The Type III Inositol 1,4,5-Trisphosphate Receptor Preferentially Transmits Apoptotic Ca\textsuperscript{2+} Signals into Mitochondria*

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There are three isoforms of the inositol 1,4,5-trisphosphate receptor (InsP\textsubscript{3}R), each of which has a distinct effect on Ca\textsuperscript{2+} signaling. However, it is not known whether each isoform similarly plays a distinct role in the activation of Ca\textsuperscript{2+}-mediated events. To investigate this question, we examined the effects of each InsP\textsubscript{3}R isoform on transmission of Ca\textsuperscript{2+} signals to mitochondria and induction of apoptosis. Each isoform was selectively silenced using isoform-specific small interfering RNA in Chinese hamster ovary cells, which express all three InsP\textsubscript{3}R isoforms. ATP-induced cytosolic Ca\textsuperscript{2+} signaling patterns were altered, regardless of which isoform was silenced, but in a different fashion depending on the isoform. ATP also induced Ca\textsuperscript{2+} signals in mitochondria, which were inhibited more effectively by silencing the type III InsP\textsubscript{3}R than by silencing either the type I or type II isoform. The type III isoform also co-localized most strongly with mitochondria. When apoptosis was induced by activation of either the extrinsic or intrinsic apoptotic pathway, induction was reduced more effectively by silencing the type III InsP\textsubscript{3}R. These findings provide evidence that the type III isoform of the InsP\textsubscript{3}R plays a special role in induction of apoptosis by preferentially transmitting Ca\textsuperscript{2+} signals into mitochondria.

Cytosolic Ca\textsuperscript{2+} (Ca\textsubscript{\textsuperscript{2+}}\textsubscript{cyt}) is a versatile second messenger that can simultaneously regulate multiple processes within an individual cell (1). This complex regulatory action of Ca\textsuperscript{2+} is thought to result in part from the varied spatial and temporal patterns of Ca\textsuperscript{2+} signals that can occur (1). These signaling patterns in turn are thought to result from special properties of the inositol-1,4,5-trisphosphate (InsP\textsubscript{3}) receptor (InsP\textsubscript{3}R), which is the principal intracellular Ca\textsuperscript{2+} release channel in most types of cells (2). The InsP\textsubscript{3}R is gated by InsP\textsubscript{3} and is localized to the endoplasmic reticulum (3) and to a lesser extent the nucleus (4, 5). There are three isoforms of the InsP\textsubscript{3}R, each of which has a different affinity for InsP\textsubscript{3} (6) and distinct functional properties at the single channel level (7, 8). Each

InosP\textsubscript{3}R isoform also has distinct effects on Ca\textsuperscript{2+} signaling patterns in intact cells (9, 10). Although some cells and tissues express a single or predominant isoform of the receptor, most cells instead express two or all three InsP\textsubscript{3}R isoforms (9, 11–13). The presence of multiple isoforms within an individual cell suggests that Ca\textsuperscript{2+} released from each isoform might mediate distinct cellular events.

Apoptosis is an Ca\textsuperscript{2+}-mediated event that may be influenced differently by each InsP\textsubscript{3}R isoform. Morphologically, apoptosis is characterized by membrane blebbing, chromatin condensation, DNA fragmentation, and eventually the formation of apoptotic bodies, which are phagocytosed by neighboring cells (14). Studies at the molecular level suggest that the InsP\textsubscript{3}R plays an important role in the development of apoptosis (15–17). Initial evidence suggested that Ca\textsuperscript{2+}-dependent apoptotic death was mediated by the type III InsP\textsubscript{3}R (15), but subsequent studies have shown that the type I isoform can also mediate apoptosis (16–19). The specific role of the type II InsP\textsubscript{3}R in mediating apoptosis has not been examined. Moreover, the relative role that each isoform plays in cells co-expressing all three InsP\textsubscript{3}R isoforms has not been examined, although most cells express two or all three isoforms. The goal of this work was to examine the relative role of each InsP\textsubscript{3}R isoform in mediating apoptosis in a model cell line that expresses all three isoforms of the receptor.

