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Caspase-3-truncated type 1 inositol 1,4,5-trisphosphate receptor enhances intracellular Ca^{2+} leak and disturbs Ca^{2+} signalling

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Abstract

Background information—The IP_3R (inositol 1,4,5-trisphosphate receptor) is a tetrameric channel that accounts for a large part of the intracellular Ca^{2+} release in virtually all cell types. We have previously demonstrated that caspase-3-mediated cleavage of $\text{IP}_3\text{R1}$ during cell death generates a C-terminal fragment of 95 kDa comprising the complete channel domain. Expression of this truncated IP_3R increases the cellular sensitivity to apoptotic stimuli, and it was postulated to be a constitutively active channel.

Results—In the present study, we demonstrate that expression of the caspase-3-cleaved C-terminus of $\text{IP}_3\text{R1}$ increased the rate of thapsigargin-mediated Ca^{2+} leak and decreased the rate of Ca^{2+} uptake into the ER (endoplasmic reticulum), although it was not sufficient by itself to deplete intracellular Ca^{2+} stores. We detected the truncated $\text{IP}_3\text{R1}$ in different cell types after a challenge with apoptotic stimuli, as well as in aged mouse oocytes. Injection of mRNA corresponding to the truncated $\text{IP}_3\text{R1}$ blocked sperm factor-induced Ca^{2+} oscillations and induced an apoptotic phenotype.

Conclusions—In the present study, we show that caspase-3-mediated truncation of $\text{IP}_3\text{R1}$ enhanced the Ca^{2+} leak from the ER. We suggest a model in which, in normal conditions, the increased Ca^{2+} leak is largely compensated by enhanced Ca^{2+} -uptake activity, whereas in situations where the cellular metabolism is compromised, as occurring in aging oocytes, the Ca^{2+} leak acts as a feed-forward mechanism to divert the cell into apoptosis.

Keywords

apoptosis; calcium leak; calcium oscillation; caspase 3; inositol 1,4,5-trisphosphate receptor (IP_3R)

Introduction

Many physiological processes are regulated by changes in free $[Ca^{2+}]_i$ (intracellular Ca^{2+} concentration). Various Ca^{2+} channels, pumps, exchangers and buffers are responsible for the accurate control of $[Ca^{2+}]_i$ (Berridge et al., 2000). Perturbations in the $[Ca^{2+}]_i$ homeostasis are considered to be of great importance in initiating and modulating both apoptotic (programmed) and necrotic cell death (Berridge et al., 1998, 2003; Hajnoczky et al., 2003; Orrenius et al., 2003; Rizzuto et al., 2003). The caspase family of cysteine proteases represents one of the key players in apoptotic cell-death processes (Degterev et al., 2003; Yuan et al., 2003). The two major signalling pathways of caspase activation, the death-receptor pathway (extrinsic pathway) (Curtin and Cotter, 2003; Micheau and Tschopp, 2003) and the intrinsic or mitochondria-mediated pathway (Schafer and Kornbluth, 2006), eventually lead to the activation of so-called effector caspases, including caspase 3. The latter mediates the proteolytic processing of a wide variety of cellular targets, including many that directly control or affect intracellular Ca^{2+} homeostasis (Verbert et al., 2007). Cleavage by caspase 3 can induce a gain- or a loss-of-function of these substrates, possibly leading to the stimulation of aberrant Ca^{2+} signalling that can influence the execution of the apoptotic process.

The IP_3R (inositol 1,4,5-trisphosphate receptor), a large tetrameric intracellular Ca^{2+} -release channel mainly located in the ER (endoplasmic reticulum) and represented by three isoforms (types 1, 2 and 3), is one of the targets of caspase 3. There is increasing evidence that the IP_3R plays a pivotal role in apoptotic programmed cell-death pathways (Joseph and Hajnoczky, 2007). IP_3R -knockout cells are less sensitive to apoptotic stimuli (Sugawara et al., 1997; Assefa et al., 2004), and anti-sense knockout of IP_3R1 blocks cell death in Jurkat T-lymphoma cells (Jayaraman and Marks, 1997). IP_3R activity is also shown to be regulated by cytochrome *c* (Boehning et al., 2005), GAPDH (glyceraldehyde- 3-phosphate dehydrogenase) (Patterson et al., 2005) and members of the Bcl-2 family of proteins (Chen et al., 2004; Oakes et al., 2005; White et al., 2005; Pinton and Rizzuto, 2006), which are all well-known key players in cell-death processes. Moreover, IP_3R1 is specifically cleaved by caspase 3 in Jurkat T-lymphoma cells upon induction of apoptosis by STS (staurosporin) or anti-Fas IgM (Hirota et al., 1999), thereby generating a truncated 95-kDa protein containing all of the transmembrane domains and the channel pore. The consensus site for caspase-3-dependent cleavage of mouse IP_3R1 was identified at D¹⁸⁸⁸EVD* (* indicates cleavage site) (Hirota et al., 1999). The role of IP_3R1 truncation during apoptosis has been investigated in DT40 cells deficient in all three IP_3R isoforms [IP_3RKO (IP_3R knockout) cells]. IP_3RKO cells expressing a caspase-3-non-cleavable IP_3R1 mutant were less sensitive to apoptotic stimuli, whereas cells expressing full-length IP_3R1 or the truncated IP_3R1 displayed an increased propensity to undergo apoptosis. In addition, we have found previously that caspase 3 cleavage of IP_3R1 was related to an increase in $[Ca^{2+}]_i$ during the execution phase of apoptosis (Assefa et al., 2004). Until now, the functional properties of this truncated IP_3R1 have not been elucidated. Therefore, the aim of the present study was to further investigate the formation and the Ca^{2+} -leak properties of this channel in different cell types and in well-defined conditions. Our results demonstrate that stable or transient expression of the truncated IP_3R1 induced a modest Ca^{2+} leak from the ER. However, this Ca^{2+} leak was not sufficient to deplete the ER Ca^{2+} stores in normal conditions. Nevertheless, our results emphasize the physiological importance of the generation of a truncated IP_3R , as it was consistently found in several cell types undergoing apoptotic cell death, including aged mouse oocytes. Furthermore, expression of the C-terminal fragment in fresh oocytes led to severe perturbations in the Ca^{2+} signalling and resulted in cellular fragmentation. Proteolytic cleavage of IP_3R1 and generation of a long-lived channel domain may therefore represent a feed-forward pathway in the execution of cell death, particularly when intracellular Ca^{2+} -removal mechanisms become inadequate.

