Caspase-3-induced Truncation of Type 1 Inositol Trisphosphate Receptor Accelerates Apoptotic Cell Death and Induces Inositol Trisphosphate-independent Calcium Release during Apoptosis*

Received for publication, April 7, 2004, and in revised form, July 16, 2004
Published, JBC Papers in Press, July 28, 2004, DOI 10.1074/jbc.M403872200

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Inositol 1,4,5-trisphosphate receptor-deficient (IP₃R-KO) B-lymphocytes were used to investigate the functional relevance of type 1 inositol 1,4,5-trisphosphate receptor (IP₃R1) and its cleavage by caspase-3 in apoptosis. We showed that inositol 1,4,5-trisphosphate receptor-deficient cells were largely resistant to apoptosis induced by both staurosporine (STS) and B-cell receptor (BCR) stimulation. Expression of either the wild-type IP₃R1 or an N-terminal deletion mutant (Δ1-225) that lacks inositol 1,4,5-trisphosphate-induced Ca²⁺ release activity restored sensitivity to apoptosis and the consequent rise in free cytosolic Ca²⁺ concentration ([Ca²⁺]ᵢ). Expression of caspase-3-non-cleavable mutant receptor, however, dramatically slowed down the rate of apoptosis and prevented both Ca²⁺ overload and secondary necrosis. Conversely, expression of the “channel-only” domain of IP₃R1, a fragment of the receptor generated by caspase-3 cleavage, strongly increased the propensity of the cells to undergo apoptosis. In agreement with these observations, caspase inhibitors impeded apoptosis and the associated rise in [Ca²⁺]ᵢ. Both the staurosporine- and B-cell receptor-induced apoptosis and increase in [Ca²⁺]ᵢ could be induced in nominally Ca²⁺-free and serum-free culture media, suggesting that the apoptosis-related rise in [Ca²⁺]ᵢ was primarily because of the release from internal stores rather than of influx through the plasma membrane. Altogether, our results suggest that IP₃R1 plays a pivotal role in apoptosis and that the increase in [Ca²⁺]ᵢ during apoptosis is mainly the consequence of IP₃R1 cleavage by caspase-3. These observations also indicate that expression of a functional IP₃R1 per se is not enough to generate the significant levels of cytosolic Ca²⁺ needed for the rapid execution of apoptosis, but a prior activation of caspase-3 and the resulting truncation of the IP₃R1 are required.

Apoptosis is a highly regulated and evolutionarily conserved form of cell death that plays an important role in normal embryonic development and maintenance of adult tissue homeostasis (1). Apoptotic cell death involves a characteristic sequence of morphological and biochemical features (2, 3). Most, but not all, forms of apoptotic cell death processes are characterized by the activation of a family of aspartate-specific cysteine proteases called caspases that cleave a wide range of cellular proteins leading to the manifestation of the major phenotypes in apoptosis (4).

Early studies of glucocorticoid-induced cell death suggested that an increase in the cytosolic free Ca²⁺ concentration ([Ca²⁺]ᵢ) was a key component of the apoptotic process (5). Various reports have since then established that a prolonged and up-regulated intracellular Ca²⁺ signal is a general feature of apoptosis (6–9). Apoptosis-related cleavage of a range of regulatory proteins and ion channels seems to be common to many apoptotic paradigms. During apoptosis, caspase-3, the main effector caspase, cleaves a wide array of cellular proteins including many that play significant roles in intracellular Ca²⁺ regulation such as the Ca²⁺/calmodulin-dependent protein kinase IV (10), plasma membrane Ca²⁺ ATPase (11, 12), Na⁺/Ca²⁺ exchanger (13), and the β-subunit of the Na⁺/K⁺-ATPase (14). Caspase cleavage could bring about a gain or loss of function on the target proteins leading to aberrant intracellular Ca²⁺ regulation that can directly influence the commitment of cells to apoptosis.

Inositol 1,4,5-trisphosphate (IP₃)³ receptors (IP₃Rs) are ubiquitous intracellular Ca²⁺ release channels, and their involvement in apoptosis has been demonstrated in different cell types. It was initially reported that the mRNA and protein levels of IP₃R3 increase during apoptosis in lymphocytes, with no change in the IP₃R1 level (15). Also, expression of an antisense cDNA construct of IP₃R3 blocked the dexamethasone-induced apoptosis and increase in [Ca²⁺]ᵢ, whereas that of IP₃R1 had no effect. Subsequently, however, it was reported that Jurkat cells deficient in IP₃R1 were resistant to apoptosis induced by Pas, dexamethasone, and γ-irradiation despite the presence of IP₃R3 (16). IP₃Rs are functionally redundant in chicken B-lymphocytes as apoptosis induced by B-cell receptor (BCR) stimulation was significantly inhibited only in cells deficient of all three receptors (17). The reason for these discrepancies is a matter of speculation, but together the reports

* This work was supported in part by Fonds voor Wetenschappelijk Onderzoek-Vlaanderen Grants G.3.0210.03 and G.0140.02, by Program on Interuniversity Poles of Attraction Grant F/505, by Concerted Actions of the Katholieke Universiteit Leuven Grant 99/08, and by Research Council of Katholieke Universiteit Leuven Grant VIS/02/006. 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.

