The role of inositol 1,4,5-trisphosphate receptors (IP3R) in caspase-3 activation and cell death was investigated in DT40 chicken B-lymphocytes stably expressing various IP3R constructs. Both full-length type-I IP3R and a truncated construct corresponding to the caspase-3 cleaved “channel-only” fragment were able to support staurosporine (STS)-induced caspase-3 activation and cell death even when the IP3R construct harbored a mutation that inactivates the pore of the Ca2+ channel (D2550A). However, a full-length wild-type IP3R did not promote caspase-3 activation when the 159-amino acid cytosol-exposed C-terminal tail was deleted. STS caused an increase in cytosolic free Ca2+ in DT40 cells expressing wild-type or pore-dead IP3R mutants. However, in the latter case all the Ca2+ increase originated from Ca2+ entry across the plasma membrane. Caspase-3 activation of pore-dead DT40 cells was also more sensitive to extracellular Ca2+ chelation when compared with wild-type cells. STS-mediated release of cytochrome c into the cytosol and mitochondrial membrane potential depolarization could also be observed in DT40 cells lacking IP3Rs or containing the pore-dead mutant. We conclude that nonfunctional IP3Rs can sustain apoptosis in DT40 lymphocytes, because they facilitate Ca2+ entry mechanisms across the plasma membrane. Although the intrinsic ion-channel function of IP3Rs is dispensable for apoptosis induced by STS, the C-terminal tail of IP3Rs appears to be essential, possibly reflecting key protein-protein interactions with this domain.

Apoptosis is an essential process required for normal development and tissue homeostasis and can be activated by diverse stimuli, including cytotoxic drugs, DNA damage, irradiation, withdrawal of growth factors, and activation of death receptors by ligands such as tumor necrosis factor or Fas. A requirement for elevated levels of Ca2+ has been implicated in many of these models of apoptosis (1–3). Several key enzymes activated during apoptosis such as endonucleases (4), phospholipase-A2 (5), and calpains (6) are known to be stimulated by Ca2+. However, the exact steps in apoptotic cascades that are affected by Ca2+ and the mechanisms resulting in the perturbation of Ca2+ homeostasis are poorly understood.

Elevations of cytosolic Ca2+ ([Ca2+]c) are achieved in cells by mobilization of intracellular Ca2+ stores and/or enhancement of Ca2+ entry across the plasma membrane. Inositol 1,4,5-trisphosphate receptors (IP3Rs)2 are a family of three intracellular Ca2+-release channels, mainly located in the ER membrane, which are primarily responsible for the agonist-mediated release of Ca2+ from intracellular stores. A number of different experimental approaches have implicated IP3Rs as pro-apoptotic regulators. The targeted deletion of all three IP3R isoforms in the chicken DT-40 lymphocyte cell line renders the cells more resistant to apoptotic stimuli induced by anti-IgM or staurosporine (STS) (7, 8). Decreasing the expression of the type-III (9) or the type-I (10) IP3R isoforms has been shown to confer resistance to various apoptotic stimuli in Jurkat T-cell lines. A selective role for the type-III IP3R in apoptosis was also reported in a study using small interference RNA to suppress expression of individual IP3R isoforms (11). However, mice deficient in both type-II and III IP3R isoforms do not show defects in developmental apoptosis suggesting the presence of significant redundancy in the requirement for individual IP3R isoforms (12).

The intrinsic pathway of apoptosis involves the release of cytochrome c (Cyt c) from the mitochondrial intermembrane space and the initiation of a cascade of events that ultimately leads to the activation of caspase-3. Although the exact transport pathway utilized for Cyt c release during apoptosis or necrosis is controversial, it is well established that enhanced accumulation of Ca2+ by the mitochondria sensitizes the Cyt c release pathway to stimulation by apoptotic agents (3, 13). The close proximity of ER to mitochondria suggests that the Ca2+ channel function of IP3Rs could be important in modulating the kinetics and sensitivity of apoptotic pathways (3, 13, 14). Further evidence for the pro-apoptotic role of IP3Rs is indicated by the finding that a number of key components of apoptotic cascades appear to interact with and regulate IP3R function. The anti-apoptotic proteins Bcl-2 and Bcl-xL have been shown to bind IP3Rs, but the studies differ as to whether this results is an

---

2 The abbreviations used are: IP3-R, inositol 1,4,5-trisphosphate receptor; ER, endoplasmic reticulum; CHAPS, (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate; Cyt c, cytochrome c; aa, amino acids(s); Ab, antibody; mAb, monoclonal antibody; STS, staurosporine; HA, hemagglutinin; TKO, triple knockout; CO, channel-only; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetrakis (acetoxymethyl ester).

---

1 To whom correspondence should be addressed: Dept. of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, 1020 Locust St., JAH 230A, Philadelphia, PA 19107. Tel.: 215-503-1222; Fax: 215-923-6813; E-mail: sureshjoseph@mail.tju.edu.

