 5′-GGCGGAGGTGAGTGTCCAGAGGAGG-3′; D2550A, 5′-GGCGGAGGTGAGTGTCCAGAGGAGGTA-3′. The cassette was then excised and subcloned back into Koz-III(+) and reconfirmed by automated sequencing.

**Human SERCA-2b**—The cDNA encoding the human isoform of the SERCA-2b ATPase in pCDNA 3.1 was the kind gift of Dr. Jonathan Lytton (University of Calgary, Alberta, Canada) and Dr. David H. MacLennan (University of Toronto, Ontario, Canada).

**Cell Culture and Transfection**

COS-7 SV40-transformed African Green monkey kidney fibroblasts (ATCC CRL 1651) were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Life Technologies, Inc.) in a 37 °C humidified incubator in an atmosphere of 5% CO2. DNA for each construct was purified on a CaCl2 gradient as described elsewhere (25). Cells were plated at a density of 1.5 × 10^6 cells/75-cm^2 flask and transfected in serum-free medium as described previously (14). Cells were exposed to DNA-lipid complexes for 5 h before replacing the medium with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were processed after 48 h as described below. In all cases, the total amount of each expression construct DNA was 14 µg/flask. In those instances where one only cDNA was being transfected, pcDNA 3.1 vector DNA was added to adjust the total DNA concentration to 28 µg.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**

Cells were solubilized in 150 mM NaCl, 50 mM Tris-HCl, pH 7.8, 1% (w/v) Triton X-100, 1 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM Complete protease inhibitor cocktail (Roche Molecular Biochemicals) for 5 min on ice and spun at 12,000 × g, and 20 µg of the resultant supernatant was run out on a 5% SDS-polyacrylamide gel and subsequently electrotransferred to nitrocellulose membranes (Bio-Rad). Nitrocellulose sheets were then probed with the antibodies listed below and developed with chemiluminescent substrates (Pierce). In those cases where a single blot was probed sequentially with more than one antibody, the nitrocellulose was stripped at 60 °C for 1 min in stripping buffer (2% SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris-HCl) prior to probing with the next antibody.

**Antibodies**

Type I IP_3 R-specific polyclonal antibody raised against amino acids 2731–2749 has been described previously (26). Type II isoform-specific polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Type III isoform-specific monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Monoclonal antibody to SERCA-2 ATPase (2a, 2b, and species-cross-reactive) was purchased from Affinity Bioreagents (Golden, CO).

**Measurement of Ca^2+ Release in Permeabilized Cells**

COS-7 cells were grown in 75-cm^2 flasks and transfected with pcDNA 3.1 vector DNA, SERCA-2b, Koz-III(+), or co-transfected with SERCA and Koz-III(+). Cells were harvested by trypsinization and washed with 10 ml of buffer A (0.25 mM sucrose, 1 mM Mg-dithiothreitol, 1 mM magnesium acetate, 1.6 mM Na_2SO_4, 10 mM HEPES-KOH, pH 7.2, 0.5 mM phenylmethanesulfonyl fluoride). Cells were resuspended in 200 µl of buffer A and stored on ice until use. Measurement of IP_3 responses was performed in 200 mM sucrose, 50 mM KCl, 0.3 mM Mg_2+, 20 mM Tris, pH 7.2, 10 mM phosphocreatine, and 10 units/ml creatine kinase at 37 °C.
37 °C with 200 μg of protein using a Ca²⁺-selective minielectrode to measure changes in [Ca²⁺]_i. Each experiment was initiated by adding 1 mM MgATP followed by 50 μM of saponin. Filling of intracellular Ca²⁺ stores was allowed to proceed until steady state was attained (15 min). Cells were then challenged with varying doses of IP₃. At the end of each experiment, a calibrating dose of 0.4 nmol of Ca²⁺ was added.

Preparation of Microsomal Vesicles

75-cm² flasks were transfected as described above and washed with phosphate-buffered saline. Cells were released from the flask by incubation with 7 ml of PBE (5 mM EDTA, 0.5% bovine serum albumin in phosphate-buffered saline) for 10 min at room temperature. Cells were washed again with phosphate-buffered saline and swollen in hypotonic solution (10 mM Tris, pH 7.5, 0.05 mM MgCl₂) for 10 min on ice. Phenylmethylsulfonyl fluoride (0.5 mM) was added, and the cells were homogenized by 30 strokes in a tightly fitting Dounce homogenizer. Cell disruption was confirmed by trypan blue exclusion. Cells were then homogenized by 30 strokes in a tightly fitting Dounce homogenizer.