RESULTS

Increased ER Ca²⁺ leak in cells stably expressing the truncated IP₃R1

We have first investigated the Ca²⁺-leak properties of the truncated IP₃R1 independently of plasma-membrane fluxes. Therefore, we have performed unidirectional Ca²⁺ fluxes in permeabilized DT40 cells. IP₃RKO cells, deficient in all three IP₃Rs, were stably transfected with the caspase 3-truncated channel domain (Δ 1–1891)IP₃R1 as described previously (Assefa et al., 2004). We measured the [Ca²⁺] in a permeabilized cell suspension with fluo 3 and monitored the leak after inhibition of the SERCA (sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase) by TG (thapsigargin; 4 μ M) (Figure 1A). After permeabilization of the cells with digitonin, Ca²⁺ was actively sequestered into the ER. Once a steady-state [Ca²⁺] was reached, we monitored the increase in [Ca²⁺] in the cuvette upon addition of TG. The total Ca²⁺ store content was estimated by the application of the Ca²⁺ ionophore A23187. To directly compare the Ca²⁺-leak properties in IP₃RKO cells and (Δ 1–1891)IP₃R1 cells, we measured the Ca²⁺-leak rate ($d[Ca^{2+}]/dt$) as a function of time (Figure 1B). The rate of Ca²⁺ leak was significantly higher in (Δ 1–1891)IP₃R1 compared with IP₃RKO cells during a large part of the release process ($P < 0.05$). Hence, these data indicate that expression of (Δ 1–1891)IP₃R1 enhances the Ca²⁺ leak from the ER in response to TG. (Δ 1–1891)IP₃R1 therefore represents a leaky channel. Furthermore, the A23187-releasable Ca²⁺ was not significantly affected by the presence of the truncated IP₃R1. This finding indicates, in contrast with previous observations (Nakayama et al., 2004), that the presence of (Δ 1–1891)IP₃R1 was not sufficient to reduce or deplete the total amount of stored Ca²⁺. This observation was confirmed in intact DT40 cells using the ratiometric Ca²⁺ probe fura 2/AM (fura 2 acetoxymethyl ester), where no significant differences in TG-releasable store content were detected (Figure 1C).

Altered kinetics of ER Ca²⁺ uptake and release in transiently transfected cells

Since stable cell lines might have developed compensatory mechanisms for a reduced store content, we determined the free [Ca²⁺]_{ER} (Ca²⁺ concentration in the ER) in HEK-293 cells (human embryonic kidney cells) transiently transfected with the truncated IP₃R1 channel. HEK-293 were chosen as they express low levels of endogenous IP₃R1 and have a high efficiency of transfection. The cells were cotransfected with the bioluminescent protein erAEQ (ER-targeted aequorin) and an empty vector as a control or with erAEQ and the truncated (Δ 1–1891)IP₃R1. Co-transfection conditions, using a cDNA ratio for (Δ 1–1891)IP₃R1/erAEQ of 3:1, were checked by immunocytochemistry using an antibody against the HA (haemagglutinin) tag of the erAEQ (Brini et al., 1995), and also an antibody against the C-terminal part of IP₃R1 (Rbt04) to detect the truncated channel. More than 90% of the erAEQ-expressing cells also expressed the truncated channel (data not shown). AEQ reconstitution was carried out in Ca²⁺-depleting conditions. Ca²⁺ uptake in the ER was measured by perfusion of the cells with a KRB (Krebs–Ringer Buffer) supplemented with 1 mM CaCl₂ (Figure 2A). Average curves ($n = 9–10$) representing uptake of Ca²⁺ into the ER were plotted as [Ca²⁺]_{ER} against time. Expression of the C-terminal fragment in HEK-293 cells significantly decreased the initial rate of Ca²⁺ uptake into the ER ($P < 0.05$) as compared with control cells transfected with an empty vector (Figure 2B). However, steady-state [Ca²⁺]_{ER} values were slightly, but not significantly, lower in the cells transfected with the truncated channel ($163 \pm 15 \mu\text{M}$, $n = 10$, compared with $183 \pm 21 \mu\text{M}$, $n = 9$, in control conditions) (Figure 2A), indicating that the stores could be adequately refilled, even in the presence of the truncated channel.

Alternatively, using cytAEQ (cytosol-targeted AEQ), we observed a significantly faster increase in [Ca²⁺]_i upon application of TG (1 μ M) in (Δ 1–1891)IP₃R1-expressing HEK-293 cells (Figure 3A). During the first 30 s, the rate of TG-induced Ca²⁺ release was

significantly higher ($P < 0.05$) than in control cells (Figure 3B), indicating an enhanced Ca^{2+} leak from the ER. In addition, the small, but not significant, difference in maximal amplitude between ($\Delta 1$ –1891)IP₃R1-expressing cells and control cells ($1.62 \pm 0.05 \mu\text{M}$, $n = 18$, compared with $1.35 \pm 0.14 \mu\text{M}$, $n = 16$, in control cells), may be caused by an enhanced store-operated Ca^{2+} influx, which is known to be stimulated by an enhanced Ca^{2+} release from the ER.

Taken together, these data establish that the ER Ca^{2+} store was not fully depleted upon expression of the caspase 3-cleaved IP₃R1 C-terminus.

IP₃R1 truncation in apoptotic cells

We have shown previously that a truncated IP₃R1 channel is formed in IP₃RKO cells expressing full-length IP₃R1 after treatment with STS or anti-IgM to activate apoptotic pathways (Assefa et al., 2004). We now further investigated this phenomenon in other cell types to elucidate whether the proteolytic cleavage of IP₃R1 is of more general importance. Expression levels of IP₃R1 and its caspase 3-cleavage product were assayed in SHSY-5Y, HeLa, L15 and Lvec cells by Western-blot analysis (Figure 4A). In all cell types, we observed high levels of the 95-kDa C-terminal fragment after treatment with $1 \mu\text{M}$ STS for 8 h. As shown in Figure 4(B), there was a concomitant increase in caspase 3 activity upon STS treatment. It was remarkable that the increase in caspase 3 activity was much higher in L15 cells that have a very high [approx. 8-fold compared with control Lvec cells (Mackrill et al., 1996)] overexpression of IP₃R1. These data reveal that IP₃R1 cleavage by caspase 3 during apoptosis occurred both in cells overexpressing this type of receptor (L15 cells), as well as in cells that endogenously expressed IP₃R1 at various levels (HeLa, Lvec and SHSY-5Y cells) (Wojcikiewicz, 1995; Missiaen et al., 2004; Nadif Kasri et al., 2005).