**This work was supported by National Institutes of Health Grants R01-DK34804 and R29-NOS051822 (to S. K. J.) and Training Grant T32-AA07463 (to Z. T. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.**
IP₃R and Apoptosis

inhibition or activation of IP₃R channel activity (15, 16). Mouse embryonic fibroblast cell lines obtained from Bax/Bak double knock-out mice show an increased phosphorylation of the IP₃R and an enhanced Ca²⁺ leak across the ER membrane (17). This has been attributed to the effect of unrestrained Bcl-2 on IP₃Rs (17). The type-I IP₃R isoform contains a consensus caspase-3 cleavage site and has been shown to be a substrate for this enzyme in various models of apoptosis (8, 18–20). The C-terminal “channel-only” fragment produced from caspase-3 cleavage has been reported to form a constitutively open channel and to contribute to the Ca²⁺ leak pathway across ER membranes (8, 21). Cyt c released from the mitochondria has been found to bind and activate IP₃Rs by decreasing the feedback inhibition of these channels by Ca²⁺ (22). Finally, IP₃Rs have been found to be substrates for the important anti-apoptotic regulatory protein Akt kinase (23). The highly conserved phosphorylation site for Akt kinase, as well as the binding sites for Bcl-xL and Cyt c, are all localized to the short, cytosol-exposed C-terminal tail of the receptor (16, 23, 24).

With the exception of Akt kinase phosphorylation, a common feature of all the aforementioned modes of IP₃R regulation during apoptotic signaling is that they are considered to modulate the Ca²⁺ channel function of the intact or caspase-cleaved IP₃R ion channel. To better understand the role of IP₃Rs in regulating apoptosis we have examined the effect of introducing an ion channel-defective IP₃R mutant into the IP₃R DT-40 triple knock-out lymphocytes. Surprisingly, this cell line continued to respond to cytotoxic apoptotic stimuli in a similar manner as wild-type DT-40 cells. Our studies reveal an ion-channel independent role of IP₃Rs in apoptosis, which may involve protein–protein interactions, possibly with the C-terminal tail of the receptor.

EXPERIMENTAL PROCEDURES

Reagents—Pfu polymerase was from Stratagene (Madison, WI). Protogel-stabilized acrylamide solution was from National Diagnostic (Atlanta, GA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Biosciences. Enhanced chemiluminescent substrate was obtained from Pierce. Mouse anti-chicken IgM (clone-M4) was from Southern Biotech (Birmingham, AL). RPMI 1640 culture media and G418 sulfate (Geneticin) were from Mediatech (Herndon, VA). Staurosporine, cyclosporine, FK506, and media and G418 sulfate (Geneticin) were from Mediatech (Herndon, VA). Staurosporine, cyclosporine, FK506, and ER. Proteogel-stabilized acrylamide solution was from Invitrogen-Molecular Probes (Eugene, OR).

DNA Constructs—The rat type I IP₃R construct containing a Kozak sequence and subcloned into pcDNA 3.1 has been described previously (26). The splice variants used in this study were SI(−), SII(+), and SIII(−). The channel-only (aa 1892–2749) portion of IP₃R was generated using PCR. The forward and reverse primers used were 5’-TCGAATTCCACCAT-GCGGGATGC CCCATC CCCGAAAG-3’ and 5’-GCTTATG-GTTTCTAGATTCGGG-3’. These primers encoded EcoRI/XbaI sites (underlined), which were utilized to clone the PCR product into similarly digested pcDNA 3.1 vector. The product was confirmed by automated sequencing (Nucleic Acid Facility, Thomas Jefferson University, Philadelphia, PA). The S2681A and S2681E point mutants in full-length IP₃R were constructed as described earlier (23). The S2681A, S2681E, and the D2550A pore mutation in full-length receptors were transferred to the channel-only IP₃R construct using BstBI/XbaI restriction sites. Constructs encoding deletions in the C-terminal tail were generated as described by Schug and Joseph (27).

Cells and Stable Transfection—DT40 cells (wild-type and triple knock-out (TKO) of IP₃R isoforms) were a kind Gift of Dr. T. Kuroasaki (Kansai Medical University, Moriguchi, Japan). Stable DT40 cells expressing the rat type I IP₃R were a gift from Dr. Kevin Foskett (University of Pennsylvania, PA). DT40 cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 1% chicken serum, and 100 units/ml penicillin, 100 μg/ml streptomycin, and maintained at 37 °C in 5% CO₂ atmosphere. The stable cell lines expressing various IP₃R mutants were prepared by electroporation of 0.5 ml of DT40 TKO cells (10⁶ cells/ml) with 40 μg of XbaI linearized DNA using a Gene pulser apparatus (Bio-Rad, 340 V and 950 microfarads). The cells were grown in 30 ml of RPMI for 24 h and were then serially diluted in a volume of 1:10, 1:100, and 1:1000 in RPMI containing 1.5 mg/ml G418. The diluted cells were transferred to 96-well plates and incubated for 1–2 weeks in a CO₂-incubator at 37 °C. Positive clones were identified by screening for expression of IP₃Rs by immunoblotting.