Results

Expression of Recombinant IP₃R Isoforms in COS-7 Cells—To ensure high levels of IP₃R expression, each construct had its 5’-untranslated sequence removed and a Kozak sequence inserted 5’ of the start codon (17). The levels of expressed type I SIII(−) and SIII(+) isoforms were equivalent to endogenous levels of expression found in cerebellum (Fig. 1, compare lanes 3–7 with lane 9). COS cells contain endogenous type II and type III IP₃Rs but no detectable type I IP₃R (5). Importantly, overexpression of recombinant IP₃R isoforms was not associated with up-regulation of endogenous type II or type III IP₃R proteins (for example, compare lane 3 in the first three panels). Our previous studies demonstrated that recombinant IP₃R channels expressed in COS-7 cells form tetramers, bind IP₃, localize to the endoplasmic reticulum, and do not form heterotetramers with the endogenous receptor population (28).
**Functional Expression of IP₃Rs**

versus 0.48 ± 0.06 nmol/mg protein in SERCA-2b alone at 300 nm IP₃; see Fig. 3). The size of the total releasable pool measured with 10 μM IP₃ nearly doubled from 1.62 ± 0.05 nmol/mg protein in SERCA-2b-expressing cells to 2.84 ± 0.06 in cells expressing both type I SII(+) and SERCA-2b (Fig. 3). IP₃-induced Ca²⁺ release from cells expressing SERCA-2b alone was not significantly different from that observed in cells transfected with vector alone (Figs. 2 and 3). Enhanced Ca²⁺ release was probably not due to a direct physical association between the IP₃R and SERCA-2b as determined by co-precipitation assays (data not shown). Furthermore, SERCA-2b expression did not increase the number of functional IP₃ binding sites (Fig. 9B).

**45Ca²⁺ Release from Microsomal Vesicles**—Microsomal vesicles prepared from many cell types can accumulate 45Ca²⁺ in the presence of MgATP but are unresponsive to IP₃ unless GTP and polyethylene glycol are added (32–34). It is thought that IP₃ Rs and SERCA pumps segregate to different vesicle populations during microsome preparation, and the addition of GTP and polyethylene glycol promotes fusion of the vesicles restoring the sensitivity to IP₃. We hypothesized that overexpression of IP₃ Rs and SERCA pumps in combination would result in co-segregation of both proteins during vesicle preparation, resulting in vesicles responsive to IP₃. To test this hypothesis, the time course of 45Ca²⁺ uptake by microsomal vesicles was determined using preparations in which SERCA-2b was expressed alone or in conjunction with the type I SII(+) IP₃ R (cells not expressing SERCA-2b did not accumulate significant levels of 45Ca²⁺ in our assay system; data not shown). 45Ca²⁺ accumulation achieved equilibrium by 40 min, and the addition at 60 min of the potent nonhydrolyzable IP₃ R agonist AdA (1 μM) elicited a 33.0 ± 0.04% reduction in the size of the A23187-releasable pool within 30 s (Fig. 4). No reduction in the size of the A23187-releasable pool was observed upon the AdA addition to microsomes expressing SERCA-2b alone (n = 10). Similar results were obtained when 10 μM IP₃ was used instead of AdA (data not shown).

**45Ca²⁺ Uptake in the Presence of Potassium Oxalate**—The addition of potassium oxalate (Kox) when measuring SERCA Ca²⁺ uptake activity allows an almost 50-fold increase in 45Ca²⁺ uptake, due to the fact that Kox acts as an intravesicular Ca²⁺ sink (compare Figs. 4 and 5). SERCA-dependent uptake in the presence of Kox is linear during the first 15 min, and the slope of this line is a direct measure of the initial rate of SERCA-dependent 45Ca²⁺ uptake (Fig. 6) (35). The addition of IP₃ reliably reduced the initial rate of 45Ca²⁺ uptake, which provided an indirect measure of IP₃ R-mediated Ca²⁺ release (36, 37). The IP₃-dependent decrease in SERCA activity was fully reversible by the addition of the IP₃ R antagonist heparin, further suggesting that the effects of IP₃ are due to the gating of IP₃ R channels (Fig. 6). 45Ca²⁺ uptake in microsomal vesicles prepared from cells transfected with SERCA alone or in combination with IP₃ R cDNAs demonstrated that there was no IP₃-dependent reduction in the initial rate of uptake unless both recombinant IP₃ R and SERCA pumps were present (Fig. 7).