EXPERIMENTAL PROCEDURES

Materials, Reagents, and Cells—Staurosporine and Nonidet P-40 were purchased from Calbiochem. Glycochenodeoxycholic acid (GCDCA), 4′,6-diamidino-2-phenylindole (DAPI), and ATP were from Sigma. Alexa-488 and Alexa-568 secondary antibodies, Mitotracker Red, ProLong Antifade Kit, Fluo-4/AM, and rhod-2/AM were from Molecular Probes, Inc. (Eugene, OR). The Silencer siRNA construction kit was from Ambion (Austin, TX). RNAi Fect transfection reagent and Effectene transfection kit were from Qiagen (Valencia, CA). Monoclonal antibody against InsP\textsubscript{3}R-III was purchased from Transduction Laboratories (Lexington, KY), whereas polyclonal antibody against InsP\textsubscript{3}R-I was purchased from Affinity BioReagents (Golden, CO). Polyclonal antibody against InsP\textsubscript{3}R-II was prepared as previously described (11). Peroxidase-conjugated secondary antibody was purchased from Amersham Biosciences. The plasmid pNTCP-GFP was kindly provided by Jonathan A. Dranoff (Yale University). CHO and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640, supplemented with 1.6% l-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% fetal bovine serum at 37°C in 5% CO\textsubscript{2} (21). All other reagents were of the highest quality commercially available.
Real Time Polymerase Chain Reaction—Real time PCR analysis was performed using an Applied Biosystems 7700 sequence detector (Foster City, CA) as described (22). Sets of primers were chosen for each InsP3R isoform and for glyceraldehyde-3-phosphate dehydrogenase to give PCR products less than 100 base pairs in length. Dual labeled fluorogenic probes complementary to a sequence within each PCR product were added to the PCRs. Primers and probes were custom synthesized by Applied Biosystems. Reverse transcription was performed on 5 μg of CHO total RNA with SuperScript II Ribonuclease H2 reverse transcriptase from Invitrogen according to the manufacturer’s protocol. Complementary DNA was amplified in a 50-μl volume containing 25 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 100 nmol/liter probe (Applied Biosystems), and 300 nmol/liter each primer. After a denaturing step at 95 °C for 10 min, 40 cycles were performed at 95 °C for 15 s and then 60 °C for 1 min. Mathematical analysis of the results was performed as recommended by the manufacturer.

Reverse Transcription-PCR—Reverse transcription-PCR was performed to determine whether CHO cells express the ryanodine receptor. Total RNA from CHO cells was isolated using Trizol reagent. First strand cDNA was synthesized using the primer oligo-(dT)16 and Moloney murine leukemia virus reverse transcriptase. RNA samples were then subjected to DNase- and RNase-free treatment to extract any possible genomic DNA. A negative control was carried out in which RNA but no reverse transcriptase was added (RNA control). Degenerate primers were designed to amplify a 530-base pair product from a portion of the 3’ region common to all three ryanodine receptor isoforms (23). PCR amplification then was performed in a PTC-100 automated thermocycler (MJ Research, Watertown, MA) using 2 μl of the first strand cDNA reaction, 200 nM each primer, 200 μM dNTPs, 2.5 mM

FIGURE 1. CHO cells express all three InsP3R isoforms. Real time quantitative PCR was used to measure the relative mRNA distribution for each InsP3R isoform. The type II isoform is most abundant in CHO cells, present in an InsP3R/glyceraldehyde-3-phosphate dehydrogenase ratio of 2.43. Isoforms I and III are present in ratios of 1.29 and 1.04, respectively.

FIGURE 2. Silencing of specific InsP3R isoforms. CHO (A–C) or HEK293 (D) cells were transfected with 40 nM siRNA for InsP3R-I, InsP3R-II, InsP3R-III, or scrambled InsP3R-III, followed by a 48-h incubation. Immunoblot of whole-cell protein from CHO cells demonstrates that siRNA for InsP3R-I (A), InsP3R-II (B), and InsP3R-III (C) specifically knocks down expression of each InsP3R isoform. Expression of β-actin serves as a loading control. Densitometric analysis confirms reduction of InsP3R-I expression by 99 ± 0.3% (A), InsP3R-II by 70 ± 0.6% (B), and InsP3R-III by 97 ± 0.6% (C), compared with control. Each result is representative of four individual experiments. D, Immunoblot of whole-cell protein from HEK293 cells demonstrates that siRNA for InsP3R-I, InsP3R-II, and InsP3R-III specifically knocks down expression of each InsP3R isoform in this cell type as well. Each result is representative of three individual experiments.
FIGURE 3. Subcellular localization of InsP3R isoforms in CHO cells. Confocal immunofluorescence with isoform-specific antibodies demonstrates that each isoform is distributed heterogeneously in the cytosol, with variable nuclear staining (scale bar, 10 μm). Nonspecific staining by secondary antibody was not seen (not shown). Expression of each isoform is specifically silenced by its respective siRNA. Results are representative of five independent experiments.

Preparation of siRNA—Potential target sites within the rat InsP3R genes were selected and then searched with NCBI BlastN to confirm specificity for each InsP3R isoform. The siRNAs for the type I, II, and III InsP3R, and an siRNA containing the same nucleotides for type III InsP3R but in a scrambled sequence were prepared by a transcription-based method using the Silencer kit according to the manufacturer’s instruction. The sense and antisense oligonucleotides of siRNAs were, respectively, as follows: type I, 5′-AAAGTTGTAGCTGCTTGTTGCTCTGTCCCTC-3′ and 5′-AAAGCACACAGCTACACTCCTCTGCCTCTCC-3′; type II, 5′-AAAGCTAAACATCAAGATCTCCTGCTC-3′ and 5′-AAAGGAGATCTTGATTAGGCTGCCCTGCTCTC-3′; type III, 5′-ATGGTGTCGGAAACTCGTTCCGTTGCTC-3′ and 5′-AAACAAGTTGGCCAGCACCATCTCGCTCTC-3′; scrambled type III, 5′-AACAGTCAAAAGCTCTCTGCACTCGCTCTC-3′ and 5′-TGACGAGAAGCTTGTAGCTGCCTGTCTC-3′.