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The abbreviations used are: IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; BCR, B-cell antigen receptor; ICRC, IP₃-induced Ca²⁺ release; IP₃-RKO cells, DT40 cells lacking all three IP₃Rs isoforms; STS, staurosporine; WT, wild-type; F, farad; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; PI, propidium iodide; z-, benzylxoycarbonyl; fmk, fluoromethyl ketone.
indicate that Ca\(^{2+}\) flux through IP\(_3\)R plays a fundamental role in apoptotic cell death induced by various stimuli. A more direct involvement of IP\(_3\)R in apoptosis was demonstrated by reports that identified IP\(_3\)R as a substrate of caspase-3 during apoptosis (18, 19). IP\(_3\)R1, but not IP\(_3\)R2 or IP\(_3\)R3, contains a single DEVD-specific cleavage site for caspase-3 at amino acids 1888–1891 (mouse sequence), and this sequence is conserved in rat and human proteins. Caspase-3-mediated degradation may actually explain a previous observation that the IP\(_3\)R1 level shows a dramatic decrease following dexamethasone from the extracellular medium. Our data also indicate that IP3R1 substrate could have a far reaching physiological significance. There-

direct involvement of IP3Rs in apoptosis was demonstrated by in apoptotic cell death induced by various stimuli. A more

DNA Constructs and Transfection—Mouse cerebellum IP\(_3\)R1 cDNA (a kind gift from Dr. K. Miki) (Tokyo, Japan) in pcDNA3.3(−) vector was used as a template to generate different mutants of the receptor. Mutagenesis was carried out using the QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. To construct the caspase-3-non-cleavable mutant of IP3R1 (IP\(_3\)R1(ΔC)), a fragment containing the region 2129–6819 of the full-size IP\(_3\)R1 cDNA, flanked by two BamHI restriction sites, was first subcloned into the pBlueScript II SK(+) vector. This construct was then used as template for the insertion of the mutations using 5′-GGGAA-AACAAAAAGGATGCAAGTGTCGGAGGATGCCCCGTCG-3′ as a forward primer and 5′-GGGGGGGACTTGGCCACTGATGATGTTTC-

**EXPERIMENTAL PROCEDURES**

**Reagents**—The polyclonal antibody (Rb04) raised against amino acids 2735–2749 of mouse IP\(_3\)R1 has been described previously (21). STS was purchased from Sigma. Mouse anti-chicken IgM (clone M-4) was from Southern Biotech (Birmingham, AL). Caspase inhibitors and colorimetric caspase-3 substrate were obtained from Bachem (Bubendorf, Switzerland).

**Cells and Culture Conditions**—The DT40 chicken B-lymphocytes lacking all three IP\(_3\)R isoforms (IP\(_3\)R-KO) were a kind gift from Dr. T. Kurokosi (Tokyo, Japan). IP3R-KO cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, 1% chicken serum, 50 μM 2-mercapto-

**Cleavage of IP\(_3\)R1 by Recombinant Caspase-3**—For in vitro assay of IP\(_3\)R cleavage by caspase-3, microsomes (200 μg) were incubated with purified active recombinant human caspase-3 (Pharmingen, BD Bio-

**Preparation of Cell Lysates and Microsomes**—Following treatments, cells were harvested and washed in ice-cold PBS before preparation of lysates as described previously (23). Total microsomes were prepared as described previously with minor modifications (21). Briefly, cells were harvested by centrifugation for 5 min at 400 × g and washed twice with ice-cold PBS without Ca\(^{2+}\) and Mg\(^{2+}\). Cell pellets were then resuspended in homogenization buffer (10 mM Tris/Cl, pH 7.4, 1 mM EDTA, 0.8 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 μM leupeptin, 0.5 μM iodoacetatin, 0.5 μM pepstatin A) and homogenized on ice with a probe sonicator (MSE Ltd., UK). Total microsomes were obtained by centrifugation of 125,000 × g for 20 min. A crude mitochondrial pellet was then resuspended in end medium (20 mM Tris/Cl, pH 7.4, 300 mM sucrose, 0.8 mM benzdiamide, and 0.2 mM phenylmethylsulfonyl fluoride). Cell lysates and microsomal preparations were frozen in liquid nitrogen and stored at −80°C. Protein concentrations were de-

**Clathrin-IP\(_3\)R Complex**—To prepare the clathrin-IP\(_3\)R complex for electron microscopy, clathrin-coated pits were isolated from mouse brain cortex as described (54). After extensive washing, clathrin-coated pits were lysed in a detergent-containing buffer containing 1% Triton X-100 to solubilize the clathrin, 10 μM EGTA, 2.5 mM ATP, 40 μM BODIPY-fluorescein, and 0.5 μg/ml of anti-clathrin antibodies. Lysates were then incubated for 2 h at 4°C. Lysates and pellets were analyzed by Western blotting for clathrin and IP3R1 expression.
Capase-3 Cleavage of IP<sub>3</sub>R1 Enhances Apoptosis