($\Delta 1$ –1891)IP₃R1 is present in aged mouse MII (second meiotic division)-stage eggs and leads to perturbed Ca^{2+} signals

Apoptosis is a process that is often linked with neurodegeneration and aging. Hence, we investigated if ($\Delta 1$ –1891)IP₃R1 formation plays a role in the well-defined conditions of oocyte aging. Indeed, in crude lysates from aged mouse MII-stage eggs [34 h post-hCG (human chorionic gonadotropin)] we detected a 95-kDa fragment that was absent in lysates from fresh MII-stage eggs (14 h post-hCG), as assessed by Western blotting (Figure 5A). To investigate further the effect of a truncated IP₃R1 with respect to Ca^{2+} signalling and apoptosis, ($\Delta 1$ –1891)IP₃R1 mRNA ($1 \mu\text{g}/\mu\text{l}$) was injected into fresh MII-stage eggs. Expression level of the truncated channel was verified on Western blot (Figure 5B). These eggs displayed an increased fragmentation rate 6 h after ($\Delta 1$ –1891)IP₃R1 mRNA injection (Figures 5C and 5D), a phenomenon that resembles apoptotic blebbing. Ca^{2+} oscillations in response to sperm factor ($0.5 \mu\text{g}/\mu\text{l}$) were measured with the ratiometric fluorescent Ca^{2+} probe fura 2/AM, and plotted as ratios of the fluorescence intensity at 340 nm/380 nm wavelengths as a function of time (Figures 5E and 5F). In contrast to control fresh MII-stage eggs (Figure 5E), eggs expressing ($\Delta 1$ –1891)IP₃R1 failed to establish persistent oscillatory responses (Figure 5F). Furthermore, the TG-induced Ca^{2+} leak into the cytosol in the absence of extracellular Ca^{2+} was significantly decreased (Figure 5G). The Ca^{2+} -store content thus was decreased, but not completely depleted. Together, these data confirm that the formation of the truncated IP₃R1 represents a leaky Ca^{2+} channel that disturbs cellular Ca^{2+} signalling.

DISCUSSION

In the present study we have performed a detailed characterization of the properties of the caspase-3-truncated IP₃R1. On the basis of data obtained from different expression systems

[stable transfection of DT40 cells lacking all three IP₃Rs, transient transfection, mRNA injection of (Δ1–1891)IP₃R1 in oocytes], different techniques (fluo 3 in permeabilized cells; erAEQ, cytAEQ and fura 2 in intact cells) and several cell types, we conclude that the truncated IP₃R1 creates a modest leak, but, by itself, does not lead to an empty ER Ca²⁺ store. These data are in contrast with the study by Nakayama et al. (2004), in which it was shown that overexpression of an EGFP (enhanced green fluorescent protein)-tagged form of the caspase-3-truncated IP₃R1 in fura-2-loaded COS and HeLa cells almost completely depleted ER Ca²⁺ stores, as no increase in [Ca²⁺]_i was observed in response to TG. The latter findings are also not compatible with our previous study (Assefa et al., 2004), in which a stable cell line expressing the truncated IP₃R showed normal store filling and where an additional apoptotic trigger (STS) was required to induce an increased Ca²⁺ response over a prolonged time period. The reason for these discrepancies is not clear.

We experienced technical difficulties with the use of EGFP in combination with an UV-excitable Ca²⁺ indicator, such as fura 2. Since EGFP and fura 2 have partially overlapping spectral properties, as documented by Bolsover et al. (2001), we observed a significant decrease of the fura 2 ratio signal as a function of EGFP expression in COS cells, as well as in HEK-293 cells (data not shown). We therefore choose to evaluate the role of the (Δ1–1891)IP₃R1 expression on Ca²⁺ signalling in HEK-293 cells co-transfected with the Ca²⁺-sensitive bioluminescent protein AEQ. This technique avoids the possible fluorescence artifacts and, furthermore, also allows us to study [Ca²⁺]_i as well as [Ca²⁺]_{ER} changes. Using erAEQ, we found that the steady-state [Ca²⁺]_{ER} was only slightly, but not significantly, decreased in cells expressing the truncated channel. Nonetheless, the lower initial rate of Ca²⁺ refilling clearly indicated the presence of a significant additional Ca²⁺-leak pathway. Hence, in transiently transfected HEK-293 cells, the active Ca²⁺ pumps could, to a large extent, counteract the Ca²⁺ leak through the truncated channel. These data are entirely consistent with our observations using cytAEQ, where inhibition of the Ca²⁺ pumps with TG revealed a significantly faster Ca²⁺ leak into the cytosol in cells co-transfected with (Δ1–1891)IP₃R1. Similar effects were observed for the TG-induced Ca²⁺ leak in cell suspensions of a DT40 IP₃RKO cell line stably expressing the (Δ1–1891)IP₃R1.

Our findings of only small effects on ER Ca²⁺ content in cells that have undergone caspase cleavage of IP₃R1 or that overexpress a truncated version of IP₃R1 are compatible with several studies. For example, Hirota et al. (1999) reported previously that a significant reduction in IP₃ (inositol 1,4,5-trisphosphate)-induced Ca²⁺-release activity required caspase-3-mediated cleavage of at least 80% of the IP₃R1. As shown in Figure 4(A), and as described in Assefa et al. (2004), STS treatment of different cell types did not yield more than 35% of truncated channels in endogenously IP₃R1-expressing cells and up to 60% in IP₃R1-overexpressing cells. This finding indicates that, even under apoptotic conditions, it is likely that the intact IP₃R1 is more abundant than the 95-kDa channel and that the Ca²⁺-leak properties of the truncated channel are not preponderant. In addition, in most cell types, IP₃R2 and IP₃R3 could also contribute to normal IP₃-induced Ca²⁺-release function. Furthermore, it has been shown that IP₃Rs can exist as heterotetramers (Monkawa et al., 1995). Since the transmembrane regions and the C-terminal part of the IP₃R are the main determinants for dimerization (Boehning and Joseph, 2000; Galvan and Mignery, 2002), it is conceivable that heterotetramers are formed containing one or more truncated IP₃R1 monomers. The regulatory suppressor/coupling N-terminal domains of the other intact subunits, whether from isoform 1, 2 or 3, could then still control the opening of the channel (Devogelaere et al., 2007). Collectively, our results support the view that the insertion of a caspase-3-truncated C-terminus of IP₃R1 could introduce a constitutive (i.e. IP₃-independent), albeit modest, leak from the ER. Conceivably, given the previous findings demonstrating that a limited elevation of mitochondrial [Ca²⁺] activates ATP production which enhances Ca²⁺-pump activity (Visch et al., 2006; Giacomello et al., 2007), the