Measurement of Caspase Activation in DT40 Cells—For fluorometric assays, DT40 cells were collected and lysed in a buffer containing 20 mM Tris/Hepes, pH 7.4, 0.1% CHAPS, 5 mM EDTA. Lysates containing 10 μg of protein were incubated with 25 μM DEVD-aminomethyl coumarin as substrate for 30 min at 37 °C. Fluorescence of the aminomethyl coumarin product was measured at 380 nm excitation and 460 nm emission wavelength. Flow cytometry detection of caspase-3 was carried out according to manufacturer’s instructions using a caspase-3 reagent (FLICA, Invitrogen-Molecular Probes). After apoptosis induction, the DT40 cells were analyzed on a Flow cytometer with 488 nm excitation using 530 nm band pass and 670 nm long pass emission filters.

Cell Viability Assays—The assay using CellTitre 96 Aqueous One reagent from Promega was done as described by the manufacturer with some modifications. Briefly, 0.5 × 10⁶ DT40 cells were grown in 1 ml of media (in 24-well plates). The cells were treated with STS for 6 h, 20 μl of CellTitre reagent was added to each well, and the mixture was incubated for another 2 h. For a blank, 20 μl of CellTitre reagent was added to 1 ml of RPMI media. The supernatants were transferred in Eppendorf tubes and centrifuged on a microcentrifuge for 2 min. The supernatant was read at 490 nm against the media blank.
**IP$_3$R and Apoptosis**

**Measurement of [Ca$^{2+}$]$_i$ in DT40 Cells**—Changes in cytosolic [Ca$^{2+}$] in individual DT40 cells plated on coverslips coated with poly-d-lysine was measured by digital imaging fluorescence microscopy as previously described (28). The cells were loaded with Fura-2 by incubating with 2 µM Fura-2AM in Hepes buffer, pH 7.4 (10 mM Hepes, 120 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 6 mM glucose, and 1 mM glutamine) for 20 min at room temperature. The coverslips were washed with the same buffer for 10 min to de-esterify Fura-2 and were transferred to a chamber with 1 ml of HEPES buffer and mounted onto the stage of an inverted microscope thermostatically maintained at 37 °C. Fluorescence images with a 4-s delay were recorded alternately at excitation wavelengths of 340 and 380 nm with an emission wavelength of 460–600 nm using a charge-coupled device imaging system. Changes in cytosolic calcium were measured in response to STS or anti-IgM in the presence or absence of extracellular calcium and are expressed as the ratio of Fura-2 fluorescence at 340 nm and 380 nm ($F_{340}$/ $F_{380}$). Typically 50–60 cells were monitored per experiment, and traces represent the average of three different experiments.

**Cyt c Release, Annexin V Assays, and Measurement of Mitochondrial Membrane Potential**—Cyt c release into the cytosol was measured in DT40 cells after fractionation using the ApoAlert cell fractionation kit (Clontech, Mountain View, CA). Western blotting of lysates was carried out on 15% SDS-PAGE gels with immunoblotting for Cyt c using a monoclonal Ab from Zymed Laboratories Inc. Annexin V fluorescein isothiocyanate conjugate (BioSource, Camarillo, CA) was added together with propidium iodide (5 µg/ml), and staining was analyzed on a flow cytometer. Changes in mitochondrial membrane potential were assessed using the MitoProbe$^\text{TM}$ DiIC$_{18}$ (5) assay kit as described by the manufacturer (Invitrogen-Molecular Probes). The stain intensity decreases when reagents disrupt mitochondrial membrane potential. The cells were analyzed on a flow cytometer with 633 nm excitation using emission filters for far red (658 nm). Histograms were analyzed using WinMDI Software.