In this study, we aimed to investigate the exact role of the IP<sub>3</sub>R1 channel activity and the significance of this cleavage in apoptotic cell death. For this purpose, we first generated a caspase-non-cleavable mutant (IP<sub>3</sub>R1acasp) by introducing the mutations D1888I and D1891A into the wild-type receptor (Fig. 1B) using mouse IP<sub>3</sub>R1 as a template. IP<sub>3</sub>R1acasp possesses the same level of IICR activity as that of the WT-IP<sub>3</sub>R1 in stably expressing cell lines (data not shown). We also generated a deletion mutant lacking the first 225 amino acids (Δ(1–225)IP<sub>3</sub>R1), and then by substituting Asp-1888 with isoleucine and Asp-1891 with alanine, C and D, representing the (Δ(1–225))IP<sub>3</sub>R1, lacking the N-terminal 225 residues, and the (Δ1–1891)IP<sub>3</sub>R1 mutant, containing only the C-terminal channel domain of IP<sub>3</sub>R1 generated during its cleavage by caspase-3. E and F, subcellular localization of (Δ1–1891)IP<sub>3</sub>R1 mutant in D740 cells. Shown are confocal microscopic images of DT40 cells stably expressing (Δ1–1891)IP<sub>3</sub>R1 mutant stained with Rbt04 antibody and organelle-specific dyes BODIPY-thapsigargin (TH) for the ER (E) or MitoTracker for mitochondria (F).

RESULTS

Expression of Different IP<sub>3</sub>R1 Constructs in IP<sub>3</sub>R-deficient Cells—The basic structure of IP<sub>3</sub>Rs depicted the three functionally distinct regions, the IP<sub>3</sub>-binding, regulatory/transducing, and channel domains. Also shown is the specific cleavage site for caspase-3 (DEVD) that is present in IP<sub>3</sub>R1. No cleavage sites for other known caspases are found in IP<sub>3</sub>R1. B, the mutations introduced into the wild-type receptor by substituting Asp-1888 with isoleucine and Asp-1891 with alanine. C and D, representations of (Δ(1–225))IP<sub>3</sub>R1, lacking the N-terminal 225 residues, and the (Δ1–1891)IP<sub>3</sub>R1 mutant, containing only the C-terminal channel domain of IP<sub>3</sub>R1 generated during its cleavage by caspase-3. E and F, subcellular localization of (Δ1–1891)IP<sub>3</sub>R1 mutant in DT40 cells. Shown are confocal microscopic images of DT40 cells stably expressing (Δ1–1891)IP<sub>3</sub>R1 mutant stained with Rbt04 antibody and organelle-specific dyes BODIPY-thapsigargin (TH) for the ER (E) or MitoTracker for mitochondria (F).

Cleavage of IP<sub>3</sub>R1 in Vitro and in Cells Undergoing Apoptosis—The first data demonstrating a possible involvement of IP<sub>3</sub>R1 in cell death were published a few years ago showing that cells deficient in IP<sub>3</sub>R1 were resistant to apoptosis (16).
This study, together with reports that caspase-3 mediates the cleavage of IP₃R1 (18, 19), prompted us to examine the relevance of IP₃R1 cleavage to the cell death processes and its effect on intracellular Ca²⁺ distribution. First, to examine whether the cleavage process could occur in vitro as well as in stable cell lines, crude microsomal preparations from WT-IP₃R1 and IP₃R1/H₉004 casp cells were incubated with recombinant human caspase-3. In addition, we also studied IP₃R1 degradation in cells treated with 50 nM STS to induce apoptosis. The extent of receptor degradation was then analyzed by Western blotting. As shown in Fig. 2A, recombinant caspase-3 cleaved IP₃R1 in vitro in a dose-dependent manner generating exactly the same 95-kDa fragment as in cells undergoing STS-induced apoptosis. No such cleavage product could be detected in microsomal preparations from IP₃R1/H₉004 casp cells. Pretreatment of WT-IP₃R1 cells with 100 µM z-VAD-fmk (a pan-caspase inhibitor) or z-DEVD-fmk (a specific inhibitor of caspase-3) completely inhibited the degradation of IP₃R1 during STS-induced apoptosis (data not shown). These results confirm that caspase-3 was responsible for the cleavage of IP₃R1 in cells undergoing apoptosis. The same 95-kDa fragment was also generated in (Δ1–225)IP₃R1 cells induced to undergo apoptosis by either 50 nM STS or BCR cross-linking using 15 µg/ml anti-chicken IgM (Fig. 2B). Again, no cleavage product was detected in IP₃R1/H₉004 casp cells. These results suggest that the process of IP₃R1 cleavage by caspase-3 is probably common to a wide range of apoptosis-inducing agents. Importantly, the results from the (Δ1–225)IP₃R1 cells indicate that the initial caspase-3 activation during both STS- and anti-chicken IgM-induced apoptosis does not require IICR activity.

Role of Caspase-3-mediated Cleavage of IP₃R1 in the Process of Apoptotic Cell Death—Previous studies have shown that the degradation of IP₃R1 by caspase-3 inhibits IICR activity in microsomal preparations from cerebellum (18) and in digitonin-permeabilized A7r5 cells. However, the point at which Ca²⁺ is involved in apoptosis and the possible contribution of the IP₃R1 and its cleavage to the process of cell death are not yet thoroughly investigated. We addressed these points by inducing apoptosis by 50 nM STS or by BCR stimulation with 15 µg/ml anti-chicken IgM for 30 h. Cell lysates were prepared and subjected to Western blotting using Rbt04 antibody to determine the extent of cleavage. Arrowheads in A and B indicate the intact receptor, whereas the arrows show the 95-kDa caspase-3 cleavage product.