**Ca²⁺ Sensitivities of Recombinant Type I SII(–), Type I SII(+), and Type III IP₃ R Isoforms**—A fundamental regulatory property of IP₃ Rs is a biphasic dependence of channel activity on cytoplasmic Ca²⁺ concentration (38, 39). We used the flux assay shown in Fig. 6 to measure the Ca²⁺ sensitivities of the two type I splice variants and the type III receptor isoforms. Flux assays were performed in oxalate-containing buffers, which had been calibrated to the indicated [Ca²⁺]free as described under “Experimental Procedures” in the presence or absence of 1.0 μM IP₃. All three isoforms demonstrated a biphasic dependence upon [Ca²⁺]free (Fig. 8). The Ca²⁺ dependence of the recombinant type I SII(+) channel is similar to that previously reported for the endogenous channels of cerebellum (39). As summarized in Table I, calculated values of half-maximal activation by Ca²⁺ (Kₘₐₓ) and inhibition (Kₖᵢₙ) were 0.014 and 1.4 μM [Ca²⁺]free, respectively, for the type I SII(+) splice variant. The Hill coefficient for activation was 1, whereas for inactivation it was 2.5. The Ca²⁺ sensitivity of the SII(–) variant was shifted to the left with respect to the SII(+) channel (Fig. 8, Table I). Kₘₐₓ and Kₖᵢₙ for the SII(–) splice variant were 0.0025 and 0.27 μM, respectively. The activation curve for the SII(–) receptor showed greater cooperativity with an Hₘᵢₙ value of 1.8 providing a good fit to the data (Table I). Conversely, Ca²⁺ dependent inhibition was less cooperative, with an Hₖᵢₙ of 1.0 (Table I). The Ca²⁺ sensitivity of the type III

**FIG. 2.** Ca²⁺ release in saponin permeabilized cells. COS-7 cells were transfected with either pcDNA3.1 (control, black trace), human SERCA-2b (SERCA, green trace), type I IP₃ R SII(+) (Type SII(+), red trace), or a combination of SERCA and type I IP₃ R (Type I SII(+)SERCA, blue trace). Cells were then trypsinized, washed with phosphate-buffered saline, and resuspended in buffer A. 200 μg of cells were then placed in a Ca²⁺-selective minilactrode and permeabilized with saponin, and intracellular stores were loaded with Ca²⁺ in the presence of MgATP for 10 min (not shown). Cells were then challenged sequentially with 150 nM, 300 nM, and 10 μM IP₃. A calibrating dose of 0.4 nmol of Ca²⁺ was added at the end of each experiment.

**FIG. 3.** Functional changes associated with transient type I IP₃ R overexpression. A–C show the amount of Ca²⁺ release from saponin-permeabilized cells at concentrations of IP₃ of 150 nM, 300 nM, and 10 μM, respectively. The bars represent the pooled data from experiments illustrated in Fig. 2 performed at least three times in triplicate. Note that the axis scaling is different in all three panels.* significance of p < 0.05 versus SERCA or control.
A series of experiments were undertaken to determine the feasibility of utilizing this assay system to screen for the Ca\textsuperscript{2+} release activity of expressed IP\textsubscript{3}Rs with engineered point mutations. We hypothesized that the aspartic acid residue at position 2550 may be important for Ca\textsuperscript{2+} ion permeation through the channel.