**RESULTS**

STS-induced Caspase-3 Activation and Cell Death in DT40 Cell Lines Containing Functional and Non-functional IP$_3$R—DT40 cells containing a targeted deletion of all 3 IP$_3$R isoforms (TKO) were transfected with a number of IP$_3$R mutants and stable cells lines were established. Fig. 1A shows the basic architecture of IP$_3$ receptors with an N-terminal ligand-binding domain, a C-terminal channel domain, and an intervening regulatory domain. Fig. 1B shows a detail of the 159-amino acid C-terminal tail of the receptor that projects into the cytosol together with the binding sites for known proteins that interact with this region. The location of the deletion mutants (channel only, 1TM, tail-less) and point mutants (Asp-2550 and Ser-2618) used in this study are also indicated in the figure. Fig. 2A shows the expression of these mutant constructs detected by immunoblotting with an Ab to the C-terminal 18 amino acids of the type-I IP$_3$R (CT-1) or, in the case of the tail-less mutants, with a C-terminal HA tag Ab. The mutants encoding the caspase-3-cleaved fragment, referred to as “channel-only” (CO) mutants (8), were expressed as a doublet of bands. These could correspond to glycosylated and non-glycosylated bands (cf. Refs. 21 and 29). In initial experiments we measured the time course of cell death induced by 0.5 µM STS in wild-type and TKO cells and found that the biggest differences were evident at earlier time points (<6 h) (data not shown). The time course of caspase-3 activation over this period is shown in Fig. 2B. In agreement with published data (8), caspase-3 activation was substantially slowed in TKO cells. However, 80% of the caspase-3 activation of wild-type cells could be rescued by the D2550A IP$_3$R pore mutant, which has previously been shown to inactivate the channel (30). The absence of Ca$^{2+}$ mobilization in response to cross-linking of cell surface IgM receptors with anti-IgM Ab in D2550A cells is shown in Fig. 2B (inset). Several other studies have confirmed that this mutant is functionally inactive (31–33). Data on caspase-3 activation in several additional mutant cell lines is summarized in Fig. 2C and corresponding measurements of cell viability are shown in Fig. 2D. The wild-type DT40 cells contain three chicken IP$_3$R isoforms, whereas the mutant cells contain only one IP$_3$R isoform made in the background of the rat type-I IP$_3$R. For comparison, we have also used a stable DT40 cell line expressing the wild-type rat type-I IP$_3$R as a control (kindly given by Kevin Foskett). These cells showed a somewhat higher
IP₃R and Apoptosis

A. Immunoblots of cell lysates prepared from the indicated DT40 cell lines. The reason why the CO constructs express as a doublet is not known but may be due to differential glycosylation. B, a time course of caspase-3 activation in the indicated DT40 cell lines in response to 0.5 μM STS measured fluorometrically in cell lysates. The inset shows cytosolic free Ca²⁺ changes measured in WT and D2550A Fura-2-loaded cells in response to 5 μg/ml α-IgM. C, the cumulative data of caspase-3 activation in response to 0.5 μM STS for 6 h measured in the indicated cell lines and expressed relative to the activation in WT cells. Data are mean ± S.E. (n = 3–6). *, p < 0.05 relative to WT cells. D, conditions were as described in C except that cell viability was measured with a Promega 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay kit.

Cytosolic tail from full-length receptors was sufficient to suppress caspase-3 activation and cell death to the levels observed in TKO cells (Fig. 2, C and D). In independent experiments we have established that the TL-5 and TL-6 mutants are functionally inactive as IP₃-gated channels (27). Because channel-inactive mutants (e.g. D2550A or CO) can still support cell death, this implies that the C-terminal tail has a unique function in regulating apoptosis induced by STS. Similar findings were made when using anti-IgM as an inducer of apoptosis, notably, caspase-3 activation was supported by the D2550A pore-inactive full-length IP₃R or the D2550A CO mutant but not the IP₃R lacking the C-terminal tail (supplemental Fig. S1).

We have previously shown that IP₃Rs are phosphorylated by Akt kinase in the C-terminal tail at serine 2618 without altering IP₃-dependent channel activity (23). The functional role of this phosphorylation is unknown. However, we noted that the ability of IP₃Rs to support STS-induced caspase-3 activation was enhanced when the Akt phosphorylation site was inactivated by mutation to alanine (S2618A) as compared with a phosphomimetic mutant (S2618E). It was suggested that perhaps important functional responses to Akt kinase phosphorylation could be expressed after the receptor had been cleaved by caspase-3 (23). However, the data in Fig. 2 (C and D) show that the CO construct containing the S2618A or S2618E mutant did not show significant differences in their ability to support STS-induced caspase-3 activation or cell death, suggesting that the functional effects of Akt kinase phosphorylation are exerted at the level of full-length receptors.

Cyt c Release and Mitochondrial Membrane Potential Depolarization in IP₃R-defective DT40 Cell Lines—The release of Cyt c into the cytosol is a mandatory step in most models of apoptosis activated by the intrinsic pathway. We therefore measured Cyt c release in DT40 cell lines containing wild-type and mutant IP₃Rs employing a crude subcellular fractionation procedure and using immunoblotting for Cyt c detection. Surprisingly, the TKO DT40 cell line released Cyt c into the cytosol with indistinguishable kinetics from the wild-type DT40 cells (Figs. 3, A and B). Although some variability was observed in the amount of basal Cyt c released, all the nonfunctional IP₃R mutant cell lines also released Cyt c in response to STS. This included the D2550A, TL-5, and TL-6 mutants (Fig. 3C). A loss of mitochondrial membrane potential invariably accompanies Cyt c release (34). We measured membrane potential using a fluorescent cationic carbocyanine dye, and the data are shown in Fig. 4. In agreement with our data on Cyt c release, both the...
indicate that the two parameters do not have a quantitatively
linear relationship and/or that the two processes are not necessarily tightly coupled (35, 36).