presumed decrease in the Ca^{2+} -store content may be compensated by this activation. This presumption is confirmed by findings that cytosolic ATP levels were increased for up to 6 h after STS treatment. Longer incubation times with STS are needed before ATP levels drop below original levels, which coincides with the massive activation of caspase 3 and reduction of cell viability (Zamaraeva et al., 2005). This may explain why the effects of expression of the C-terminal fragment on intracellular Ca^{2+} handling and cell death were only observed after prolonged incubation with STS (Assefa et al., 2004). Apparently, an additional trigger is required, creating a double-hit mechanism to increase the $[\text{Ca}^{2+}]_i$, leading to cell death. The feed-forward effect exerted by the truncated $\text{IP}_3\text{R1}$ was particularly marked in L15 cells, probably because of the high level of $\text{IP}_3\text{R1}$ in these cells. Hence, the fact that the caspase-3-truncated C-terminus of $\text{IP}_3\text{R1}$ is a constitutively open channel should be interpreted as an IP_3 -independent Ca^{2+} -leak pathway that under normal conditions is largely compensated by an enhanced SERCA-mediated Ca^{2+} -uptake activity, but that in situations in which the cellular metabolism is compromised may lead to the execution of the apoptotic cascade.

The physiological relevance of our observations was demonstrated in aging mouse oocytes. Aged oocytes endogenously expressed higher levels of the $\text{IP}_3\text{R1}$ C-terminal 95-kDa fragment. Strikingly, injection of ($\Delta 1$ –1891) $\text{IP}_3\text{R1}$ mRNA in fresh mouse oocytes prematurely terminated sperm factor-induced Ca^{2+} oscillations, decreased the TG-releasable Ca^{2+} -store content and induced morphological changes that resemble apoptotic blebbing. In aged oocytes, the presence of the leaky C-terminal 95-kDa $\text{IP}_3\text{R1}$ fragment, in combination with the decreased SERCA activity as a result of the decreased intracellular [ATP] (Takahashi et al., 2000; Gordo et al., 2002; Igarashi et al., 2005), may lead to the observed disturbed Ca^{2+} homeostasis (Fissore et al., 2002; Gordo et al., 2002), with fertilization often resulting in the activation of caspases and fragmentation, rather than the normal event of oocyte activation and embryo development (Gordo et al., 2002).

There is as yet no consensus about the exact mechanisms by which Ca^{2+} signals and particularly IP_3 -independent Ca^{2+} signals contribute to the cell-death pathways (Rizzuto et al., 2003; Joseph and Hajnoczky, 2007). On the basis of our findings, we hypothesize that caspase-3-mediated truncation of $\text{IP}_3\text{R1}$ creates a modest Ca^{2+} leak from the ER. This Ca^{2+} leak becomes an important part of the cell-death programme, if accompanied by a decrease in the activity of ATP-dependent Ca^{2+} -removal mechanisms. These changes will result in depletion of the ER Ca^{2+} store that evokes a persistent increase in store-operated Ca^{2+} entry, leading to a prolonged cellular Ca^{2+} overload that diverts the normal cellular functions towards cell death. Caspase-3-mediated cleavage of $\text{IP}_3\text{R1}$ can thereby act as a feed-forward mechanism in the execution of the apoptotic process.

Materials and methods

Reagents

The polyclonal antibody (Rbt04) raised against amino acids 2735–2749 of mouse $\text{IP}_3\text{R1}$ has been described previously (Parys et al., 1995). STS, TG and A23187 were purchased from Sigma.

Cells and culture conditions

$\text{IP}_3\text{R1}$ -overexpressing L15 and control Lvec fibroblasts were a gift from Dr K. Mikoshiba (Division of Molecular Neurobiology, University of Tokyo, Tokyo, Japan). DT40 chicken B-lymphocytes lacking all three IP_3Rs (IP_3RKO) were a gift from Dr T. Kurosaki (Laboratory for Lymphocyte Differentiation, RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan). IP_3RKO cells were stably transfected with pcDNA(+)-

IP₃R1 (wild-type IP₃R1) or pcDNA(+)-(Δ1–1891)IP₃R1 [(Δ1–1891)IP₃R1], as described previously (Assefa et al., 2004). DT40-derived cells were maintained in RPMI 1640 medium (Gibco) containing 10% (v/v) FCS (fetal calf serum) (Sigma), 1% (v/v) chicken serum (Sigma), 50 μM 2-mercaptoethanol, 85 units/ml penicillin, 85 μg/ml streptomycin and 3.5 mM L-alanyl-L-glutamine (Glutamax™, Gibco) in a humidified incubator at 5% CO₂ and 37°C. The culture medium of the transfected IP₃RKO cells was supplemented with 1.5 mg/ml G418 for selection.

HEK-293, HeLa, Lvec and L15 cells were grown in DMEM (Dulbecco's modified Eagle's medium) (Gibco) containing 10% (v/v) FCS, 4 mM Glutamax™, 100 units/ml penicillin, 100 μg/ml streptomycin and MEM (minimal essential medium) with NEAAs (non-essential amino acids) (Gibco) in a humidified incubator at 10% (v/v) CO₂ and 37°C. For L15 cells this medium was supplemented with G418 (400 μg/ml). SHSY-5Y cells were grown in DMEM/ Ham's F12 medium containing 15% FCS, 4 mM Glutamax™, 100 units/ml penicillin, 100 μg/ml streptomycin and MEM with NEAAs.

[Ca²⁺] measurements in DT40 cell suspensions

Cell pellets were resuspended in intracellular medium (120 mM KCl, 30 mM Hepes, pH 7.4, and 1 mM MgCl₂) in the presence of 1 mM ATP, 25 mM phosphocreatine, 50 units/ml creatine kinase and 5 μM fluo 3 (Molecular Probes) and transferred into a 4 ml fluorescence quartz cuvette that was thermostatically maintained at 37°C. Cell density was 5×10^7 cells/ml.

Mild treatment of the cells with digitonin (50 μM) disrupted the plasma membrane. The fluo 3 fluorescence ($\lambda_{\text{excitation}} = 503$ nm and $\lambda_{\text{emission}} = 530$ nm) was measured with an Aminco-Bowman® Series 2 spectrometer (Spectronic Unicam). A23187 (8 μM) was added at the end of each experiment to measure the total releasable Ca²⁺. The fluorescence signal (F) was calibrated by addition of 0.5 mM CaCl₂ (F_{max}) followed by 5 mM EGTA (F_{min}). The free [Ca²⁺] was calculated using the following equation: $[\text{Ca}^{2+}] = K_d \times (F - F_{\text{min}})/(F_{\text{max}} - F)$ (Grynkiewicz et al., 1985), with a K_d value of 864 nM, as determined in cytosol-like medium at 37°C (Missiaen et al., 1991). Ca²⁺-leak rate ($d[\text{Ca}^{2+}]/dt$) was determined by adding TG (4 μM).