This residue is adjacent to a sequence in the IP\textsubscript{3}R that is analogous to the selectivity filter in potassium channels (underlined in Fig. 9) (40). We made three point mutations in which the aspartic acid was mutated to alanine (D2550A), asparagine (D2550N), and glutamic acid (D2550E). These mutations did not affect the ability of the channel to express at high levels (Fig. 1) or form oligomers, as determined by their ability to co-precipitate with an epitope-tagged type III IP\textsubscript{3}R (data not shown). Furthermore, all three mutants bound IP\textsubscript{3} at levels comparable with wild type (Fig. 9B). Mutating aspartic acid to either asparagine or alanine eliminated responsiveness of 45Ca\textsuperscript{2+} fluxes to IP\textsubscript{3} (Fig. 9A). However, the conservative mutation to glutamate preserved IP\textsubscript{3}-induced Ca\textsuperscript{2+} release activity (Fig. 9A). Taken together, these data suggest that a negatively charged residue at position 2550 is necessary for Ca\textsuperscript{2+} ion permeation through the IP\textsubscript{3}R channel.
In the present study, we have described a system for measurements of the Ca\(^{2+}\) release activity of recombinant IP\(_3\)R channels in the absence of a contribution from the endogenous channel population, which can be used for determining structure-function relationships of IP\(_3\)R channels. A key feature of this assay system is the necessity to transfect SERCA pumps together with IP\(_3\)Rs in order to observe IP\(_3\) responses in microsomal vesicles. We interpret this result to indicate that under these circumstances the recombinant IP\(_3\)Rs and SERCA pumps co-segregate into the same compartments. An implication of this result is that recombinant and endogenous IP\(_3\)Rs may reside in different subcellular compartments of the endoplasmic reticulum. Although we have not directly addressed the subcellular localization of IP\(_3\)Rs and SERCA pumps, our studies lend support to the view that IP\(_3\)Rs are not uniformly distributed in intracellular membranes (reviewed in Ref. 41).

Various strategies have been employed to reduce the contribution of endogenous channels in functional studies of recombinant IP\(_3\)R channel activity. In one approach, IP\(_3\)R channels were overexpressed in cell lines that have a low density of endogenous IP\(_3\)R channels. Microsomes or proteoliposomes enriched in IP\(_3\)Rs were prepared from these cells and incorporated into planar lipid bilayers (12, 17). An alternative nuclear patch clamp approach employed IP\(_3\)R mRNA injection into Xenopus oocytes at low levels of endogenous IP\(_3\)R channel activity were detected (18). The advantage of the assay system described in this study is that it is relatively rapid and simple and allows for the measurement of recombinant IP\(_3\)R Ca\(^{2+}\) release activity in native endoplasmic reticulum membranes. In contrast to single-channel analyses, it measures global Ca\(^{2+}\) flux, which is important when considering how a population of channels responds to stimulation with IP\(_3\). A potential disadvantage of this approach is that it does not permit detailed biophysical analyses of channel properties. In all expression systems used to date, a possible limitation is the likelihood that transfected receptors may form heterotetramers with the endogenous channels, which could complicate interpretation of the data. However, we have shown that recombinant IP\(_3\)R channels do not associate significantly with the endogenous IP\(_3\)R population in the COS cells used in the present study (28). We therefore consider the methodology described in this study well suited for investigating the regulatory properties of recombinant IP\(_3\)Rs, as well as for preliminary screening of the functionality of IP\(_3\)R mutations prior to more laborious electrophysiological analyses.

**Table I**

|          | K\(_{\text{act}}\) | K\(_{\text{inh}}\) | Hill\(_{\text{act}}\) | Hill\(_{\text{inh}}\) |
|----------|----------------|----------------|----------------|----------------|
| Type I SH(−) | 0.0925 | 0.2718 | 1.8069 | 0.9631 |
| Type I SH(+) | 0.0143 | 1.4403 | 0.9088 | 2.4635 |
| Type III | 0.0332 | 0.9973 | 1.1019 | 1.7686 |

**DISCUSSION**

In the present study, we have described a system for measurements of the Ca\(^{2+}\) release activity of recombinant IP\(_3\)R channels in the absence of a contribution from the endogenous channel population, which can be used for determining structure-function relationships of IP\(_3\)R channels. A key feature of this assay system is the necessity to transfect SERCA pumps together with IP\(_3\)Rs in order to observe IP\(_3\) responses in microsomal vesicles. We interpret this result to indicate that under these circumstances the recombinant IP\(_3\)Rs and SERCA pumps co-segregate into the same compartments. An implication of this result is that recombinant and endogenous IP\(_3\)Rs may reside in different subcellular compartments of the endoplasmic reticulum. Although we have not directly addressed the subcellular localization of IP\(_3\)Rs and SERCA pumps, our studies lend support to the view that IP\(_3\)Rs are not uniformly distributed in intracellular membranes (reviewed in Ref. 41).