Transfection of pNTCP-GFP and siRNA—CHO cells were maintained in culture at 37 °C in an atmosphere of 5% CO2 for 24 h prior to transfections. For studies of bile acid-induced apoptosis, cells were transfected using Effectene with 0.5 μg of pNTCP-GFP, a construct encoding a green fluorescent protein (GFP)-tagged bile acid transporter (24). For siRNA studies, the cells were washed and supplied with 1 ml of fresh tissue culture medium, and then 1 μg of siRNA and 3 μl of transfection reagent were mixed with 100 μl of tissue culture medium. The mixture was incubated for 15 min at room temperature for complex formation, and then 900 μl of tissue culture medium was added to the mixture, and this solution was placed dropwise onto the cells, resulting in a final siRNA concentration of 40 nM. The cells were incubated at 37 °C in an atmosphere of 5% CO2 for 48 h prior to use.

Immunoblotting—Standard methods were used for immunoblots (25, 26). Briefly, cells grown in 35-mm dishes were washed three times with phosphate-buffered saline (PBS) and solubilized in 80 μl of Nonidet P40 containing a protease inhibitor mixture (Roche Applied Science). The protein concentration was determined spectrophotometrically, and 40 μg of protein was separated by electrophoresis in a 6% polyacrylamide gel and then transferred to an Immobilon polyvinylidene membrane. The membrane was blocked with 5% skim milk in PBS (1% Tween 20) for 60 min and then incubated with primary antibody. The primary antibodies used were as follows: rabbit anti-InsP3R-I polyclonal antibody (1:1000), rabbit anti-InsP3R-II polyclonal antibody (1:150), mouse anti-InsP3R-III monoclonal antibody (1:500), and rabbit anti-α-tubulin monoclonal antibody (1:400). This incubation was carried out for 2 h at room temperature. After three washes with PBS, the membrane was incubated with peroxidase-conjugated secondary antibody (1:5000) for 1 h at room temperature. Bands were revealed by enhanced chemiluminescence (ECL plus; Amersham Biosciences). The film was scanned with a GS-700 imaging densitometer (Bio-Rad), and then quantitative analysis was performed using Multi-Analyt software (Bio-Rad).

Immunofluorescence—Confocal immunofluorescence was performed as described previously (25, 26). Cells were fixed with 4% paraformaldehyde in PBS for 10 min and then washed three times in PBS. The cells were incubated for 1 h in blocking solution (PBS containing 1% bovine serum albumin, 0.5% Triton, 5% goat serum), and then incubated for 2 h at room temperature in PBS with 1% bovine serum albumin containing one of the following primary antibodies: anti-InsP3R-I polyclonal antibody (diluted 1:100), anti-InsP3R-II polyclonal antibody (diluted 1:25), or anti-InsP3R-III monoclonal antibody (diluted 1:100). The cells were washed three times in PBS and incubated for 1 h in PBS with 1% bovine serum albumin containing secondary antibody conjugated to Alexa-488 (diluted 1:500). Negative controls were stained with secondary antibody alone. Cells were washed six times, and coverslips were mounted with an antifade reagent. Immunofluorescence images were obtained with a Zeiss LSM 510 confocal microscope (Thornwood, NY) using a ×63 water immersion objective. The 488-nm line of an argon laser was used to excite Alexa-488 and emission was collected between 505 and 530 nm. To ensure specificity of staining, images were obtained using confocal machine settings at which no Alexa-488 fluorescence was detectable in negative control specimens labeled with the secondary antibody alone. InsP3R immunofluorescence was determined, keeping the pinhole and detector gain setting identical while analyzing wild type CHO cells and CHO cells transfected with InsP3R siRNA. For mitochondrial co-localization studies, CHO cells were incubated with 500 nm Mitotracker Red for 20 min at room temperature and then processed and examined as described above, using excitation at 543 nm with observation at 560 nm to detect Mitotracker Red. Co-localization of the mitochondrial label and InsP3R staining was quantified using Image-J software (available on the World Wide Web at rsb.info.nih.gov/ij).
frames/s. Neither autofluorescence nor background signals were detectable at the machine settings used.

**Induction of Apoptosis**—Apoptosis was induced in two separate ways. To induce apoptosis via the intrinsic pathway, CHO cells were treated with 0.5 μM staurosporine at 37 °C in 5% CO₂ for 12 h (27). To induce apoptosis via the extrinsic pathway, CHO cells first were transiently transfected with the GFP-tagged bile acid transporter, Na⁺/H⁻taurocholate co-transport polypeptide (NTCP), and then were treated with 50 nM of the hydrophilic bile acid GCDCA at 37 °C in 5% CO₂ for 2 h (28). In either case, apoptosis was quantified by loading the cells with 1 μM of the nuclear binding dye DAPI (28), and then chromatin condensation and nuclear fragmentation were assessed by confocal microscopy.

**Statistical Analysis**—All experiments were performed in at least triplicate, and results are expressed as mean ± S.E. Statistical analyses were performed using PRISM statistical software (GraphPad; San Diego, CA). Groups of data were compared using one-way analysis of variance. A value of p < 0.05 was considered to indicate a statistically significant difference.