Free [Ca²⁺]_i was also measured in intact cells (1.5×10^7 cells/ml) loaded with fura 2/AM (Teflabs). After de-esterification, cells were resuspended in modified KRB (135 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 11.6 mM Hepes, pH 7.3, and 11.5 mM glucose) containing 1.5 mM CaCl₂. The [Ca²⁺]_i was monitored at excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm.

AEQ measurements

The constructs for erAEQ and cytAEQ were provided by Dr R. Rizzuto (Department of Experimental and Diagnostic Medicine, University of Ferrara, Ferrara, Italy). HEK-293 cells, seeded on to 13 mm (diameter) gelatine-coated coverslips, were transfected with the constructs using the GeneJuice® transfection reagent (Novagen). Experiments were performed 2 days after transfection with a confluent cell layer at 37°C.

Prior to measuring erAEQ signals, the culture medium was replaced with KRB (135 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 20 mM KH₂PO₄, 20 mM Hepes and 5.5 mM glucose, pH 7.4) containing 600 μM EGTA. Cells were then incubated for 1 h with 5 μM ionomycin (Sigma) for Ca²⁺ depletion, and with 5 μM coelenterazine-n (Molecular Probes) for reconstitution of the active AEQ at 4°C. The cells were then washed extensively with KRB supplemented with 2% BSA (Sigma) and 1 mM EGTA. After superfusion of the cells with KRB supplemented with 100 μM EGTA, the stores were loaded by perfusion with a KRB

solution containing 1 mM CaCl₂. Finally, digitonin (100 µM in water containing 10 mM CaCl₂) was added to lyse the cells and to take up the remaining AEQ.

Reconstitution of cytAEQ with coelenterazine-w (Molecular Probes) was performed in culture medium for 1 h at 37°C. The cells were washed with KRB containing 1 mM CaCl₂. After a 2 min perfusion with the same solution, TG-induced Ca²⁺ release was measured (1 µM TG in KRB supplemented with 1 mM CaCl₂). Calibration was performed in a similar manner to erAEQ measurements.

The light signal was collected by a low-noise photomultiplier tube with a built-in amplifier–discriminator, and conversion into [Ca²⁺] values was carried out as described previously (Brini et al., 1995; Rizzuto et al., 1995).

Preparation of cell lysates

Suspension cells were harvested (400 g for 5 min at 4°C) and lysed in RIPA buffer (25 mM Hepes, pH 7.5, 0.3 mM NaCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 20 mM β-glycerolphosphate, 10% glycerol, 1 mM Na₃VO₄, 1% Triton X-100, 1 mM PMSF, 20 µg/ml aprotinin and 20 µg/ml leupeptin). After 15 min of incubation on ice, the lysates were cleared by centrifugation (400 g for 15 min at 4°C). Adherent cells were first scraped with a rubber policeman into ice-cold PBS before lysis with RIPA. Protein concentrations were determined according to Lowry et al. (1951).

Western-blotting analysis

Whole-cell lysates (25 µg) from the various cell types were subjected to electrophoresis on Tris/acetate SDS polyacrylamide gradient gels (3–8% gel) (Invitrogen), transferred on to a PVDF membrane. The blot was blocked with PBS containing 0.1% Tween 20 and 5% skimmed dried milk and subsequently incubated with the primary antibody Rbt04 against the C-terminus of IP₃R1 (dilution, 1:3000). Detection of the immunoreactive bands was performed by exposure to Hyperfilm ECL (GE healthcare) using a horseradish-peroxidase-conjugated secondary antibody (dilution, 1:2000) and ECL substrate (GE healthcare). The Hyperfilm was developed using an X-Omat 1000 (Kodak).

Evaluation of caspase activity

Caspase 3 activity in STS-treated cells was measured in whole-cell lysates using a colorimetric CaspACE™ Assay System (Promega). Briefly, 50 µg of protein was incubated with 200 µM Ac-DEVD-*p*-nitroaniline (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline; caspase 3 substrate) for 4 h at 37°C. A₄₀₅ was measured using a 96-well plate reader (Corning Incorporated).

Recovery of eggs and culture conditions

MII-stage eggs were collected from the oviducts of 6- to 8-week-old CD-1 female mice. Females were superstimulated with an injection of 5 units of PMSG (pregnant mare serum gonadotropin; Sigma). *In vivo* MII-stage eggs were recovered in TL-Hepes (Hepes-buffered tyrode/lactate solution), supplemented with 5% heat-treated FCS (Gibco), 12–14 h after injection of 5 units of hCG, which was administered 45–48 h after PMSG stimulation. After the removal of cumulus cells, eggs were transferred into 50 µl drops of KSOM (potassium simplex optimized medium; Speciality Media) and cultured under paraffin oil at 36.5°C in a humidified atmosphere containing 6% CO₂.

Preparation of mouse MII-stage egg crude lysates and Western blotting

Cell lysates from 50 MII-stage eggs were mixed with 15 μ l of 2 $\times$ Laemmli sample buffer, boiled and loaded on to NuPAGE Novex Tris/acetate 3–8% gels (Invitrogen). After electrophoresis, proteins were transferred on to nitrocellulose membranes (Micon Separations) and blocked with PBS containing 0.05% Tween 20 supplemented with 6% skimmed dried milk and incubated overnight with Rbt04 antibody (dilution, 1:3000). The membranes were subsequently washed and incubated for 1 h with a goat anti-rabbit secondary antibody coupled to horseradish peroxidase. Membranes were washed and incubated for 1 min in chemiluminescence reagent (NEN Life Science Products) and developed according to the manufacturer's instructions. Each nitrocellulose membrane was digitally captured and quantified using an Imaging Station 440 CF (Kodak).

Fluorescence recordings and $[Ca^{2+}]_i$ determinations in mouse MII-stage eggs

Measurements of $[Ca^{2+}]_i$ were performed using fura 2/AM (Molecular Probes) as reported previously (Kurokawa et al., 2005). Eggs were monitored in drops of TL-HEPES under mineral oil. Up to 10 eggs were monitored simultaneously using the software SimplePCI (C-Imaging System), which controls a high-speed filter wheel rotating between excitation wavelengths of 340 and 380 nm. Illumination was provided by a 75 W xenon arc lamp, and the light emitted above 510 nm was collected by a cooled Photometrics SenSys CCD (charge-coupled-device) camera (Roper Scientific). Fluorescence ratios of 340/380 nm were obtained every 20–30 s.

