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

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The type 1 inositol 1,4,5-trisphosphate receptor (IP₃R1) is an intracellular Ca²⁺ channel protein that plays crucial roles in generating complex Ca²⁺ signalling patterns. IP₃R1 consists of three domains: a ligand-binding domain, a regulatory domain and a channel domain. In order to investigate the function of these domains in its gating machinery and the physiological significance of specific cleavage by caspase 3 that is observed in cells undergoing apoptosis, we utilized various IP₃R1 constructs tagged with green fluorescent protein (GFP). Expression of GFP-tagged full-length IP₃R1 or IP₃R1 lacking the ligand-binding domain in HeLa and COS-7 cells had little effect on cells' responsiveness to an IP₃-generating agonist ATP and Ca²⁺ leak induced by thapsigargin. On the other hand, in cells expressing the caspase-3-cleaved form (GFP-IP₃R1-casp) or the channel domain alone (GFP-IP₃R1-ES), both ATP and thapsigargin failed to induce increase of cytosolic Ca²⁺ concentration. Interestingly,

store-operated (-like) Ca²⁺ entry was normally observed in these cells, irrespective of thapsigargin pre-treatment. These findings indicate that the Ca²⁺ stores of cells expressing GFP-IP₃R1-casp or GFP-IP₃R1-ES are nearly empty in the resting state and that these proteins continuously leak Ca²⁺. We therefore propose that the channel domain of IP₃R1 tends to remain open and that the large regulatory domain of IP₃R1 is necessary to keep the channel domain closed. Thus cleavage of IP₃R1 by caspase 3 may contribute to the increased cytosolic Ca²⁺ concentration often observed in cells undergoing apoptosis. Finally, GFP-IP₃R1-casp or GFP-IP₃R1-ES can be used as a novel tool to deplete intracellular Ca²⁺ stores.

Key words: calcium, caspase, green fluorescent protein (GFP), inositol 1,4,5-trisphosphate receptor (IP₃R), store-operated calcium entry.

INTRODUCTION

All eukaryotic cells must control their cytosolic Ca²⁺ concentration ([Ca²⁺]_i) and pattern according to extracellular conditions. Most cells utilize Ca²⁺ influxes from the extracellular space and Ca²⁺ release from intracellular stores to generate these signals [1,2]. Two families of channels that mediate the intracellular Ca²⁺ release, the inositol 1,4,5-trisphosphate (IP₃) receptor (IP₃R) family and the ryanodine receptor (RyR) family, have been identified. These intracellular channels are mainly localized to the endoplasmic reticulum (ER), which is believed to be the major Ca²⁺ store and sink.

There are three members of the IP₃R family (types 1 to 3), each of which is encoded by a different gene [3]. Murine type 1 IP₃R (IP₃R1) contains 2749 amino acid residues [4]. The receptor channel is tetrameric, with each subunit containing three functionally distinct domains: the ligand-binding domain at the N-terminal region, the channel domain at the C-terminal end and the large regulatory domain (also called the transducing/modulatory domain) between them, which is believed to convey conformational changes from the ligand-binding domain to the channel domain [3]. This domain contains more regulatory sites,

including those for phosphorylation and calmodulin binding, than other domains [3,5]. Hamada et al. [6] recently showed in an electron-microscopic study that IP₃R1 occurs in two states, 'windmill' and 'square', that are mainly attributable to differences in the structure of the regulatory domain [6]. However, it remains unknown whether these two states correspond to the 'open' and 'closed' states as a channel. One of the important and unsolved questions is the 'default' state of the channel domain of IP₃R: does IP₃ induce a conformational change that opens the channel domain or does IP₃ 'unlock' certain machinery that has kept the channel domain closed?