**Ca^{2+} Changes Mediated by STS in Wild-type and Mutant IP_{3R} Cell Lines**—To explore the Ca^{2+} dependence of apoptosis induced by STS we initially measured cytosolic [Ca^{2+}] changes in Fura-2-loaded DT40 cells. STS elicited a Ca^{2+} signal (Fig. 5A), which is in agreement with a previous study in DT40 cells (8), and observations in other cell types (22). In the presence of extracellular Ca^{2+} the wild-type DT40 cells gave the largest Ca^{2+} response, the TL-6 and TKO cells generated much smaller responses, and the TL-5 and pore mutant cell line produced an intermediate-sized Ca^{2+} signal (Fig. 5B). When extracellular Ca^{2+} was removed the STS response of wild-type cells was partially inhibited indicating that a component of the Ca^{2+} signal originated from intracellular Ca^{2+} release in these cells. However, in the absence of extracellular Ca^{2+} the response of the pore-mutant, TKO, TL-5 and TL-6 cells were almost eliminated (Fig. 5C). The data suggest that the Ca^{2+} signal seen in response to STS in the pore-defective IP_{3R} mutant is entirely derived from Ca^{2+} entry across the plasma membrane. A maintained coupling of the pore-defective IP_{3R} to Ca^{2+} entry could explain the residual apoptosis seen in the pore-defective DT40 cells. The dependence of STS-induced caspase-3 activation on extracellular Ca^{2+} chelation in selected cell lines that maintain high (wild-type, pore-mutant, CO) and low (TKO cells) caspase-3 activation is shown in Fig. 5D. Chelation of extracellular Ca^{2+} had a partial inhibitory effect (40%) on caspase-3 activation induced by STS in wild-type cells, which is in agreement with previous findings in DT40 cells in which apoptosis was quantified in a different manner (37). By contrast extracellular Ca^{2+} chelation inhibited caspase-3 activation in the pore-mutant by 80%. This is consistent with our hypothesis that the pore-defective cell line relies to a greater extent on extracellular Ca^{2+} to supply the Ca^{2+} required for apoptosis. The CO cell line behaved similarly to wild-type cells, although the inhibitory effect of extracellular Ca^{2+} chelation was somewhat larger (60%). The blockade of intracellular Ca^{2+} mobilization (and coupled Ca^{2+} entry processes) with BAPTA-AM totally suppressed caspase-3 activation in all cell lines.

**DISCUSSION**

Many studies using different experimental models have concluded that IP_{3R}s enhance the rate of apoptosis (reviewed in Ref. 14). The key conclusion that emerges from the present study is that IP_{3R}s do not necessarily have to function as intracellular Ca^{2+} release channels to fulfill this role. This conclusion is based on studies with a DT40 cell line containing defective IP_{3R} mutants and using STS as the apoptosis-inducing stimulus. The results do not imply that intracellular Ca^{2+} release from functional IP_{3R}s have no role in apoptosis induced in wild-type cells. STS-induced caspase-3 activation in both wild-type and pore-defective mutant cell lines is Ca^{2+}-dependent, as shown by its blockade by BAPTA loading in both types of cells (Fig. 5D). STS induces an increase of cytosolic Ca^{2+} in the pore-mutant cells, but this increase is entirely prevented by chelation of extracellular Ca^{2+} (Fig. 5C). Therefore, we suggest that the Ca^{2+} requirements for apoptosis in the pore-mutant cells is met exclusively by Ca^{2+} entry across the
**IP$_3$R and Apoptosis**

### FIGURE 4. Measurements of mitochondrial membrane potential depolarization.

A, DT40 cells were incubated with STS (0.5 μM) for 6 h to induce apoptosis. 50 nm Mitoprobe (DiIC$_1$ (5)) was added for 15 min. The cells were centrifuged and washed twice in PBS before analysis on a flow cytometer as described under "Experimental Procedures." The first panel shows complete mitochondrial uncoupling induced by 0.5 μM CCCP for 3 min. The second and third panels show the data for WT and TKO cells treated with STS. B, the number of cells having fluorescence values in the range observed for carbonyl cyanide m-chlorophenyl hydradrene (CCCP) treated cells was considered to have depolarized mitochondria. This was quantitated from the flow cytometry data in three experiments (mean ± S.E.) for the various DT40 cell lines after treatment with 0.5 μM STS for 6 h. *, p < 0.05; significantly different from WT DT40 cells.