**Fig. 8.** Ca\(^{2+}\) dependence of type I SH(−), type I SH(−), and type III IP\(_3\)Rs. Uptake in microsomes prepared from cells expressing type I (SH(−)), type I (SH(−)), or type III IP\(_3\)R in conjunction with SERCA was assayed exactly as described in Fig. 6. The effect of IP\(_3\) on inhibiting flux at each [Ca\(^{2+}\)] was expressed as 1−(r\(_{\text{IP3/r}}\)) and is plotted on the ordinate. Assays were performed at Ca\(^{2+}\) concentrations of 1.9 nm, 6.5 nm, 55 nm, 200 nm, 1.0 \(\mu\)M, and 3.0 \(\mu\)M. The data were fit to a biphasic equation assuming independent Ca\(^{2+}\)-activating and -inhibiting sites (27). Parameters for fitting the equation are listed in Table I. SERCA-2b activity was monophasically dependent upon [Ca\(^{2+}\)] free with a K\(_{\text{act}}\) of 0.06 \(\mu\)M and a Hill coefficient of 2 (data not shown).

**Fig. 9.** Effect of amino acid substitutions at aspartic acid 2550. Above A is shown the aligned sequences of the three IP\(_3\)R isoforms and type I RyR flanking amino acid 2550 (in boldface type) of the rat type I IP\(_3\)R sequence. Amino acids 2545–2549 (rat type I) are similar to the selectivity filter TVGYGD of potassium-selective voltage-gated channels (solid bar). A shows the effect on \(^{45}\)Ca\(^{2+}\) flux when aspartic acid 2550 is substituted for glutamic acid (D2550E), asparagine (D2550N), and alanine (D2550A). The inhibition of the rate of \(^{45}\)Ca\(^{2+}\) uptake in the presence of 1 \(\mu\)M IP\(_3\) is represented as a percentage of the control rate of uptake. *, not significantly different from control uptake (p > 0.1). B shows the IP\(_3\) binding capacity of the recombinant type I SH(−) IP\(_3\)R (in the presence and absence of SERCA-2b), D2550E, D2550N, and D2550A. There was negligible binding in vector-transfected cells (Mock). IP\(_3\) binding to cell lysates was done exactly as described previously (57). Rat IP\(_3\)-I, IP\(_3\)-II, and IP\(_3\)-III and rabbit RyR-I sequences were obtained from GenBankTM accession numbers J05510, X61677, L06096, and X15209, respectively.
A fundamental regulatory property of IP$_3$R channels is their biphasic dependence upon cytoplasmic Ca$_{2+}$ concentration (38, 39). This property is presumed to be one of the underlying mechanisms by which IP$_3$R channels are capable of generating and propagating Ca$_{2+}$ waves (42). We show here that the recombinant type I SII(+) or type I SII(−) and type III IP$_3$Rs are all biphasically regulated by [Ca$^{2+}$_i]$_{out}$. The type I SII(+) receptor displays a Ca$_{2+}$ sensitivity similar to that found previously (39). The behavior of the type I SII(−) splice variant was analyzed for the first time in the present study. The type I SII(−) splice variant was activated by lower Ca$_{2+}$ concentrations and with greater cooperativity when compared with type III or type I SII(+). This splice variant was also more sensitive to Ca$_{2+}$-dependent inhibition. Therefore, alternative splicing of the type I transcript appears to have significant effects on channel regulation by Ca$_{2+}$. It has been proposed that deletion of the SII region creates an additional binding site for calmodulin (14). It is therefore possible that an additional calmodulin binding site is responsible for shifting the Ca$_{2+}$ sensitivity to the left. Further characterization of the effects of calmodulin on IP$_3$R channels is necessary to validate these conclusions.