The results from the (Δ1–225)IP₃R1 cells indicate that the initial caspase-3 activation during both STS- and anti-chicken IgM-induced apoptosis does not require IICR activity.
The level of caspase activation in IP₃R₁/H₉₀₀₄ casp cells, however, was considerably lower (only about 1.5-fold higher than the control cells). The annexin V-FITC/PI apoptosis detection kit was used to determine the induction of apoptosis in cells treated with either STS or anti-chicken IgM by flow cytometry. Analysis of the percentage of apoptotic cells after treatment with STS (12 h) or anti-chicken IgM (48 h) revealed a similar pattern whereby WT-IP₃R₁, (Δ₁-225)IP₃R₁, and (Δ₁-1891)IP₃R₁ cells showed a significant increase in sensitivity both to STS and BCR stimulation (Fig. 3B). The extent of apoptosis in IP₃R₁Δcasp and IP₃R₁-deficient cells was very low and practically indistinguishable at these time points (Fig. 3B). It is noteworthy that (Δ₁-1891)IP₃R₁ showed enhanced responses both at the level of caspase-3 activity and the percentage of apoptotic cells. Pretreatment of the cells with 100 μM z-VAD-fmk or z-DEVD-fmk substantially inhibited both caspase-3 activation and apoptosis in all of the cells (data not shown).

**Fig. 3.** Caspase-3 cleavage of IP₃R₁ is required for optimal caspase-3 activation and apoptosis. A, IP₃R-KO cells expressing the indicated constructs of IP₃R₁ were treated with 50 nM STS for the indicated time periods before harvesting for colorimetric caspase-3 activity assay using DEVD-p-nitroaniline as substrate. Values were expressed as fold of the basal activity present in untreated cells. B, cells were treated with either 15 μg/ml anti-chicken IgM for 48 h or 50 nM STS for 12 h, and the level of apoptosis was then determined by the annexin V-FITC apoptosis detection kit using flow cytometry. For each case, 15,000 cells were analyzed, and the percentage of cells positive only for annexin V-FITC is shown. In both A and B, the values indicated are averages of three independent experiments, and the error bars represent the standard deviation from the mean.
Elevation of \([\text{Ca}^{2+}]_i\) during Apoptosis Requires the Cleavage of IP_3R1 by Caspase-3—

We next examined whether the alterations in \([\text{Ca}^{2+}]_i\) that usually accompany apoptotic cell death are influenced by the cleavage status of the IP_3R1 constructs. Following the treatment of the cells with 50 nM STS, the induction of apoptosis and changes in \([\text{Ca}^{2+}]_i\) were monitored in parallel by flow cytometry using annexin V-FITC and \([\text{Ca}^{2+}]_i\)-binding dyes Fluo-3/AM and Fura red/AM. Cell populations known from preliminary experiments to be positive for PI staining, indicative of the loss of plasma membrane integrity, were excluded during analysis. The increase in \([\text{Ca}^{2+}]_i\) was observed as a shift in the peak in the cell number versus fluorescence intensity distribution. As depicted in Fig. 4, A and B, the increase in \([\text{Ca}^{2+}]_i\) was indicated by a shift to the right in Fluo-3 fluorescence (higher intensity upon binding \([\text{Ca}^{2+}]_i\)) and to the left in Fura red fluorescence (lower intensity upon binding \([\text{Ca}^{2+}]_i\)). The data presented here are representative of at least three independent experiments with similar results.

Predictably, the largest increase in the percentage of cells with elevated \([\text{Ca}^{2+}]_i\), was observed in cells expressing \((\Delta 1-1891)\text{IP}_3\text{R}1\), the channel-only fragment, which also had a considerably higher percentage of apoptotic cells. The rise in the level of \([\text{Ca}^{2+}]_i\) showed a pattern of time-dependent increase, commencing at 6–8 h after the addition of STS (data not shown), suggesting that it was the consequence of the induction of apoptosis and caspase-3 activation (see Fig. 3A). The results depicted in Fig. 5 confirm that the apoptosis-associated increase in \([\text{Ca}^{2+}]_i\) was indeed caused by the activity of caspases. Pretreatment of cells with 100 μM z-VAD-fmk blocked the STS-induced increase in \([\text{Ca}^{2+}]_i\). Similar results were obtained with the specific caspase-3 inhibitor z-DEVD-fmk (data not shown). Taken together, the results indicate that the increase in \([\text{Ca}^{2+}]_i\), measured here was the consequence rather than the primary cause of apoptosis as it requires caspase-mediated cleavage of IP_3R1. These results also establish that the truncation of IP_3R1 by caspase-3 was a crucial requirement for the rise in \([\text{Ca}^{2+}]_i\), in cells undergoing apoptosis. However, it should be noted that the cleavage of IP_3R1 by caspase-3, although absolutely necessary, is not sufficient by itself to induce a spontaneous rise in