plasma membrane, whereas the wild-type cells could potentially use both intracellular Ca$^{2+}$ release or Ca$^{2+}$ entry mechanisms. Van Rossum et al. (31) have shown that agonist-mediated Ca$^{2+}$ entry can be activated in DT40 cells containing the D2550A IP$_3$R pore-defective mutant and have suggested that IP$_3$ binding to a pore-defective channel is sufficient to activate a Ca$^{2+}$ entry mechanism in the plasma membrane. The activation of this type of Ca$^{2+}$ entry mechanism by STS could account for the continued caspase-3 activation and cell death responses observed in the pore-defective mutant cells. The exact Ca$^{2+}$ entry channels involved during apoptosis of DT40 cells are not known. Vasquez et al. (38) have shown that IP$_3$R expression is required to measure the activity of endogenous TRPC7 channels in isolated patches derived from DT40 cells. However, the necessity for IP$_3$R channel function for this effect was not tested. There have also been reports of IP$_3$R operating directly as Ca$^{2+}$ entry channels in the plasma membrane of DT40 cells, but these are unlikely to be involved in the apoptotic responses being examined here, because these channels are inactivated by pore mutations (33). Interestingly, functionally inactive mutant ryanodine receptor channels also remain coupled to a Ca$^{2+}$ entry pathway in dyspedic myotubes (39), and the presence of ryanodine receptors has been documented in DT40 cells (40). A possible role of ryanodine receptors in coupling to apoptotic mutant cells. In both types of cells these increases were derived from intracellular Ca$^{2+}$ release and were blocked by pre-treatment with the general caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone (8), consistent with the idea that the Ca$^{2+}$ changes are secondary to the generation of the CO fragment. In our experiments, the STS-induced increase in cytosolic Ca$^{2+}$ was equal to the pore-defective mutant seen in the pore-defective mutant cells was derived exclusively from extracellular Ca$^{2+}$ and was not blocked by the specific caspase-3 inhibitor DEVD-CHO at concentrations that inhibit STS-induced caspase-3 activation (data not shown). The reasons for these discrepancies are presently not clear, although the use of different non-functional IP$_3$R mutants and other methodological differences may be involved. Further work is necessary to clarify the issue of whether the caspase-3 cleavage fragment of type-I IP$_3$R plays an important primary role in apoptotic signaling.

The release of Cyt c from the mitochondrial intermembrane space is a key step involved in the downstream activation of effector caspases. Although, the exact mitochondrial mechanism involved in Cyt c release during apoptosis is controversial, it is generally accepted that the process is stimulated by elevations of cytosolic Ca$^{2+}$ in the local environment of the mitochondria (3, 34). Such an environment would be provided at the open mouth of either IP$_3$R or Ca$^{2+}$ influx channels (41, 42). The pathways in IP$_3$R-inactive DT40 cells lines remains to be explored.

Assefa et al. (8) reported that a non-functional IP$_3$R mutant missing 225 amino acids from the N terminus could still support STS or anti-IgM-induced apoptosis in DT40 cells. Because this mutant can be cleaved by caspase-3 to generate the CO fragment, the result was interpreted as indicating an important role for this fragment in supplying the Ca$^{2+}$ required for apoptosis. We found no evidence that the CO cell lines encoded constitutively leaky ER channels based on measurements of thapsigargin-releasable Ca$^{2+}$ (data not shown). Furthermore, a CO domain containing the inactivating pore mutation was equally effective in supporting STS-induced apoptosis (Fig. 2). This suggests that the IP$_3$R CO fragment is unlikely to be directly involved in promoting an ER Ca$^{2+}$ leak, but does not exclude the possibility that the proteolytic fragment could regulate a separate leak mechanism.

Our data differ in one additional respect from the study of Assefa et al. (8). This study noted that STS induced an increase of cytosolic Ca$^{2+}$ in both wild-type cells and non-functional IP$_3$R 1–225 deletion mutant cells. In both types of cells these increases were derived from intracellular Ca$^{2+}$ release and were blocked by pre-treatment with the general caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone (8), consistent with the idea that the Ca$^{2+}$ changes are secondary to the generation of the CO fragment. In our experiments, the STS-induced increase in cytosolic Ca$^{2+}$ seen in the pore-defective mutant cells was derived exclusively from extracellular Ca$^{2+}$ and was not blocked by the specific caspase-3 inhibitor DEVD-CHO at concentrations that inhibit STS-induced caspase-3 activation (data not shown). The reasons for these discrepancies are presently not clear, although the use of different non-functional IP$_3$R mutants and other methodological differences may be involved. Further work is necessary to clarify the issue of whether the caspase-3 cleavage fragment of type-I IP$_3$Rs plays an important primary role in apoptotic signaling.

The release of Cyt c from the mitochondrial intermembrane space is a key step involved in the downstream activation of effector caspases. Although, the exact mitochondrial mechanism involved in Cyt c release during apoptosis is controversial, it is generally accepted that the process is stimulated by elevations of cytosolic Ca$^{2+}$ in the local environment of the mitochondria (3, 34). Such an environment would be provided at the open mouth of either IP$_3$R or Ca$^{2+}$ influx channels (41, 42). The
finding that Cyt c binds to the C-terminal tail of IP₃Rs and prevents Ca²⁺ inhibition of the channel has suggested an additional amplification mechanism by which IP₃R channels could potentiate Cyt c release (22, 24). However, our studies show that STS-induced Cyt c release and mitochondrial membrane depolarization occur in TKO cells, as well as in cell lines containing non-functional IP₃Rs. Indeed, the kinetics of Cyt c release is not markedly different between STS-stimulated wild-type and TKO cells. Cyt c release has previously been observed in TKO cells stimulated with anti-IgM (22). This suggests that IP₃Rs are not essential to elicit or sustain Cyt c release in DT40 cells. Interestingly, recent studies have shown that Ca²⁺ entry may actually direct the movement of mitochondria to the cell periphery in Jurkat T-lymphocytes (43).