**RESULTS**

**Expression and Knockdown of InsP₃R Isoforms**—CHO cells were used for these studies, because this epithelial cell line expresses all three InsP₃R isoforms (4). Real time PCR (Fig. 1) confirmed previous studies at the protein level indicating that CHO cells express all three isoforms (4) and suggests that the InsP₃R-II is most highly expressed, followed by the type I and then the type III isoform (Fig. 1). PCR additionally showed that CHO cells do not express the ryanodine receptor (data not shown), the other intracellular Ca²⁺ release channel that is present in some epithelial cells (29, 30). Together, these data show that InsP₃R is the principal intracellular Ca²⁺ release channel in CHO cells. The data also confirm that all three isoforms of InsP₃R are expressed in CHO cells and show that the type II InsP₃R is the predominant isoform at the RNA level. Next, siRNA constructs were used to silence each isoform of the InsP₃R. Immunoblot analysis using isoform-specific antibodies demonstrated that the siRNA constructs were able to specifically and selectively knock down InsP₃R-I (Fig. 2A), InsP₃R-II (Fig. 2B), or InsP₃R-III (Fig. 2C), respectively. In contrast, scrambled siRNA for InsP₃R-III had

![Figure 4](image-url)

**FIGURE 4. Knockdown of each InsP₃R isoform affects cytosolic Ca²⁺ signaling.** Shown are serial confocal images of CHO cells loaded with fluo-4, before and during stimulation with ATP (2 μM). Shown is cytosolic Ca²⁺ increase in wild type CHO cells (A), cells transfected with siRNA for InsP₃R-I (B), cells transfected with siRNA for InsP₃R-II (C), and cells transfected with siRNA for InsP₃R-III (D). The top images in each panel show the pattern of Ca²⁺ increase. The images are pseudocolored according to the color scale shown in A. At the bottom is the tracing of fluo-4 fluorescence over time, of the cell indicated by the arrow. To the right is the summary of the different patterns of Ca²⁺ signaling when cells were stimulated with ATP. An oscillatory pattern of Ca²⁺ signaling predominates in wild type cells. Compared with these controls, an increase in the fraction of cells responding with a plateau pattern of Ca²⁺ signaling is observed when cells were transfected with siRNA for InsP₃R-I or InsP₃R-III. On the other hand, an increase in the number of cells responding with a transient pattern of Ca²⁺ signaling is observed when cells were transfected with siRNA for InsP₃R-II. Results are representative of those seen in three separate experiments (n = 70 cells) for control CHO cells, three separate experiments (n = 70 cells) for cells transfected with siRNA for InsP₃R-I, three separate experiments (n = 70 cells) for cells transfected with siRNA for InsP₃R-II, and three separate experiments (n = 70 cells) for cells transfected with siRNA for InsP₃R-III (*, p < 0.001 relative to control cells).
no effect on InsP$_3$R gene expression (Fig. 2C). Densitometric analysis confirmed reduction of InsP$_3$R-I expression by 99% (Fig. 2A), InsP$_3$R-II by 70% (Fig. 2B), and InsP$_3$R-III by 97% (Fig. 2C), each compared with nontransfected control. The effect of each siRNA was specific for the respective InsP$_3$R isoform, since the amount of other InsP$_3$R isoforms or β-actin was unaffected (Fig. 2, A–C). To further evaluate the siRNA constructs, they were used to knock down each InsP$_3$R isoform in HEK293 cells, which also express all three receptor isoforms (Fig. 2D).

Densitometric analysis of isoform-specific immunoblots showed reduction of InsP$_3$R-I expression by 88%, InsP$_3$R-II by 81%, and InsP$_3$R-III by 70% (Fig. 2D), each compared with nontransfected control. Efficacy of each siRNA was slightly lower in HEK293 cells than in CHO cells, which may reflect the fact that the siRNAs were designed for rodent InsP$_3$R sequences, whereas HEK293 cells are of human origin. As in CHO cells, however, the effect of each siRNA was specific for the respective InsP$_3$R isoform. The effects of each siRNA construct were then confirmed by examining the subcellular localization of InsP$_3$Rs in CHO cells using confocal immunofluorescence (Fig. 3). CHO cells were labeled with the same InsP$_3$R-I, II, or III antibodies used for immunoblots. Both InsP$_3$R-I and InsP$_3$R-II were distributed heterogeneously throughout the cell (Fig. 3, left and middle). On the other hand, InsP$_3$R-III was distributed mainly in the cytosol (Fig. 3, right).

Immunofluorescence also showed that each siRNA specifically knocked down InsP$_3$R-I, II, or III (Fig. 3), consistent with immunoblot results. Together, these findings demonstrate that InsP$_3$R isoforms are distributed in heterogeneous patterns in CHO cells and that the siRNA constructs used here are highly efficient and specific for silencing each InsP$_3$R isoform.