When compared with type I SII(+) or type III IP$_3$R channel in Xenopus oocytes (18). These observations are in contrast to other reports, which have demonstrated that both type II and type III IP$_3$R channels are not biphasically regulated by Ca$_{2+}$ when measured in planar lipid bilayers (43, 44). These studies utilized tissues that are specifically enriched in either the type II receptor (ventricular cardiac myocytes) or type III receptors (RIN-m5F cell line) (43, 44). Hagar et al. (44) suggested that those cells that have predominately type II or type III IP$_3$Rs would not support Ca$_{2+}$ oscillations due to a lack of Ca$_{2+}$-dependent inhibition. However, studies using targeted IP$_3$R knockouts in DT40 cells have shown that those cells that are expressing only type II IP$_3$Rs can support oscillations (45). Furthermore, Swatton et al. (46) showed that IP$_3$-mediated Ca$_{2+}$ release in permeabilized RIN-m5F cells is biphasically regulated by Ca$_{2+}$. Therefore, the lack of Ca$_{2+}$-dependent inhibition of the type II and type III receptor observed in some studies may have resulted from the loss of accessory proteins after incorporation into planar lipid bilayers, as has been demonstrated with the type I receptor from cerebellum (47). Swatton et al. (46) have suggested that Ca$_{2+}$-dependent inhibition of type III IP$_3$Rs in RIN-m5F cells might result from hetero-oligomer formation with the low levels of endogenous type I receptor. We consider this an unlikely explanation to account for the Ca$_{2+}$ sensitivity of recombinant type III IP$_3$Rs in our system, since we have no evidence for the occurrence of hetero-oligomerization under our experimental conditions (28). Hetero-oligomerization was similarly ruled out in studies of the type III channel in nuclear patch clamp studies (18). Hence, we conclude that a general property of the type III IP$_3$R is a biphasic dependence upon cytoplasmic Ca$_{2+}$ concentration.

The success of our approach for studying the functionality of recombinant IP$_3$R isofoms suggested that it will be useful for future structure-function studies of mutant IP$_3$R channels. We explored this possibility in initial experiments to determine which residues are important for IP$_3$R channel permeation. The transmembrane topology of IP$_3$R channels is similar to voltage-gated potassium channels with six transmembrane-spanning segments and a pore-forming region between segments 5 and 6 (48–50). The crystal structure of a potassium channel from Streptomyces lividans (KcsA) has highlighted the importance of the highly conserved sequence GYG within the pore-forming region (40). The carbonyl oxygens of these three amino acids function as a selectivity filter by precisely coordinating potassium ions (40). The IP$_3$R is not as ion-selective as K$^+$ channels (51, 52), and it is therefore likely that there will be differences between the selectivity filters of the two channels. However, this region of IP$_3$Rs, RyRs, and K$^+$ channels does show a high degree of homology. In particular, the aspartate residue immediately following the GYG sequence in potassium channels is highly conserved, being GVGD in IP3Rs and GIGD in RyRs (Fig. 9). Based on the crystal structure of KcsA, the aspartate 2550 to uncharged aspartate or alanine inhibits IP$_3$-stimulated 45Ca$_{2+}$ fluxes. However, preserving a negative charge at this position by replacing aspartate with glutamate retained channel function. We conclude that a negative charge at position 2550 is required for channel function. This is a result that is consistent with analogous mutations in Shaker (53, 54) and Kv2.1 (55) potassium channels, where a negative charge is absolutely required at this position. Furthermore, this residue has been shown to be important for ion selectivity in potassium channels (55) and has been proposed to increase K$^+$ ion occupancy in the channel pore (54). Nevertheless, the corresponding mutation in cardiac RyRs (D4829A) failed to block caffeine-mediated Ca$_{2+}$ release, although ryanodine binding was inhibited (56). Although future mutagenesis studies will be required to clarify these differences and to investigate the mechanism of ion permeation by IP$_3$R channels, the results described in the present study demonstrate the utility of this system in furthering such efforts.