In regard to the default state of IP₃R, it is interesting to note that there is a specific cleavage site for caspase 3 in the C-terminal part of the regulatory domain of IP₃R1 [7,8] and, because of this, cleavage by caspase 3 generates a truncated 'channel-domain only' IP₃R1. IP₃R1 has been demonstrated to be cleaved by caspase 3 in Jurkat, MCF-7 [7] and SH-SY5Y [8] cells undergoing apoptosis, and an *in vitro* assay showed that the cleaved IP₃R1 loses its responsiveness to IP₃ [7]. However, it is unknown whether the caspase-3-cleaved IP₃R1 is constitutively closed or is leaking Ca²⁺ continuously. Although the precise roles of the cytosolic Ca²⁺ signalling pattern in the process of

Abbreviations used: BSS, balanced salt solution; [Ca²⁺]_i, cytosolic Ca²⁺ concentration; CRT, calreticulin; ER, endoplasmic reticulum; GFP, green fluorescent protein; IICR, IP₃-induced Ca²⁺ release; IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; IP₃R-TKO, IP₃R triple knock-out; RyR, ryanodine receptor; TG, thapsigargin.

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apoptosis are a matter of controversy and appear to be highly dependent on the stimuli, conditions and cell types [1], several studies have implicated IP₃R in the process of apoptosis. For example, decreasing the amount of IP₃R either by antisense RNA in a Jurkat cell [9] or by gene deletion in a DT40 chick B-cell [10] leads to significant inhibition of apoptosis. Type 3 IP₃R (IP₃R3) is selectively increased during apoptosis of lymphocytes, and their apoptosis is blocked by expression of IP₃R3 antisense RNA [11,12]. Bcl-X_L down-regulates IP₃R1 and IP₃R3, and the down-regulation may be part of its anti-apoptotic action [13]. Therefore it is very likely that IP₃R and its cleavage by caspase 3 play an important role in the signal transduction essential to apoptosis under certain conditions.

In the present study, we expressed various IP₃R1 proteins tagged with green fluorescent protein (GFP) and performed real-time Ca²⁺ imaging experiments in order to identify the role of each domain in the regulation of Ca²⁺ ion permeation. GFP tagging is helpful not only in identifying cells expressing exogenous protein, but also in excluding cells in which the exogenous protein mislocalizes. In the present paper, we present strong evidence that the default state of the channel domain of IP₃R1 is open (at least leaky) and the large regulatory domain of IP₃R1 is necessary to keep the channel domain closed.

EXPERIMENTAL

Antibodies

The anti-IP₃R1 monoclonal antibody 18A10 has been characterized previously [14]. Anti-calreticulin (CRT) polyclonal antibody was purchased from Affinity BioReagents (Golden, CO, U.S.A.). Anti-GFP was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Alexa 594-conjugated anti-rabbit IgG was from Molecular Probes (Eugene, OR, U.S.A.).

Plasmids

To construct the expression vector for GFP-IP₃R1-N (see Figure 1A), the 5'-untranslated region (containing Kozak's sequence) and coding sequence of enhanced GFP was amplified by PCR using *Pfu* turbo (Stratagene, La Jolla, CA, U.S.A.) with primers 5'-TCCGCTAGCGCTACCGGTC-3' and 5'-GAGCTT-AAGCTTGTACAGCTCGTCCATG-3' (the underlined regions indicate *Nhe*I and *Afl*II restriction endonuclease sites respectively), using pEGFP-C3 (Clontech, Palo Alto, CA, U.S.A.) vector as a template. All the PCR was performed at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min for a total of 35 cycles. The 5'-part (1.25 kb) of mouse IP₃R1 cDNA was amplified by PCR with primers 5'-CTTAAGATGTCTGACAAAATGTCGAG-3' (the underlined region indicates *Afl*II site) and 5'-GCTTCCTTGCTCCTTCAG-3'. Likewise, the 3'-part (0.5 kb) of mouse IP₃R1 cDNA was amplified with primers 5'-ATCGAATTCTTTGCTGACCTGAGGAGTGAG-3' and 5'-TTACTCGAGCTAGGCCGGCTGCTGTGGG-3' (the underlined regions indicate *Eco*RI and *Xho*I restriction enzyme sites respectively). These fragments and other parts of mouse IP₃R1 cDNA were sequentially subcloned into pcDNA3.1/Zeo+ (Invitrogen, Carlsbad, CA, U.S.A.). GFP-IP₃R1-C expression vector (see Figure 1A) was constructed essentially in the same fashion. Primers used to amplify the 5'-part (1.3 kb) of mouse IP₃R1 cDNA (with 22 bp of 5'-untranslated sequence) were 5'-TTTGCTAGCGTCAGGCTTTCCAACACGG-3' (the underlined region indicates a *Nhe*I site) and 3'-GCTTCCTTGCTCCTTCAG-5'. Primers used to amplify the 3'-part of IP₃R1 cDNA (without stop codon, 0.5 kb) were 5'-ATCGAATTCTTTGCTGACCTGAGGAGTGAG-3' and 5'-