Each InsP$_3$R Isoform Affects Ca$^{2+}$ Signaling Patterns in CHO Cells—Although each of the three InsP$_3$R isoforms acts as an InsP$_3$-gated Ca$^{2+}$ channel, the isoforms are not uniformly sensitive to InsP$_3$ (6). Isoform-specific differences in tissue expression and subcellular distribution further suggest that the various InsP$_3$Rs serve distinct roles in Ca$^{2+}$ signaling. Since there is no selective antagonist for each InsP$_3$R isoform, we used the isoform-specific siRNAs to test this. Ca$^{2+}$ signaling was induced by extracellular stimulation of the cells with ATP (2 μM), since this nucleotide binds to P2Y nucleotide receptors expressed on the plasma membrane of CHO cells, and since activation of these receptors initiates the InsP$_3$-induced Ca$^{2+}$ signaling cascade (31). Ca$^{2+}$ signaling was monitored in individual CHO cells loaded with the Ca$^{2+}$-sensitive fluorescent dye fluo-4/AM using time lapse confocal microscopy (Fig. 4). ATP stimulation of control cells untreated with siRNA resulted in Ca$^{2+}$ oscillations in 79.9 ± 7.5% (mean ± S.E. of measurements made in 12 cells for each isoform). Few cells showed sustained (5.3 ± 2.4%) or transient (6.5 ± 3.9%) Ca$^{2+}$ increases upon ATP stimulation. After knockdown of InsP$_3$R-I (n = 70 cells), Ca$^{2+}$ oscillations remained the most frequent Ca$^{2+}$ signaling pattern (61.3 ± 12.1%), but there was a significant increase in the number of cells in which a sustained increase in Ca$^{2+}$ was observed (30.1 ± 9.1%; p < 0.001 relative to control cells; Fig. 4B). On the other hand, few cells (2.3 ± 1.1%) responded with a transient Ca$^{2+}$ signal (Fig. 4B). After knockdown of InsP$_3$R-II (n = 70 cells), the majority of cells once again responded to ATP with Ca$^{2+}$ oscillations (56.3 ± 5.8%), although there was a significant increase in the number of cells responding with a transient increase in Ca$^{2+}$ (36.3 ± 63%; p < 0.001 relative to control cells; Fig. 4C). Only a small number of cells (5.7 ± 3.0%) responded with a sustained increase in Ca$^{2+}$. Similar to what was observed when InsP$_3$R-1 was silenced, knockdown of the type III isoform (n = 70 cells) increased the fraction of cells responding to ATP with a sustained increase in Ca$^{2+}$ (27.0 ± 2.0%; p < 0.001 relative to control cells; Fig. 4D). The majority of these cells nonetheless responded with an oscillatory pattern of Ca$^{2+}$ increase (68.0 ± 3.0%), and only a

![FIGURE 5. Co-localization of InsP$_3$R isoforms and mitochondria in CHO cells. A, cells were double-labeled with isoform-specific InsP$_3$R antibodies (green) and Mitotracker Red (red) to identify mitochondria. The subcellular distribution of each InsP$_3$R isoform, along with mitochondria, was determined by confocal immunofluorescence. B, areas of co-localization appear yellow in the merged image (best appreciated in the magnified region of cytosol at the right). The fraction of the pixels within the cytosol that are yellow was quantitated for each InsP$_3$R isoform (bottom) and revealed that co-localization with mitochondria follows a rank order of InsP$_3$R-III > InsP$_3$R-II > InsP$_3$R-I (p < 0.001 among groups by analysis of variance). Results are mean ± S.E. of measurements made in 12 cells for each isoform.](image-url)
rhod-2 co-localized nearly completely with the mitochondrial dye Mitofiluor Green (Fig. 6A), indicating that rhod-2 was specifically loaded into mitochondria. \( \text{Ca}^{2+} \) signals, as measured by rhod-2 fluorescence, were quantified in both control and siRNA-treated cells stimulated with 2 \( \mu \)M ATP (Fig. 6B). Rhod-2 fluorescence increased by 1230 \( \pm \) 83% in control cells stimulated with ATP (n = 62 cells). Similarly, rhod-2 fluorescence increased by 1173 \( \pm \) 102% in cells pretreated with InsP3R-I siRNA (n = 36; p > 0.05 relative to nontransfected controls) and by 1104 \( \pm \) 95% in cells pretreated with InsP3R-II siRNA (n = 43; p < 0.05 relative to controls). In contrast, silencing InsP3R-III led to a fluorescence increase of only 838 \( \pm \) 55% (n = 62; p < 0.01 relative to controls). This represents only 68% of the increase that was observed in nontransfected controls (Fig. 6C). To examine the generality of these findings, mitochondrial \( \text{Ca}^{2+} \) signals also were observed in HEK293 cells treated with each type of siRNA and then stimulated with 2 \( \mu \)M ATP. As in CHO cells, ATP-induced increases in \( \text{Ca}^{2+} \) were reduced by 41% relative to controls in HEK293 cells pretreated with InsP3R-III siRNA but were not reduced in cells pretreated with siRNA for InsP3R-I or InsP3R-II (p < 0.001; Fig. 6D). Thus, knockdown of InsP3R-II but not InsP3R-I or II significantly reduces mitochondrial \( \text{Ca}^{2+} \) signals, and the findings suggest that this is because InsP3R-III is the isoform in closest proximity to mitochondria in these cell types.