**Fig. 4.** Apoptosis-related increase in \([\text{Ca}^{2+}]_i\) by caspase. IP_3R-KO cells or those expressing the indicated constructs of IP_3R1 were left untreated or incubated with 50 nM STS for 8 h. About 90 min before the end of the incubation period, aliquots of 0.5 x 10^6 cells were loaded with either 3 μM Fluo-3/AM (A) or 6 μM Fura red/AM (B) at 37 °C. For each sample, 15,000 cells were analyzed on a Coulter Epics flow cytometer for changes in fluorescence of the dyes using the standard filters following a 488 nm excitation using an argon laser. The fluorescence intensity of Fluo-3 increases (shifts to the right), and that of Fura red decreases (left shift) upon binding \([\text{Ca}^{2+}]_i\); the extent of the shifts indicates the changes in \([\text{Ca}^{2+}]_i\). The data presented here are representative of at least three independent experiments with similar results.
normal resting [Ca\(^{2+}\)]\(_j\) prior to the induction of apoptosis.

STS-induced Elevation of [Ca\(^{2+}\)]\(_j\), in Nominally Ca\(^{2+}\)-free Medium—To determine whether the rise in [Ca\(^{2+}\)]\(_j\) was because of the release of calcium from intracellular stores or because of an influx of extracellular calcium, changes in intracellular calcium were determined in cells treated with STS in serum-free medium with no added calcium. Incubation of the cells in this nominally Ca\(^{2+}\)-free medium did not change the basal level of intracellular calcium in untreated control cells as compared with those cells cultured in normal medium. Treatment of the cells with STS induces a similar level of apoptotic cell death (data not shown) and rise in [Ca\(^{2+}\)]\(_j\), (Fig. 6) whether or not extracellular calcium was present. These results indicate that the elevated calcium level in STS-treated cells is derived mainly from the intracellular stores. It is conceivable that an influx of extracellular Ca\(^{2+}\) triggered by the depletion of intracellular stores could further contribute to the sustained increase in [Ca\(^{2+}\)]\(_j\). At a later stage of apoptosis, Ca\(^{2+}\) influx may also increase as a result of the reduced plasma membrane integrity.

Excessive Intracellular Free Ca\(^{2+}\) Enhances the Rate of Apoptosis Leading to a Secondary Necrosis—Cells were incubated with 50 nM STS for a prolonged period (24 h), and the percentage of apoptotic, necrotic, and secondary necrotic cells was determined by flow cytometry using the annexin V-FITC/PI apoptosis detection kit. Secondary necrosis can be regarded as a postapoptotic event that is observed particularly during apoptosis in cultured cells. Secondary necrotic cells readily stain both with annexin V-FITC and PI. Fig. 7 shows the percentage of cells that undergo secondary necrosis following treatment with 50 nM STS for 24 h. The proportion of cells in this phase of cell death ranged from about 40% in WT-IP\(_3\)R1 cells to over 65% in (Δ1–1891)IP\(_3\)R1 cells. The number of secondary necrotic cells in IP\(_3\)R-KO and IP\(_3\)R1Δcasp cells did not differ appreciably and was less than 15% on average. The results suggest that caspase cleavage of IP\(_3\)R1 and the resulting rise in [Ca\(^{2+}\)]\(_j\), augment the rate of apoptotic cell death to the point where it switches into necrosis because of Ca\(^{2+}\) overload. The deficiency of IP\(_3\)R1 or mutation of its caspase-3 cleavage site precludes an excessive increase in [Ca\(^{2+}\)]\(_j\), thereby resulting in an exceedingly slow rate of apoptosis.

**DISCUSSION**

Changes in Ca\(^{2+}\) homeostasis in general and elevation of [Ca\(^{2+}\)]\(_j\), in particular are an integral part of the process of cell death in many systems. Several mechanisms have been proposed regarding the contribution of cytosolic Ca\(^{2+}\) to apoptosis (13, 30). Because all mammalian cell types express at least one of the three IP\(_3\)Rs, it can be presumed that these receptors play a central role in the regulation of [Ca\(^{2+}\)]\(_j\), during apoptosis. In this study, we showed that IP\(_3\)Rs are required for STS- and BCR-induced apoptosis because IP\(_3\)R-KO cells were mostly resistant to cell death induced by both stimuli. Expression of IP\(_3\)R1 was sufficient to restore the susceptibility of the cells to apoptosis and a consequent rise in [Ca\(^{2+}\)]\(_j\). Surprisingly, expression of a deletion mutant that lacks IICR activity renders the cells even slightly more sensitive to apoptotic cell death than those expressing the wild-type receptor. One conclusion that could be drawn from these results is that the IICR activity of IP\(_3\)R1 per se was not required to mediate either the cell death or the associated rise in [Ca\(^{2+}\)]\(_j\). Significantly, cells expressing a mutant IP\(_3\)R1 resistant to caspase-3 cleavage underwent extremely slow apoptotic cell death. Conversely, expression of the channel-only domain of the IP\(_3\)R1 strongly increased the propensity of the cells to undergo apoptosis. Our results indicate that IP\(_3\)R1 plays an important role in apoptosis and that the disturbance in intracellular Ca\(^{2+}\) homeostasis during apoptosis is mainly caused by IP\(_3\)R1 cleavage by caspase-3. This essential role of caspase-3 in this process was substantiated by the observation that the expression of a caspase-non-cleavable mutant of IP\(_3\)R1 or the treatment of cells with caspase inhibitors strongly impedes such a rise in [Ca\(^{2+}\)]\(_j\). We also observe that rapid execution of apoptosis does not require the expression of a functional IP\(_3\)R1 per se but a prior activation of caspase-3 and the resulting cleavage of the receptor. The requirement for some level of caspase activity illustrates that the elevation in [Ca\(^{2+}\)]\(_j\), was the consequence but not the cause of the initiation of STS- or BCR-induced apoptosis. As such, increased [Ca\(^{2+}\)]\(_j\), may function as an additional stress that sustains and amplifies the apoptosis signals rather than serving as an obligatory messenger for the induction of death. Both apoptosis and the accompanying increase in [Ca\(^{2+}\)]\(_j\), could be induced in nominally Ca\(^{2+}\)-free culture medium, suggesting that the primary source of increased [Ca\(^{2+}\)]\(_j\), was the intracellular store, in agreement with a previous report (16). The finding is also compatible with the subcellular localization of the IP\(_3\)R1 in DT40 cells as well as other cell types (20), which was coincident with an ER marker. The strong dependence of a sustained [Ca\(^{2+}\)]\(_j\), increase on caspase-3 truncation suggests that it is primarily caused by an increased leak from the ER, with conceivably a consequent activation of store-operated Ca\(^{2+}\) influx. However, the cleavage of other regulators of intracellular Ca\(^{2+}\),