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REFERENCES

1. Patel, S., Joseph, S. K., and Thomas, A. P. (1999) Cell Calcium 25, 247–264
2. Mignery, G. A., Newton, C. L., Archer, B. T., III, and Sudhof, T. C. (1990) J. Biol. Chem. 265, 12679–12685
3. Sudhof, T. C., Newton, C. L., Archer, B. T., III, Ushkaryov, Y. A., and Mignery, G. A. (1991) EMBO J. 10, 2135–2144
4. Blondel, O., Takeda, J., Janssen, H., Seino, S., and Bell, G. I. (1993) J. Biol. Chem. 268, 11356–11363
5. Wojciechowski, R. J. H. (1995) J. Biol. Chem. 270, 11678–11683
6. Wojciechowski, R. J. H., and He, Y. (1995) Biochim. Biophys. Res. Commun. 213, 813–820
7. Monkawa, T., Miyawaki, A., Sigiyama, T., Yoneshima, H., Yamamoto-Hino, M., Furutachi, T., Saruta, T., Hasegawa, M., and Mikoshiba, K. (1995) J. Biol. Chem. 270, 14700–14704
8. Joseph, S. K., Lin, C., Pierson, S., Thomas, A. P., and Maranto, A. P. (1995) J. Biol. Chem. 270, 23310–23316
9. Nurisora, F. C., Sharp, A. H., Milgram, S. L., and Ross, C. A. (1996) Mol. Biol. Cell 7, 949–960
10. Newton, C. L., Mignery, G. A., and Sudhof, T. C. (1994) J. Biol. Chem. 269, 29615–29619
11. Danoff, S. F., Ferris, C. D., Denath, C., Fischer, G. A., Munemitsu, S., Ullrich, A., Snyder, S. H., and Ross, C. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2951–2955
12. Blinks, O., Takeda, J., Janssen, H., Seino, S., and Bell, G. I. (1993) J. Biol. Chem. 268, 11356–11363
13. Challiss, R. A. J. (1996) J. Gen. Physiol. 105, 23310–23316
14. Lin, C., Widjaja, J., and Joseph, S. K. (2000) J. Gen. Physiol. 115, 241–255
15. Maeda, N., Niinobe, M., and Mikoshiba, K. (1990) J. Biol. Chem. 265, 21356–21361
16. Liu, J. P., Chen, S. W., and Numa, S. (1991) J. Biol. Chem. 266, 18783–18789
17. Miyawaki, A., Furuichi, T., Maeda, N., and Mikoshiba, K. (1990) EMBO J. 9, 2135–2144
18. Wolery, P. F., Baronah, J. M., Dupratpstone, S., Wilson, V. S., and Snyder, S. H. (1987) J. Biol. Chem. 262, 12132–12136
19. Kuznetsova, E., Vavilina, D., and Sokolov, S. V. (1996) J. Gen. Physiol. 111, 847–856
20. Mak, D. D., McBride, S., Rahagurum, V., Yue, Y., Joseph, S. K., and Fossett, J. K. (2000) J. Gen. Physiol. 115, 241–255
21. Miyawaki, A., Furutachi, T., Maeda, N., and Mikoshiba, K. (1990) Neuron 3, 11–18
22. Davis, R. J., Challis, R. A. J., and Nahorski, S. R. (1999) Biochem. J. 341, 871–878
23. Mackrell, J. J., Wileon, R. A., Miyawaki, A., Mikoshiba, K., Nahorski, S. R., and Challis, R. A. J. (1996) Biochem. J. 318, 871–878
24. Dusour, J. P., Luthi, M., Forestier, M., and Magnino, P. (1999) Hepatology 30,
23. Catterall, W. A. (1995) *Annu. Rev. Biochem.* **64**, 493–531
24. Kozak, M. (1987) *Nucleic Acids Res.* **15**, 8125–8137
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Joseph, S. K., and Samanta, S. (1993) *J. Biol. Chem.* **268**, 6477–6486
27. Kozak, M. (1987) *Nucleic Acids Res.* **15**, 8125–8137
28. Joseph, S. K., Bokkala, S., Boehning, D., and Zeigler, S. (2000) *J. Biol. Chem.* **275**, 16084–16090
29. Mak, D. O., McBride, S., and Foskett, J. K. (1999) *J. Biol. Chem.* **274**, 22231–22237
30. Joseph, S. K., Bokkala, S., Boehning, D., and Zeigler, S. (2000) *J. Biol. Chem.* **275**, 16084–16090
31. Mak, D. D., and Foskett, J. K. (1998) *Am. J. Physiol.* **275**, C179–C188
32. Goldstein, A. N., Pheasant, D. J., and Miller, C. (1994) *Neuron* **12**, 1377–1388
33. Molina, A., Ortega-Saenz, P., and Lopez-Barneo, J. (1998) *J. Physiol. (Lond.)* **509.2**, 327–337
34. Kirsch, G. E., Pascale, J. M., and Shich, C. (1995) *Biophys. J.* **68**, 1804–1813
35. Zhao, M., Li, P., Li, X., Zhang, L., Winkfein, R. J., and Chen, S. R. W. (1999) *J. Biol. Chem.* **274**, 25971–25974
36. Joseph, S. K., Pierson, S., and Samanta, S. (1995) *Biochem. J.* **307**, 859–865