**Differential Effects of InsP3R Isoforms on Apoptosis**—The distinct effect of each InsP3R isoform on \( \text{Ca}^{2+} \) signaling suggests that each isoform may have distinct effects on \( \text{Ca}^{2+} \)-mediated events as well. To investigate this, we examined the effects of each InsP3R isoform on apoptosis. Apoptosis was induced by activation of either the extrinsic or intrinsic apoptotic pathway, since the two pathways converge at the level of \( \text{Ca}^{2+} \) signaling. Previous studies suggest that either InsP3R-I or InsP3R-II can play a role in apoptosis (15–17), but little is known about either the role of InsP3R-II or the relative effects of each InsP3R isoform on apoptosis. Apoptosis was triggered here by two separate effector mechanisms, one that involves the extrinsic or death receptor pathway and the other that includes the intrinsic or mitochondria-mediated pathway. The extrinsic pathway can be activated by uptake of the prototypical toxic bile acid GCDCA, which induces apoptosis by activation of the Fas receptor independent of the presence of Fas ligand (28). GCDCA was used to induce apoptosis in CHO cells in which individual InsP3R isoforms were knocked down. CHO cells were first transfected with a GFP-tagged form of the NTCP (24) to allow uptake of GCDCA

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**FIGURE 6. InsP3R isoforms have differential effects on mitochondrial \( \text{Ca}^{2+} \) signaling.** A, detection of \( \text{Ca}^{2+} \) in mitochondria in CHO cells. Cells were double-labeled with Mitofiluor Green (green) to identify mitochondria and the cationic \( \text{Ca}^{2+} \) dye rhod-2 (red). The left panel shows subcellular distribution of mitochondria, and the middle panel shows rhod-2 labeling. The merged image (right) demonstrates that rhod-2 co-localizes closely with Mitofiluor Green and thus loads mitochondria in this cell system. B, serial confocal images of CHO cells before and during stimulation with ATP (2 \( \mu \)M). Cells were loaded with rhod-2, and images are pseudocolored according to the color scale shown in Fig. 4. Typical mitochondrial \( \text{Ca}^{2+} \) increases in wild type CHO cells (left column) and in cells transfected with siRNA for InsP3R-I (middle left column), InsP3R-II (middle right column), and InsP3R-III (right column) are shown. At the bottom are representative tracings of rhod-2 fluorescence over time in a nontransfected control cell (left) and a CHO cell transfected with siRNA for InsP3R-III. C, summary of mitochondrial \( \text{Ca}^{2+} \) increase in CHO cells stimulated with ATP. Silencing InsP3R-I, InsP3R-II, or InsP3R-III decreased mitochondrial \( \text{Ca}^{2+} \) signaling by 5% (p > 0.05; n = 36), 10% (p > 0.05; n = 43), or 32% (p < 0.05; n = 62), respectively. Results represent mean \( \pm \) S.E. of three separate experiments. D, summary of mitochondrial \( \text{Ca}^{2+} \) increase in HEK293 cells stimulated with ATP. Silencing InsP3R-I or InsP3R-II does not decrease mitochondrial \( \text{Ca}^{2+} \) whereas silencing InsP3R-III decreases mitochondrial \( \text{Ca}^{2+} \) signaling by 41% (p < 0.001; n = 30 cells in each group). Results represent mean \( \pm \) S.E. of three separate experiments.
InsP$_3$R Isoforms, Apoptosis, and Mitochondrial Ca$^{2+}$

A

![Transmission and DAPI images of NTCP-GFP and merged channels](image)

B

![siRNA treatment effects](image)

C

![Graph showing apoptosis cell percentages](image)

FIGURE 7. Effects of InsP$_3$R isoforms on bile acid-induced apoptosis. Wild type or NTCP-GFP-transfected CHO cells were treated with the toxic bile acid GCDCA (50 nM). The cells were stained with DAPI and examined using confocal microscopy. Apoptosis was evaluated in GFP-expressing cells by assessing nuclear changes. A, top panels show non-transfected cells with normal nuclei. The middle panels show a cell expressing NTCP-GFP that has not been exposed to GCDCA and has a nonapoptotic nucleus as well. The bottom panels show an NTCP-GFP-transfected cell treated with bile acid. Note chromatin condensation and nuclear fragmentation, indicative of apoptosis. B, cells transfected with NTCP-GFP that had each isoform of InsP$_3$R knocked down. The top panels show an NTCP-GFP cell that was also transfected with siRNA for InsP$_3$R-I. Nuclear fragmentation was observed in the majority of these cells. The middle panels show an NTCP-GFP cell that was co-transfected with siRNA for InsP$_3$R-II. Nuclear fragmentation was observed in more than half of such cells. The bottom panels show an NTCP-GFP cell that was co-transfected with siRNA for InsP$_3$R-III. No chromatin condensation was observed in the majority of these cells. C, summary of the effect of silencing each InsP$_3$R isoform on bile acid-induced apoptosis. Silencing InsP$_3$R-I, InsP$_3$R-II, and InsP$_3$R-III decreased the number of apoptotic cells to 62.0 ± 0.5% (p < 0.05, n = 45), 47.1 ± 0.4% (p < 0.001, n = 53), and 30.6 ± 3.4% (p < 0.001, n = 83), respectively. These results represent the mean ± S.E. of five separate experiments.