**Fig. 5.** Caspase inhibitors block apoptosis-related increase in [Ca\(^{2+}\)]\(_j\). The indicated cells were preincubated with 100 μM z-VAD-fmk for 1 h before treatment with 50 nM STS. After incubation for 12 h, cells were harvested for analysis of changes in Fluo-3 and Fura red fluorescence by flow cytometry exactly as described in Fig. 4.
notably the plasma membrane Ca\(^{2+}\)-ATPase (12) and the Na\(^+\)/Ca\(^{2+}\) exchanger (13), can significantly contribute to the overall rise in \([\text{Ca}^{2+}]_i\) during apoptosis. In addition, we cannot exclude the possibility that an influx of extracellular Ca\(^{2+}\) could occur in the late phase of apoptosis because of the emptying of intracellular stores and/or the deterioration of cell membrane integrity.

A new impetus to the study of IP\(_3\)Rs emanated from reports implying IP\(_3\)Rs in apoptosis and IP\(_3\)R1 as a caspase-3 substrate. Structure-function relationship studies of IP\(_3\)R1 have led to the proposal that the large regulatory domain of the receptor was necessary to maintain a closed state of the channel in resting cells (29). In principle, therefore, the cleavage and removal of this region should specifically abolish the IICR, but the effect on the general properties of the channel remains mostly unclear. A recent study has demonstrated that caspase cleavage of IP\(_3\)R1 results in a constitutively leaky channel, resulting in an almost complete emptying of the stores in transiently overexpressing cells (20). The extent of leakiness of the channel and the level of this apparently unregulated release of Ca\(^{2+}\) from the ER remain to be fully characterized. We reasonably expect that the stably expressed (1–189)IP\(_3\)R1 behaves similarly to the endogenously generated channel-only domain as both have a comparable expression level and the same localization at the ER. However, it is clear that in our model system using stable expression, the assumed passive leak did not result in a considerably increased \([\text{Ca}^{2+}]_i\) in resting cells and was not sufficient by itself to trigger apoptotic cell death. Moreover, a similar level of thapsigargin-induced increase in \([\text{Ca}^{2+}]_i\), was observed in cells that stably express the truncated receptor as in those expressing the WT-IP\(_3\)R1 (data not shown), suggesting that thapsigargin-sensitive stores of these cells are sufficiently filled. Nevertheless, after treatment with suboptimal levels of either STS or anti-chicken IgM, the

![Fig. 6. Intracellular Ca\(^{2+}\) increase in normal and in nominally Ca\(^{2+}\)-free medium](image)

Apoptosis was induced using 50 nM STS in the indicated cells grown in normal medium or in those that were transferred to nominally Ca\(^{2+}\)-free medium just before treatment. The level of \([\text{Ca}^{2+}]_i\), was determined using Fluo-3 and a flow cytometer as described above, 8 h after treatment. A representative result from at least three independent experiments is shown.

![Fig. 7. Secondary necrosis in STS-treated cells](image)

The different cells were incubated for 24 h with 50 nM STS, and the level of secondary necrosis was assessed using the annexin V-FITC apoptosis detection kit and flow cytometry. For each condition, 15,000 cells were analyzed, and those that stained with both annexin V-FITC and PI were recognized as cells undergoing secondary necrosis. The values shown here are averages of three independent experiments, and the error bars represent the standard deviation from the mean.
cells expressing the channel-only domain undergo a swift apoptotic cell death with an enhanced rate of caspase-3 activation and phosphatidylinerine exposure relative to those expressing the WT-IP<sub>R</sub>1. Therefore, it seems that an enhanced response to cell death stimuli and a related rise in [Ca<sup>2+</sup>]<sub>i</sub>, require apoptosis-associated changes and/or activation of an essential factor during apoptosis as well as the caspase-3 cleavage of IP<sub>R</sub>1.