Our data suggest that the impaired caspase-3 activation seen in TKO and tail-less IP₃R mutant cell lines occurs at step(s) distal to mitochondrial membrane potential depolarization and Cyt c release. The nature of this site and how it is regulated by IP₃R remains a matter of speculation. Our working hypothesis is that IP₃Rs serve at least two critical functions in the apoptotic pathway. The first is to provide elevated cytosolic Ca²⁺ either directly from intracellular stores and/or by coupling to Ca²⁺ entry pathways. The latter mechanism obviously predominates in cell lines expressing only non-functional IP₃Rs. A second role appears to be intimately linked to the activation mechanism of caspase-3 itself. There are no indications in the literature that physiological increases of Ca²⁺ regulate key steps in caspase activation, such as apoptosome assembly or the catalytic activity of caspases. The experimental data would also suggest that Ca²⁺ elevation alone is not the primary regulatory factor, because the TL-5 and TL-6 mutant cell lines have different STS-mediated Ca²⁺ signals but are both deficient in caspase-3 activation (Fig. 2). Removal of the C-terminal tail suppresses caspase-3 activation induced by STS and is known to interact with a number of proteins, including Cyt c (24), Bcl-X₇, 4.1N (44). The C-terminal tail also contains the phosphorylation site for Akt kinase, and mutations of this site also modulate caspase-3 activity in DT-40 cells (23). One hypothesis is that this portion of the IP₃R represents a major binding site for a protein that regulates caspase-3 activation in the cytosol. Loss of this binding site would alter the partition of this regulator between ER membranes and the cytosol. Preliminary studies indicate that a deletion of 60 amino acids, which removes the coiled-coil domain and putative binding sites for Bcl-2 (Fig. 1), produces cells that retain wild-type caspase-3 responses to STS (data not shown). Additional mutagenesis to localize the required regions of the C-terminal tail is presently being carried.
IP$_3$R and Apoptosis

out. Finally, it should be noted that there are precedents for membrane-bound regulators of cytosolic caspases in the literature, including the proteins BRUCE/Apollon (45) and the integral ER protein ARMER, which inhibit the catalytic activity of caspase-9 (46).

Our studies lend weight to the argument that IP$_3$R$_{s}$ may do more than act as Ca$_{2}^{+}$ channels in cells (47, 48). More than 50 different proteins have been documented to interact with IP$_3$R$_{s}$ (47, 49), suggesting that there may be considerable heterogeneity in the types of IP$_3$R complexes present in intact cells. Mutant DT40 cell lines containing only non-functional IP$_3$R$_{s}$ may survive by “rewiring” their Ca$_{2}^{+}$ transport pathways to supply their biological requirements. We suggest that even wild-type cells may contain pools of inactive IP$_3$R complexes that subserve specific functions, such as coupling to Ca$_{2}^{+}$ entry pathways and regulation of caspase-3 activity. Further studies on the role of non-functional IP$_3$R complexes may lead to pharmacological approaches that allow a more selective approach to interfering with the biological effects regulated by IP$_3$R$_{s}$.

Acknowledgments—We thank Drs. Gyorgy Hajnoczky and Darren Boehning for reading draft versions of the manuscript.