Statistical analysis

The results are expressed as means $\pm$ S.E.M. Individual groups were compared by Student's unpaired *t* test. Statistics was performed with Origin 7.0 software (OriginLab Corporation).

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Abbreviations used

AEQ	aequorin
cytAEQ	cytosol-targeted AEQ
$[Ca^{2+}]_{ER}$	Ca^{2+} concentration in the endoplasmic reticulum
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
DMEM	Dulbecco's modified Eagle's medium
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
erAEQ	ER-targeted AEQ
FCS	fetal calf serum

fura 2/AM	fura 2 acetoxymethyl ester
hCG	human chorionic gonadotropin
HEK-293 cell	human embryonic kidney cell
IP ₃	inositol 1,4,5-trisphosphate
IP ₃ R	IP ₃ receptor
IP ₃ RKO	IP ₃ R knockout
KRB	Krebs–Ringer buffer
MII	second meiotic division
MEM	minimal essential medium
NEAA	non-essential amino acid
PMSG	pregnant mare serum gonadotropin
SERCA	sarcoplasmic/endoplasmic reticulum Ca ²⁺ -ATPase
STS	staurosporin
TG	thapsigargin
TL-Hepes	Hepes-buffered tyrode/lactate solution

References

- Assefa Z, Bultynck G, Szlufcik K, Nadif Kasri N, Vermassen E, Goris J, Missiaen L, Callewaert G, Parys JB, De Smedt H. Caspase-3-induced truncation of type1 inositol trisphosphate receptor accelerates apoptotic cell death and induces inositol trisphosphate-independent calcium release during apoptosis. *J. Biol. Chem* 2004;279:43227–43236. [PubMed: 15284241]
- Berridge MJ, Bootman MD, Lipp P. Calcium – a life and death signal. *Nature* 1998;395:645–648. [PubMed: 9790183]
- Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol* 2000;1:11–21. [PubMed: 11413485]
- Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell Biol* 2003;4:517–529. [PubMed: 12838335]
- Boehning D, Joseph SK. Direct association of ligand-binding and pore domains in homo- and heterotetrameric inositol 1,4,5-trisphosphate receptors. *EMBO J* 2000;19:5450–5459. [PubMed: 11032812]
- Boehning D, van Rossum DB, Patterson RL, Snyder SH. A peptide inhibitor of cytochrome c/inositol 1,4,5-trisphosphate receptor binding blocks intrinsic and extrinsic cell death pathways. *Proc. Natl. Acad. Sci. U.S.A* 2005;102:1466–1471. [PubMed: 15665074]
- Bolsover S, Ibrahim O, O’Luanaigh N, Williams H, Cockcroft S. Use of fluorescent Ca²⁺ dyes with green fluorescent protein and its variants: problems and solutions. *Biochem. J* 2001;356:345–352. [PubMed: 11368760]
- Brini M, Marsault R, Bastianutto C, Alvarez J, Pozzan T, Rizzuto R. Transfected aequorin in the measurement of cytosolic Ca²⁺ concentration ([Ca²⁺]_c). A critical evaluation. *J. Biol. Chem* 1995;270:9896–9903. [PubMed: 7730373]
- Chen R, Valencia I, Zhong F, McColl KS, Roderick HL, Bootman MD, Berridge MJ, Conway SJ, Holmes AB, Mignery GA, Velez P, Distelhorst CW. Bcl-2 functionally interacts with inositol 1,4,5-trisphosphate receptors to regulate calcium release from the ER in response to inositol 1,4,5-trisphosphate. *J. Cell Biol* 2004;166:193–203. [PubMed: 15263017]
- Curtin JF, Cotter TG. Live and let die: regulatory mechanisms in Fas-mediated apoptosis. *Cell Signal* 2003;15:983–992. [PubMed: 14499341]

- Degterev A, Boyce M, Yuan J. A decade of caspases. *Oncogene* 2003;22:8543–8567. [PubMed: 14634618]
- Devogelaere B, Verbert L, Parys JB, Missiaen L, De Smedt H. The complex regulatory function of the ligand-binding domain of the inositol 1,4,5-trisphosphate receptor. *Cell Calcium*. 2007 doi: 10.1016/j.ceca.2007.1004.1005.
- Fissore RA, Kurokawa M, Knott J, Zhang M, Smyth J. Mechanisms underlying oocyte activation and postovulatory ageing. *Reproduction* 2002;124:745–754. [PubMed: 12530912]
- Galvan DL, Mignery GA. Carboxyl-terminal sequences critical for inositol 1,4,5-trisphosphate receptor subunit assembly. *J. Biol. Chem* 2002;277:48248–48260. [PubMed: 12377774]
- Giacomello M, Drago I, Pizzo P, Pozzan T. Mitochondrial Ca^{2+} as a key regulator of cell life and death. *Cell Death Differ* 2007;14:1267–1274. [PubMed: 17431419]
- Gordo AC, Rodrigues P, Kurokawa M, Jellerette T, Exley GE, Warner C, Fissore R. Intracellular calcium oscillations signal apoptosis rather than activation in *in vitro* aged mouse eggs. *Biol. Reprod* 2002;66:1828–1837. [PubMed: 12021069]
- Gryniewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem* 1985;260:3440–3450. [PubMed: 3838314]
- Hajnoczky G, Davies E, Madesh M. Calcium signaling and apoptosis. *Biochem. Biophys. Res. Commun* 2003;304:445–454. [PubMed: 12729578]
- Hirota J, Furuichi T, Mikoshiba K. Inositol 1,4,5-trisphosphate receptor type1 is a substrate for caspase-3 and is cleaved during apoptosis in a caspase-3-dependent manner. *J. Biol. Chem* 1999;274:34433–34437. [PubMed: 10567423]
- Igarashi H, Takahashi T, Takahashi E, Tezuka N, Nakahara K, Takahashi K, Kurachi H. Aged mouse oocytes fail to readjust intracellular adenosine triphosphates at fertilization. *Biol. Reprod* 2005;72:1256–1261. [PubMed: 15659708]
- Jayaraman T, Marks AR. T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis. *Mol. Cell. Biol* 1997;17:3005–3012. [PubMed: 9154798]
- Joseph SK, Hajnoczky G. IP_3 receptors in cell survival and apoptosis: Ca^{2+} release and beyond. *Apoptosis* 2007;12:951–968. [PubMed: 17294082]
- Kurokawa M, Sato K, Wu H, He C, Malcuit C, Black SJ, Fukami K, Fissore RA. Functional, biochemical, and chromatographic characterization of the complete $[\text{Ca}^{2+}]_i$ oscillation-inducing activity of porcine sperm. *Dev. Biol* 2005;285:376–392. [PubMed: 16098961]
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J. Biol. Chem* 1951;193:265–275. [PubMed: 14907713]
- Mackrill JJ, Wilcox RA, Miyawaki A, Mikoshiba K, Nahorski SR, Challiss RA. Stable overexpression of the type-1 inositol 1,4,5-trisphosphate receptor in L fibroblasts: subcellular distribution and functional consequences. *Biochem. J* 1996;318:871–878. [PubMed: 8836131]
- Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 2003;114:181–190. [PubMed: 12887920]
- Missiaen L, Taylor CW, Berridge MJ. Spontaneous calcium release from inositol trisphosphate-sensitive calcium stores. *Nature* 1991;352:241–244. [PubMed: 1857419]
- Missiaen L, Van Acker K, Van Baelen K, Raeymaekers L, Wuytack F, Parys JB, De Smedt H, Vanoevelen J, Dode L, Rizzuto R, Callewaert G. Calcium release from the Golgi apparatus and the endoplasmic reticulum in HeLa cells stably expressing targeted aequorin to these compartments. *Cell Calcium* 2004;36:479–487. [PubMed: 15488597]
- Monkawa T, Miyawaki A, Sugiyama T, Yoneshima H, Yamamoto-Hino M, Furuichi T, Saruta T, Hasegawa M, Mikoshiba K. Heterotetrameric complex formation of inositol 1,4,5-trisphosphate receptor subunits. *J. Biol. Chem* 1995;270:14700–14704. [PubMed: 7782334]
- Nadif Kasri N, Bultynck G, Parys JB, Callewaert G, Missiaen L, De Smedt H. Suramin and disulfonated stilbene derivatives stimulate the Ca^{2+} -induced Ca^{2+} release mechanism in A7r5 cells. *Mol. Pharmacol* 2005;68:241–250. [PubMed: 15851651]
- Nakayama T, Hattori M, Uchida K, Nakamura T, Tateishi Y, Bannai H, Iwai M, Michikawa T, Inoue T, Mikoshiba K. The regulatory domain of the inositol 1,4,5-trisphosphate receptor is necessary to keep the channel domain closed: possible physiological significance of specific cleavage by caspase 3. *Biochem. J* 2004;377:299–307. [PubMed: 12968951]