The significance of an increased [Ca<sup>2+</sup>]<sub>i</sub>, especially downstream of caspase activation, is not clear. As an executioner caspase, the activation of caspase-3 requires upstream stimuli in the form of death receptor stimulation and/or the release of mitochondrial cytochrome <em>c</em>, depending on the apoptosis-inducing agent (4). Thus, a detectable level of caspase-3 activity would eventually lead to apoptotic cell death albeit at a rate dictated by the intensity of the upstream signal. Most likely, the late cleavage of IP<sub>R</sub>1 and the resulting buildup of cytosolic Ca<sup>2+</sup> were required to sustain and augment the apoptotic signals and the rate of cell death, thereby ensuring a speedy demise and removal of apoptotic cells. Recently, a study has shown that mitochondrial cytochrome <em>c</em> translocates to the ER early in apoptosis, selectively binds to the C-terminal tail of IP<sub>R</sub>Rs, and blocks the Ca<sup>2+</sup>-dependent inhibition of IP<sub>R</sub> function, which results in an oscillatory [Ca<sup>2+</sup>]<sub>i</sub>, increase (31). This study envisages a universal role for cytochrome <em>c</em> as an agonist of all IP<sub>R</sub>Rs leading to increased Ca<sup>2+</sup> release from the ER, apparently irrespective of the apoptosis-inducing agent. In effect, all three IP<sub>R</sub> classes would have identical roles as apoptosis signal amplifiers (through cytochrome <em>c</em>-induced Ca<sup>2+</sup> release activities), which, however, was not found in other reports (15, 16). From our observation, the lack of elevated [Ca<sup>2+</sup>]<sub>i</sub> in IP<sub>R</sub>1Δcasp-expressing cells strongly suggests that the increased Ca<sup>2+</sup> release during apoptosis was mainly because of the cleavage of the receptor by caspase-3 rather than cytochrome <em>c</em> binding. However, it is possible that cytochrome <em>c</em> released from a limited population of mitochondria can bind to IP<sub>R</sub>Rs on adjacent ER to induce a local Ca<sup>2+</sup> release that may overload the ER and/or inhibiting extrusion of the ion from the cell. The switch from Ca<sup>2+</sup> overload-induced apoptotic signals to necrosis depends, in part, on the intensity of the death-inducing signal (36). Our results suggest that such secondary necrosis events can occur downstream of caspase activation and apoptotic cell death. Cells that express caspase-cleavable IP<sub>R</sub>1 constructs and the channel-only domain invariably undergo an extensive necrosis after an initial robust apoptotic cell death, suggesting that the generation of the channel domain results in cytoplasmic and/or mitochondrial Ca<sup>2+</sup> overload when cells are challenged by apoptosis-inducing agents. The dependence of secondary necrosis on caspase activity may account for the previous observations where caspase inhibitors protected against cell death in ischemic and excitotoxic brain injury (34, 37, 38), which was also associated with Ca<sup>2+</sup> overload. Neuronal cells predominantly express IP<sub>R</sub>1 (39, 40), and dysregulation of Ca<sup>2+</sup> signaling is involved in neuronal cell death (41–43). Moreover, combined treatments with caspase inhibitors and Ca<sup>2+</sup> channel blockers synergistically protect against cerebral histotoxic hypoxia (37). Therefore, it is imperative to investigate the contribution of IP<sub>R</sub>Rs in stroke and neurodegenerative disorders.

Acknowledgments—We thank S. De Swaef, L. Bauwens, M. Crabbé, A. A. Khan, T. Ruyten, and for expert technical assistance. We are also grateful to Dr. T. Kuroasaki (Tokyo, Japan) for the IP<sub>R</sub>-KO cells and to Dr. K. Mikoshiba (Tokyo, Japan) for the p400CI plasmid containing the cDNA of mouse IP<sub>R</sub>1 and for stimulating discussions.