into the cells. Cells with visible expression of NTCP-GFP were treated for 2 h with GCDCA (50 μM), and then apoptosis was assessed by examining for nuclear changes with DAPI staining (28). No chromatin condensation or nuclear fragmentation was observed in nontransfected CHO cells (Fig. 7A, top), consistent with the fact that such cells are not able to take up GCDCA. Similarly, no nuclear changes were observed in CHO cells expressing NTCP-GFP but not incubated with GCDCA (Fig. 7A, middle). In contrast, nuclear fragmentation was observed in 73.9 ± 3.9% of cells expressing NTCP-GFP and treated with GCDCA (n = 54; Fig. 7A, bottom). The incidence of apoptotic nuclear changes was decreased in cells lacking any one of the three InsP$_3$R isoforms (Fig. 7B). Only 62.0 ± 0.5% of NTCP-GFP-transfected cells lacking InsP$_3$R-I were apoptotic (n = 45, p < 0.05 relative to control; Fig. 7C). When InsP$_3$R-II was silenced, the frequency of apoptotic cells was reduced further to 47.1 ± 0.4% (n = 53, p < 0.01 relative to control; Fig. 7C). Finally, knockdown of InsP$_3$R-III led to the greatest reduction in GCDCA-induced apoptosis, to only 30.6 ± 3.4% of cells expressing NTCP-GFP (n = 83, p < 0.001 relative to control; Fig. 7C). These results suggest that all three InsP$_3$R isoforms participate in induction of apoptosis induced by the extrinsic pathway in CHO cells, but InsP$_3$R-III plays the greatest role, followed by InsP$_3$R-II and then InsP$_3$R-I.

Finally, InsP$_3$R-III silencing conferred similar protection against apoptosis when activation of the intrinsic pathway was examined by staurosporine (Fig. 8). The percentage of cells that were apoptotic decreased to 22.6 ± 1.5 and 22.6 ± 0.9% when InsP$_3$R-I (n = 620; p > 0.05 relative to control group) and InsP$_3$R-II (n = 560; p > 0.05 relative to control group) were knocked down, respectively. However, only 15.3 ± 0.3% of cells were apoptotic after silencing InsP$_3$R-III (n = 680; p < 0.001 relative to controls). Together, these results show that InsP$_3$R-III plays a preferential role in mediating apoptosis in CHO cells, regardless of whether apoptosis is induced via the extrinsic or intrinsic pathway and although quantitative PCR suggests that InsP$_3$R-III is the least heavily expressed isoform in these cells. This finding is consistent with the observations that InsP$_3$R-III is the isoform that co-localizes most strongly with mitochondria and transmits Ca$^{2+}$ signals most effectively into mitochondria in these cells.

DISCUSSION

The versatility of Ca$^{2+}$ as a second messenger is in part due to the complex temporal pattern of cytosolic Ca$^{2+}$ signals that can occur. For example, the amplitude of Ca$^{2+}$ elevations can regulate differentiation in memory B cells (36), whereas the frequency of Ca$^{2+}$ oscillations differentially regulates expression of inflammatory cytokines in T cells (37, 38). The ability to produce patterns such as Ca$^{2+}$ oscillations results in part from unique features of the InsP$_3$R (39). However, the three isoforms of the InsP$_3$R each have differences in biophysical properties (7, 40), so that they may have distinct effects on Ca$^{2+}$ signaling as well. Indeed, Ca$^{2+}$ signaling patterns differ among DT40 cells engineered to express only one of the three InsP$_3$Rs (10). In addition, selective loss of either InsP$_3$R-I or InsP$_3$R-III has distinct effects on Ca$^{2+}$ signals, as examined in A7R5 vascular smooth muscle cells (41) and in HeLa and COS-7 cells (9). In the current study, isoform-specific siRNA revealed that InsP$_3$R-I and -III also have specific effects in CHO cells. In addition, the availability of specific siRNA for InsP$_3$R-II made it possible to evaluate the relative role of this isoform in Ca$^{2+}$ signaling. Nontransfected cells responded to nucleotide stimulation predominantly with Ca$^{2+}$ oscillations, but silencing either InsP$_3$R-I or InsP$_3$R-III led to an increase in the fraction of cells with a plateau Ca$^{2+}$ signaling pattern. In contrast, when InsP$_3$R-II was knocked down, there was an increase in the number of cells that responded to ATP with a transient pattern of Ca$^{2+}$ increase. How might this be explained? Although each of the three InsP$_3$R isoforms acts as an InsP$_3$-gated Ca$^{2+}$ channel, the isoforms are not uniformly sensitive to InsP$_3$. The relative order of affinity is type II > type I > type III (6). Since PCR further suggested that InsP$_3$R-II is the most abundant isoform in CHO cells, this isoform thus may be the one prefer...
InsP₃R knockdown on staurosporine-induced apoptosis. CHO cells were treated with staurosporine (0.5 μM) for 12 h, and then apoptosis was evaluated by assessing nuclear changes through DAPI staining. The first panel illustrates control cells incubated with tissue culture medium alone. No apoptotic nuclei were observed. The second panel illustrates CHO cells incubated with staurosporine (ST), resulting in some apoptotic nuclei. Subsequent panels illustrate CHO cells transfected with siRNA for InsP₃R-I, InsP₃R-II, and InsP₃R-III, respectively, and then treated with staurosporine. Apoptotic nuclei are indicated by the arrows. Each image is representative of what was observed in three independent experiments. Final panel, summary of the effects of InsP₃R knockdown on staurosporine-induced apoptosis. Silencing InsP₃R-I, InsP₃R-II, or InsP₃R-III decreased the fraction of cells that were apoptotic to 22.6 ± 1.5% (p > 0.05 compared with control, n = 620 cells), 22.6 ± 0.8% (p > 0.05 compared with control, n = 560 cells), and 15.3 ± 0.3% (p < 0.001 compared with control, n ~ 680 cells), respectively. These results represent the mean ± S.E. of three individual experiments.