TTTGC GGCCGCCGCGCGGCTGCTGTGGGTTG-3' (the underlined regions indicate *Eco*RI and *Not*I sites respectively). Primers used to amplify GFP sequence (without the first methionine residue) were 5'-GTCGGCGCCGCTGAGCAAGGGCGAGGAGC-3' and 5'-TTACTCGAGCTAGGCCGGCTGCTGTGGG-3' (the underlined regions indicate *Not*I and *Xho*I sites respectively). These fragments and other parts of mouse IP₃R1 cDNA were sequentially subcloned into pcDNA3.1/Zeo+. To construct the expression vector for GFP-IP₃R1-D610 (see Figure 1A), part of the cDNA (encoding amino acids 1–610) of IP₃R1 was excised from GFP-IP₃R1-N by *Afl*II/*Sac*II digestions and an oligonucleotide linker (encoding Gly-Ser-Ser-Gly) was inserted instead. To construct the expression vector for GFP-IP₃R1-ES (see Figure 1A), part of cDNA encoding amino acids 1–2216 was excised from GFP-IP₃R1-N by *Afl*II/*Eco*RI digestions, and an oligonucleotide linker (encoding Glu-Phe) was inserted instead. The expression vector for GFP-IP₃R1-casp (see Figure 1A) was constructed by adding the cDNA sequence encoding amino acids between Arg¹⁸⁹² and Glu²²¹⁶, amplified by PCR with primers 5'-GGGAATTCAGGGATGCCCCGTCTCGGAAG-3' (the underlined region indicates an *Eco*RI site) and 5'-CGAAGTTTCGATTCCTTAGTC-3' and digested with *Eco*RI, to the *Eco*RI site of GFP-IP₃R1-ES.

Cell culture and DNA transfection

COS-7 and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1.0 g/l glucose, and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO₂. For the transfection experiments, cells were grown on glass coverslips (18 mm diameter, coated with 50 µg/ml poly-L-lysine) or on glass-bottom microwell dishes (35 mm, uncoated) and transfected by using LIPOFECTAMINETM 2000 reagent (Invitrogen), according to the manufacturer's instructions.

IP₃R triple-knockout (IP₃R-TKO) DT40 chicken B-lymphoma cells [10] were cultured in RPMI 1640 supplemented with 10 % (v/v) foetal calf serum, 1 % (v/v) chicken serum, penicillin-streptomycin, 50 µM 2-mercaptoethanol and 2 mM L-glutamine. The cells were maintained at 39.5 °C in a humidified atmosphere containing 5 % CO₂. To establish IP₃R-TKO cells that stably express GFP-IP₃R1-N, the plasmid of GFP-IP₃R1-N (zeosin-resistant cassette) was replaced with neomycin-resistant cassette) was linearized and transfected by electroporation with Gene Pulser apparatus (Bio-Rad, Hercules, CA, U.S.A.) at 570 V and 25 µF, and cells were selected in the presence of 2 mg/ml G418. Several clones derived from a single colony were obtained, and expression was confirmed by immunoblotting analysis (results not shown). IP₃R-TKO cells that stably express wild-type (untagged) IP₃R1 have been described in [15].

Immunocytochemistry

Cells on coverslips were fixed with formalin for 5 min at room temperature (25 °C), by directly adding concentrated solution to the culture medium to a final concentration of 4 %. After washing twice with PBS, samples were permeabilized with 0.2 % (v/v) Triton X-100 in PBS at room temperature for 5 min, washed again twice with PBS, and incubated with PBS containing 2 % (v/v) goat serum and 0.2 % (v/v) Triton X-100 at room temperature for 1 h or at 4 °C overnight. The cells were then incubated with anti-CRT (1:800) in PBS containing 2 % (v/v) goat serum and 0.2 % (v/v) Triton X-100 for 2 h at room temperature. After washing the cells with PBS, they were incubated with Alexa-594-conjugated

anti-rabbit IgG for 1 h at 4 °C. After washing three times with PBS, the coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.).