REFERENCES

1. Arends, M. J., and Wyllie, A. H. (1991) Int. Rev. Exp. Pathol. 32, 223–254.
2. Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980) Int. Rev. Cytol. 68, 51–306.
3. Crompton, M. (1999) Biochem. J. 341, 233–249.
4. Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) Annu. Rev. Biochem. 68, 383–424.
5. Kaisar, N., and Edelman, I. S. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 638–642.
6. McCunkey, D. J., Hartzell, P., Amador-Perez, J. F., Oreinens, S., and Jondal, M. (1989) J. Immunol. 143, 1801–1806.
7. Nicotera, P., and Oreinens, S. (1998) Cell Calcium 23, 173–180.
8. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) Nature 395, 645–648.
9. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) Nat. Rev. Mol. Cell Biol. 1, 11–21.
10. McGinnis, K. M., Whitten, M. M., Gnegy, M. E., and Wang, K. K. (1998) J. Biol. Chem. 273, 19993–20000.
11. Paszty, K., Verma, A. K., Padanyi, R., Flitoe, A. G., Penniston, J. T., and Pennisto, J. T. (2002) J. Biol. Chem. 277, 8622–8629.
12. Schwab, B. L., Guerini, D., Diduszen, C., Bano, D., Ferrando-May, E., Fava, E., Tam, J., Xu, D., Xanthoudakis, S., Nicholson, D. W., Carafoli, E., and Catterall, W. A. (2002) J. Cell Biochem. 89, 818–831.
13. Oreinens, S., Zhitovskoy, B., and Nicotera, P. (2003) Nat. Rev. Mol. Cell Biol. 4, 552–565.
14. DiMambro, H., Rehm, M., Kogel, D., and Prehn, J. H. (2003) J. Cell Sci. 116, 525–536.
15. Khan, A. A., Soloski, M. J., Sharp, A. H., Schilling, G., Sabatini, D. M., Li, S. H., Rose, C. A., and Snyder, S. H. (1996) Science 273, 503–507.
16. Jayaraman, T., and Marks, A. R. (1997) Mol. Cell. Biol. 17, 3005–3012.
17. Sugawara, H., Kurokaki, M., Takata, M., and Kurokaki, T. (1997) EMBO J. 16, 3078–3088.
18. Hirota, J., Furuchi, T., and Mikoshiba, K. (1999) J. Biol. Chem. 274, 34433–34437.
19. Haug, L. S., Walaas, S. I., and Ostvold, A. C. (2000) J. Neurochem. 75, 1852–1861.
20. 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.
21. Parys, J. B., De Smedt, H., Missiaen, L., Bootman, M. D., Sienaert, I., and Castelaa, R. (1995) Cell Calcium 17, 239–249.
22. Bultynck, G., Szljufcik, K., Nadif Kasri, N., Callewaert, G., Missiaen, L., Parys, J. B., and De Smedt, H. (2004) Biochem. J. 381, 87–96.
23. Assefa, Z., Vantieghsem, A., Garmyn, M., Declercq, W., Vandendaele, P., Vanhendehe, J. R., Bouillon, R., Merlevede, W., and Agostins, P. (2000) J. Biol. Chem. 275, 21416–21421.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. F. (1951) J. Biol. Chem. 193, 265–275.
25. Vermaes, I., Haenen, and Beutleringsperger, C. (2000) J. Immunol. Methods 243, 167–190.
26. Scollock, A. B., Bortner, C. D., St. J. Bird, G., Putkey, J. W., Jr., and Cidlowski, J. A. (2000) J. Biol. Chem. 275, 30586–30596.
27. Yoshikawa, F., Morita, M., Monka, T., Michikawa, T., Furuchi, T., and Mikoshiba, K. (1996) J. Biol. Chem. 271, 18277–18284.
28. Yoshikawa, F., Uchiyama, T., Iwasaki, H., Tomonori-Sato, C., Tanaka, T., Furuchi, T., and Mikoshiba, K. (1999) Biochem. Biophys. Res. Commun. 257, 792–797.
29. Uchida, K., Miyauuchi, H., Furuchi, T., Michikawa, T., and Mikoshiba, K.
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30. Rizzuto, R., Pinton, P., Ferrari, D., Chami, M., Szabadkai, G., Magalhaes, P. J., Virgilio, F. D., and Pozzan, T. (2003) Oncogene 22, 8619–8627

31. Boehning, D., Patterson, R. L., Sedaghat, L., Glebova, N. O., Kurosky, T., and Snyder, S. H. (2003) Nat. Cell Biol. 5, 1031–1061

32. Tombal, B., Denmeade, S. R., Gillis, J. M., and Isaacs, J. T. (2002) Cell Death Differ. 9, 561–573

33. Nicotera, P., Leist, M., and Ferrando-May, E. (1998) Toxicol. Lett. 102, 139–142

34. Choi, D. W. (1995) Trends Neurosci. 18, 58–69

35. Martinou, J. C., and Green, D. R. (2001) Nat. Rev. Mol. Cell Biol. 2, 63–67

36. Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., and Nicotera, P. (1995) Neuron 15, 961–973

37. Schulz, J. B., Weller, M., Matthews, R. T., Heneka, M. T., Groscurth, P., Martinou, J. C., Lommatzsch, J., von Coelln, R., Wullner, U., Loschmann, P. A., Beal, M. F., Dichgans, J., and Klockgether, T. (1998) Cell Death Differ. 5, 847–857

38. Annunziato, L., Amoroso, S., Pannaccione, A., Cataldi, M., Pignataro, G., D'Alessio, A., Sirabella, R., Secondo, A., Sibaud, L., and Di Renzo, G. F. (2003) Toxicol. Lett. 130, 125–133

39. Furuichi, T., Simon-Chazottes, D., Fujino, I., Yamada, N., Hasegawa, M., Miyawaki, A., Yoshikawa, S., Guenet, J. L., and Mikoshiba, K. (1993) Recept. Channels 1, 11–24

40. Sharp, A. H., McPherson, P. S., Dawson, T. M., Aoki, C., Campbell, K. P., and Snyder, S. H. (1998) J. Neurosci. 13, 3051–3063

41. Mattson, M. P. (2000) Nat. Rev. Mol. Cell Biol. 1, 120–129

42. Mattson, M. P., Duan, W., Pedersen, W. A., and Culmsee, C. (2001) Apoptosis 6, 69–81

43. Mattson, M. P., LaFerla, F. M., Chan, S. L., Leissring, M. A., Shepel, P. N., and Geiger, J. D. (2000) Trends Neurosci. 23, 222–229