- Oakes SA, Scorrano L, Opferman JT, Bassik MC, Nishino M, Pozzan T, Korsmeyer SJ. Proapoptotic BAX and BAK regulate the type1 inositol trisphosphate receptor and calcium leak from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A* 2005;102:105–110. [PubMed: 15613488]
- Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. *Nat. Rev. Mol. Cell Biol* 2003;4:552–565. [PubMed: 12838338]
- Parys JB, De Smedt H, Missiaen L, Bootman MD, Sienaert I, Casteels R. Rat basophilic leukemia cells as model system for inositol 1,4,5-trisphosphate receptor IV, a receptor of the typeII family: functional comparison and immunological detection. *Cell Calcium* 1995;17:239–249. [PubMed: 7664312]
- Patterson RL, van Rossum DB, Kaplin AI, Barrow RK, Snyder SH. Inositol 1,4,5-trisphosphate receptor/GAPDH complex augments Ca^{2+} release via locally derived NADH. *Proc. Natl. Acad. Sci. U.S.A* 2005;102:1357–1359. [PubMed: 15677321]
- Pinton P, Rizzuto R. Bcl-2 and Ca^{2+} homeostasis in the endoplasmic reticulum. *Cell Death Differ* 2006;13:1409–1418. [PubMed: 16729032]
- Rizzuto R, Brini M, Bastianutto C, Marsault R, Pozzan T. Photoprotein-mediated measurement of calcium ion concentration in mitochondria of living cells. *Meth. Enzymol* 1995;260:417–428. [PubMed: 8592464]
- Rizzuto R, Pinton P, Ferrari D, Chami M, Szabadkai G, Magalhaes PJ, Di Virgilio F, Pozzan T. Calcium and apoptosis: facts and hypotheses. *Oncogene* 2003;22:8619–8627. [PubMed: 14634623]
- Schafer ZT, Kornbluth S. The apoptosome: physiological, developmental, and pathological modes of regulation. *Dev. Cell* 2006;10:549–561. [PubMed: 16678772]
- Sugawara H, Kurosaki M, Takata M, Kurosaki T. Genetic evidence for involvement of type1, type2 and type3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor. *EMBO J* 1997;16:3078–3088. [PubMed: 9214625]
- Takahashi T, Saito H, Hiroi M, Doi K, Takahashi E. Effects of aging on inositol 1,4,5-trisphosphate-induced Ca^{2+} release in unfertilized mouse oocytes. *Mol. Reprod. Dev* 2000;55:299–306. [PubMed: 10657049]
- Verbert L, Devogelaere B, Parys JB, Missiaen L, Bultynck G, De Smedt H. Proteolytic mechanisms leading to disturbed Ca^{2+} signalling in apoptotic cell death. *Calcium Binding Proteins* 2007;2:21–29.
- Visch HJ, Koopman WJ, Zeegers D, van Emst-de Vries SE, van Kuppeveld FJ, van den Heuvel LW, Smeitink JA, Willems PH. Ca^{2+} -mobilizing agonists increase mitochondrial ATP production to accelerate cytosolic Ca^{2+} removal: aberrations in human complex I deficiency. *Am. J. Physiol. Cell Physiol* 2006;291:C308–C316. [PubMed: 16554405]
- White C, Li C, Yang J, Petrenko NB, Madesh M, Thompson CB, Foscett JK. The endoplasmic reticulum gateway to apoptosis by Bcl-X(L) modulation of the InsP_3R . *Nat. Cell Biol* 2005;7:1021–1028. [PubMed: 16179951]
- Wojcikiewicz RJ. Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *J. Biol. Chem* 1995;270:11678–11683. [PubMed: 7744807]
- Yuan J, Lipinski M, Degtarev A. Diversity in the mechanisms of neuronal cell death. *Neuron* 2003;40:401–413. [PubMed: 14556717]
- Zamaraeva MV, Sabirov RZ, Maeno E, Ando-Akatsuka Y, Bessonova SV, Okada Y. Cells die with increased cytosolic ATP during apoptosis: a bioluminescence study with intracellular luciferase. *Cell Death Differ* 2005;12:1390–1397. [PubMed: 15905877]