Eventually activated in these cells. Thus, an absence of this isoform may result more often in transient Ca²⁺ signals, because this may be a weaker form of response.

A number of proteins modulate apoptosis in part through the InsP₃R or through related Ca²⁺ release mechanisms. Apoptosis generally occurs through one of two pathways. The extrinsic pathway is initiated by activation of death receptors, whereas the intrinsic pathway acts on mitochondria to induce formation of the permeability transition pore (43, 44). However, the death receptor pathway may act through mitochondria as well by inducing Bid-mediated mitochondrial permeabilization (45). Mitochondrial permeabilization, therefore, is important in each apoptotic pathway. We found that both apoptotic pathways were dependent on InsP₃R expression and were most sensitive to expression of the type III isoform in particular. This observation provides further evidence for a final common mechanistic link between the two apoptotic pathways. Apoptosis is inhibited by members of the Bcl-2 family of proteins, and their common mechanism of action involves inhibition of transmission of Ca²⁺ signals from InsP₃R to mitochondria. Bcl-2 acts in part by decreasing the size of ER Ca²⁺ stores (46), whereas Bcl-x is in part by inhibiting expression of the InsP₃R (47). In addition, cytochrome c that has leaked from mitochondria via the permeability transition pore binds to the InsP₃R, which facilitates release of toxic amounts of Ca²⁺ from the ER (18). This is thought to result in a positive feedback loop that causes further Ca²⁺ overload of mitochondria and then further leakage of cytochrome c (18). Inhibition of the interaction between cytochrome c and the InsP₃R inhibits development of apoptosis by blocking this positive feedback loop (19). How does this relate to isoform-specific effects of InsP₃R on apoptosis? Under certain circumstances, the type III InsP₃R lacks the feedback inhibition by high Ca²⁺ concentrations that is exhibited by the type I isoform (7). The type III isoform therefore may preferentially initiate the positive feedback cycle for Ca²⁺ signaling that is necessary to establish permeability transition pore formation and the associated release of cytochrome c. This may explain our observation that the type III InsP₃R preferentially mediates apoptosis in CHO cells, although it is not the predominant isoform in these cells.

Mitochondria play an integral role in Ca²⁺ signaling pathways and patterns. A subset of mitochondria are in close proximity to InsP₃Rs (48), and mitochondrial and cytosolic Ca²⁺ signals are interrelated (33, 49). Mitochondria play a key role in mediating most forms of apoptosis, and transmission of Ca²⁺ signals from InsP₃Rs to mitochondria is a critical step in this process (50). Increases in mitochondrial Ca²⁺ can directly induce formation of the permeability transition pore (32). This in turn permits leakage of cytochrome c from mitochondria, which is associated with progression to apoptosis (51). Both the type I (52) and type III (15) InsP₃R have been shown to induce apoptosis. Our findings corroborate this and show that the type II isoform can induce apoptosis as well. The current work furthermore shows that each isoform has a different propensity to induce apoptosis. This may be due in part to their differential distribution relative to mitochondria. Subpopulations of InsP₃Rs and mitochondria can be clustered in the cytosol, and this is associated with local, subcellular differences in Ca²⁺ signaling patterns (53, 54). The current findings raise the possibility that the type III InsP₃R has a particular propensity to form these subcellular clusters with mitochondria, resulting in the formation of associated signaling microdomains. InsP₃R isoforms localize to distinct regions of the cytosol in a number of cell types (12, 13, 20), but little is known about the factors that regulate subcellular targeting of each isoform (9, 42). Our findings raise the possibility that each InsP₃R isoform has a distinct propensity to target to regions near mitochondria, which in turn suggests an additional and previously unsuspected level of complexity in subcellular Ca²⁺ signaling.

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FIGURE 8. Effects of InsP₃R isoforms on staurosporine-induced apoptosis. CHO cells were treated with staurosporine (0.5 μM) for 12 h, and then apoptosis was evaluated by assessing nuclear changes through DAPI staining. The first panel illustrates control cells incubated with tissue culture medium alone. No apoptotic nuclei were observed. The second panel illustrates CHO cells incubated with staurosporine (ST), resulting in some apoptotic nuclei. Subsequent panels illustrate CHO cells transfected with siRNA for InsP₃R-I, InsP₃R-II, and InsP₃R-III, respectively, and then treated with staurosporine. Apoptotic nuclei are indicated by the arrows. Each image is representative of what was observed in three independent experiments. Final panel, summary of the effects of InsP₃R knockdown on staurosporine-induced apoptosis. Silencing InsP₃R-I, InsP₃R-II, or InsP₃R-III decreased the fraction of cells that were apoptotic to 22.6 ± 1.5% (p > 0.05 compared with control, n = 620 cells), 22.6 ± 0.8% (p > 0.05 compared with control, n = 560 cells), and 15.3 ± 0.3% (p < 0.001 compared with control, n ~ 680 cells), respectively. These results represent the mean ± S.E. of three individual experiments.
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