REFERENCES

1. Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003) Nat. Rev. Mol. Cell Biol. 4, 552–565
2. Rizzuto, R., Pinton, P., Ferrari, D., Chami, M., Szabadkai, G., Magalhaes, P. J., Di Virgilio, F., and Pozzan, T. (2003) Oncogene 22, 8619–8627
3. Hajnoczky, G., Davies, E., and Madesh, M. (2003) Biochem. Biophys. Res. Commun. 304, 445–454
4. Nagata, S. (2000) Exp. Cell Res. 256, 12–18
5. Taketo, M. M., and Sonoshita, M. (2002) Biochem. Biophys. Acta 1585, 72–76
6. Goll, D. E., Thompson, V. F., Li, H., Wei, W., and Cong, J. (2003) Physiol. Rev. 83, 731–801
7. Sugawara, H., Kuroskai, M., Takata, M., and Kuroskai, T. (1997) EMBO J. 16, 3078–3088
8. Assael, Z., Bultynck, G., Szulcik, K., Nadif, K. N., Vermassen, E., Goris, J., Missiaen, L., Callewaert, G., Parys, J. B., and De Smedt, H. (2004) J. Biol. Chem. 279, 43227–43236
9. Khan, A. A., Soloski, M. J., Sharp, A. H., Schilling, G., Sabatini, D. M., Li, S., Ross, C. A., and Snyder, S. H. (1996) Science 273, 503–506
10. Jayaraman, T., and Marks, A. R. (1997) Mol. Cell. Biol. 17, 3005–3012
11. Mendes, C. C., Gomes, D. A., Thompson, M., Souto, N. C., Goes, T. S., Goes, A. M., Rodrigues, T. A., Gomez, M. V., Nathanson, M. H., and Leite, M. F. (2005) J. Biol. Chem. 280, 40892–40900
12. Futatsugi, A., Nakamura, T., Yamada, M. K., Ebisu, E., Nakamura, K., Uchida, K., Kataguchi, T., Takahashi-Iwana, H., Noda, T., Aruga, J., and Mikoshiba, K. (2005) Science 309, 2232–2234
13. Szabadkai, G., and Rizzuto, R. (2004) FEBS Lett. 567, 111–115
14. Joseph, S. K., and Hajnoczky, G. (2007) Apoptosis 12, 951–968
15. Chen, R., Valencia, I., Zhong, F., McColl, K. S., Roderick, H. L., Bootman, M. D., Berridge, M. J., Conway, S. J., Holmes, A. B., Mignery, G. A., Velez, P., and Distelhorst, C. W. (2004) J. Cell Biol. 166, 193–203
16. White, C., Li, C., Yang, J., Petrenko, N. B., Madesh, M., Thompson, C. B., and Foskett, J. K. (2005) Nat. Cell Biol. 7, 1021–1028
17. Oakes, S. A., Scorrano, L., Opferman, J. T., Bassik, M. C., Nishino, M., Pozzan, T., and Korsmeyer, S. J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 105–110
18. Hirota, I., Furuchi, T., and Mikoshiba, K. (1999) J. Biol. Chem. 274, 34433–34437
19. Haug, L. S., Walaa, I., and Ostvold, A. C. (2000) J. Neurochem. 75, 1852–1861
20. Diaz, F., and Bourguignon, L. Y. (2000) Cell Calcium 27, 315–328
21. Nakayama, T., Hattori, M., Uchida, K., Nakamura, T., Tateishi, Y., Bannai, H., Iwai, M., Michikawa, T., Inoue, T., and Mikoshiba, K. (2004) Biochem. J. 377, 299–307
22. Boehning, D., Patterson, R. L., Sedaghat, L., Glebova, N. O., Kuroskai, T., and Snyder, S. H. (2003) Nat. Cell Biol. 5, 1051–1061
23. Khan, M. T., Wagner, L., Yule, D. I., Bhanumathy, C. D., and Joseph, S. K. (2006) J. Biol. Chem. 281, 3731–3737
24. Boehning, D., van Rossum, D. B., Patterson, R. L., and Snyder, S. H. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 1466–1471
25. Joseph, S., and Samanta, S. (1993) J. Biol. Chem. 268, 6477–6486
26. Boehning, D., and Joseph, S. K. (2000) J. Biol. Chem. 275, 21492–21499
27. Schug, Z. T., and Joseph, S. K. (2006) J. Biol. Chem. 281, 24431–24440
28. Wagner, L. E., Li, W. H., and Yule, D. I. (2003) J. Biol. Chem. 278, 45811–45817
29. Joseph, S. K., Boehning, D., Pierson, S., and Nicchitta, C. V. (1997) J. Biol. Chem. 272, 1579–1588
30. Boehning, D., Mak, D. O., Foskett, J. K., and Joseph, S. K. (2001) J. Biol. Chem. 276, 13509–13512
31. van Rossum, D. B., Patterson, R. L., Kiseliov, K., Boehning, D., Barrow, R. K., Gill, D. L., and Snyder, S. H. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2323–2327
32. Alzayady, K. J., and Wojcikiewicz, R. J. (2005) Biochem. J. 392, 601–606
33. Dells, O., Dedos, S. G., Tovey, S. C., Tauficq, U. R., Dubel, S. I., and Taylor, C. W. (2006) Science 313, 229–233
34. Kroemer, G., Galluzzi, L., and Brenner, C. (2007) Physiol. Rev. 87, 99–163
35. Bossy-Wetzel, E., Newmeyer, D. D., and Green, D. R. (1998) EMBO J. 17, 37–49
36. Samraj, A. K., Keil, E., Ueffing, N., Schulze-Osthoff, K., and Schmitz, I. (2006) J. Biol. Chem. 281, 29652–29659
37. Hawkins, T. E., Das, D., Young, B., and Moss, S. E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8054–8059
38. Pozzan, T., and Korsmeyer, S. J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 1415–1422
39. Hauser, H. P., Bardroff, M., Pyrowolakis, G., and Jentsch, S. (1998) J. Cell Biol. 141, 1415–1422
40. Liu, H. M., Chen, J., Wang, L., and Naukovski, L. (2003) Mol. Cancer Res. 1, 508–518
41. Patterson, R. L., Boehning, D., and Snyder, S. H. (2004) Annu. Rev. Biochem. 73, 437–465
42. Alzayady, K. J., and Wojcikiewicz, R. J. (2005) Biochem. J. 32990 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 282 • NUMBER 45 • NOVEMBER 9, 2007