Figure 1. (Δ1-1891)IP₃R1 enhances Ca²⁺ leak from the ER in permeabilized IP₃RKO cells
(A) Representative recording of fluo-3-monitored free $[Ca^{2+}]$ in permeabilized IP₃RKO cells ($n = 4$; grey line) and in permeabilized (Δ1-1891)IP₃R1 cells ($n = 5$; black line). After permeabilization of the plasma membrane with 50 μM digitonin, cells accumulated Ca²⁺ in their intracellular stores, resulting in a decrease in medium free $[Ca^{2+}]$. After reaching a steady-state level of Ca²⁺, the SERCA-pump inhibitor TG (4 μM) was added. At the end of each experiment, 8 μM A23187 was added to estimate the total releasable amount of Ca²⁺, which was used to calculate the Ca²⁺-store content. **(B)** The TG-induced Ca²⁺ leak in permeabilized IP₃RKO (grey line) or (Δ1-1891)IP₃R1 cells (black line) was measured as first-order derivative of free $[Ca^{2+}] = f(t)$. **(C)** IP₃RKO cells (grey line) and (Δ1-1891)IP₃R1 cells (black line) were resuspended in modified KRB containing 1.5 mM Ca²⁺ and free $[Ca^{2+}]_i$ was monitored by fura 2/AM. After stabilization of the signal, 6 mM EGTA was added for 5 min and the internal Ca²⁺-store content was determined by the addition of 4 μM TG. Means of at least 3 experiments were plotted as $(R - R_0)/R_0$, where R is the ratio $F_{340/380}$ and R_0 is the background ratio before the addition of TG. Mean values are plotted against time (error bars are omitted for clarity).

Figure 2. Decreased initial ER Ca²⁺-uptake rate in HEK-293 cells expressing (Δ1-1891)IP₃R1
(A) Averaged time course ($n = 9-10$) for the [Ca²⁺]_{ER} of erAEQ measurements in HEK-293 cells transiently transfected with the truncated IP₃R1 [(Δ1-1891)IP₃R1] (black line) or an empty vector as a control (grey line). Broken lines indicate the S.E.M. Steady-state [Ca²⁺]_{ER} was not significantly different ($P = 0.05$). **(B)** The initial uptake rate ($d[Ca^{2+}]_{ER}/dt$) during the first 30 s of CaCl₂ perfusion is plotted as a function of time. HEK-293 cells expressing (Δ1-1891)IP₃R1 (black triangles) showed a significantly decreased rate of Ca²⁺ uptake into the ER, as compared with HEK-293 cells transfected with an empty vector (grey squares) ($P < 0.05$).

Figure 3. Increased initial TG-induced Ca^{2+} -release rate in HEK-293 cells expressing $(\Delta 1-1891)IP_3R1$

(A) Averaged time course ($n = 16-18$) for the $[Ca^{2+}]_i$ of cytAEQ measurements in the presence of 1 mM $CaCl_2$ in HEK-293 cells transiently transfected with the truncated IP_3R1 $(\Delta 1-1891)IP_3R1$ (black line) or an empty vector as a control (grey line). TG (1 μM) was added as indicated. Broken lines indicate S.E.M. Maximal $[Ca^{2+}]_i$ was not significantly different ($P = 0.075$). (B) The initial release rate ($d[Ca^{2+}]_i/dt$) during the first 30 s of TG application is plotted as a function of time. HEK-293 cells expressing $(\Delta 1-1891)IP_3R1$ (black triangles) showed a significantly faster ($P < 0.05$) TG-induced rise in $[Ca^{2+}]_i$, as compared with HEK-293 cells transfected with an empty vector (grey squares).

Figure 4. Formation of a truncated IP₃R1 in several cell types treated with staurosporin
(A) Cell lysates from SHSY-5Y, HeLa, L15 and Lvec cells were harvested after 8 h of incubation with or without 1 μ M STS and subjected to Western blotting using the Rbt04 antibody. The chemiluminescent signal was detected by exposure to a light-sensitive film. Optimal exposure times were used to optimally determine the extent of IP₃R1 cleavage in each cell line. Arrowheads indicate the intact IP₃R1, whereas arrows indicate the 95-kDa caspase-3-cleavage product. **(B)** The same cell lysates as described in **(A)** were subjected to a caspase 3 activity assay using Ac-DEVD-p-nitroaniline ((*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline) as a substrate. The results are expressed as the means $\pm$ S.E.M. of the fold change from the basal activity present in untreated cells.

Figure 5. Effects of (Δ1-1891)IP₃R1 in mouse MII-stage eggs

(A) Crude lysates were prepared from both fresh (14 h post-hCG, left-hand lane) and aged (34 h post-hCG, right-hand lane) MII-stage eggs. Western blotting was carried out with the Rbt04 antibody against the C-terminus of IP₃R1. The 95-kDa degradation product (arrow) was present in aged MII-stage eggs. Full-size IP₃R1 is indicated with an arrowhead. (B) Crude lysates were prepared from fresh MII-stage eggs injected with buffer alone (left-hand lane) or with (Δ1-1891)IP₃R1 mRNA (right-hand lane) and allowed to express (Δ1-1891)IP₃R1 for 6 h. (C, D) MII-stage eggs were injected with buffer alone (C) or (Δ1-1891)IP₃R1 mRNA (1 μg/μl) (D) and allowed to express (Δ1-1891)IP₃R1 for 6 h. Expression of the truncated IP₃R1 led to fragmentation in fresh MII-stage eggs (34%, $n = 82$, fragmentation rate versus 0% in control cells, $n = 25$). (E, F) Fresh MII-stage eggs were first loaded with fura 2/AM. Ca²⁺ oscillations in response to sperm factor (SF, 0.5 μg/μl) were monitored and plotted as ratios of 340 nm/380 nm fluorescence as a function of time. SF was added at time 0. Prior to measuring responses (3 h), the eggs were injected with buffer alone ($n = 9$) (E) or with 1 μg/μl (Δ1-1891)IP₃R1 mRNA ($n = 12$) (F). The indicated traces were representative of 9/9 eggs (E) or 10/12 eggs (F). (G) Fresh MII-stage eggs were loaded with fura 2/AM and Ca²⁺ leak upon application of TG (10 μM) in nominally Ca²⁺-free medium was determined and plotted as ratios of 340/380 fluorescence as a function of time. Eggs that were injected with 1 μg/μl (Δ1-1891)IP₃R1 mRNA 6 h prior to measurements ($n = 11$; black line) were compared with control eggs (buffer injection only; $n = 7$; grey line). The results are expressed as the means ± S.E.M. for all of the eggs monitored for each group.