Ca²⁺ imaging

After 1 or 2 days following transfection, HeLa cells were incubated for 45 min with 5 μ M fura-2 acetoxymethyl ester (Molecular Probes). The fura-2-loaded cells were placed on the stage of an inverted fluorescence microscope (IX-70; Olympus, Japan) and perfused with balanced salt solution (BSS) [115 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and 20 mM Hepes (pH 7.4)], at a rate of 2.0 ml/min. Image capture and processing were performed with Argus 50/CA system (Hamamatsu Photonics, Japan) at room temperature. With alternate illumination at 340 nm and 380 nm excitation, pairs of fluorescence images (F340 and F380 respectively) were obtained every 5 s through an objective lens (UApo 20 $\times$ /340 or UplanApo 100 $\times$ Oil Iris; Olympus) and an emission filter (510–550 nm), captured with a silicon-intensified-target video camera (C2400–8; Hamamatsu Photonics), and digitized with an image processor. To identify GFP–IP₃R1-expressing cells, fluorescence images of GFP (emission 510–550 nm) with excitation at 470–490 nm were separately acquired and saved in the computer in the same optical fields as those for calcium imaging.

Measuring the Ca²⁺-releasing activity of GFP-tagged IP₃R1 using microsomal fractions and fura-2

The crude microsomal fraction was prepared from DT40 cells stably expressing GFP–IP₃R1-N. The IP₃-induced Ca²⁺ release (IICR) activity of this microsomal fraction was measured using fura-2 as described previously [16].

RESULTS

GFP-tagged IP₃R1 constructs

The GFP-tagged IP₃R1 constructs used in this study are summarized in Figure 1(A). Enhanced GFP was fused either at the N-terminus (GFP–IP₃R1-N) or the C-terminus (GFP–IP₃R1-C) of full-length mouse IP₃R1. GFP–IP₃R1-D610 (lacking 610 N-terminal amino acids) was used to investigate the contribution of the ligand-binding domain [3,17]. GFP–IP₃R1-casp was constructed in order to determine the characteristics of the truncated form of IP₃R1 that is generated by specific cleavage by caspase 3 [7,8]. GFP–IP₃R1-ES, which lacks almost the entire N-terminal cytoplasmic domain [18], was used to investigate the contribution of the regulatory domain as well as the ligand-binding domain.

HeLa cells were transiently transfected with each of these expression plasmids. Whole-cell lysates were prepared, and immunoblotting was performed. As shown in Figure 1(B), anti-IP₃R1 monoclonal antibody 18A10, which specifically recognizes the C-terminal domain of IP₃R1, detected all the GFP-tagged IP₃R1 proteins in addition to the endogenous IP₃R1. Anti-GFP antibody detected all the GFP-tagged IP₃R1 proteins (Figure 1C). No major degradation products of GFP–IP₃R1 proteins were detected, although some high-molecular-mass smearing bands were observed, especially in lysates from cells that expressed GFP–IP₃R1-casp or GFP–IP₃R1-ES (see below). Importantly, we consistently observed that GFP–IP₃R1-C was much less expressed than GFP–IP₃R1-N (Figures 1B and 1C, lanes 2 and 3). This was not a cell-type-specific event, since the same results were obtained when COS-7, 293T and DT40 (IP₃R1-TKO) cells were used (results not shown).

Previous studies demonstrated that the channel domain alone is sufficient for IP₃R1 to localize to the ER [18–20]. To confirm that the GFP-tagged IP₃R1 proteins used in this study were targeted to the intracellular Ca²⁺ stores, HeLa or COS-7 cells were transfected with each of the expression plasmids and their subcellular localization was examined with a confocal fluorescence microscope. The Ca²⁺ stores were visualized simultaneously by staining with anti-CRT antibody. GFP–IP₃R1-N (Figures 1D–1I) and all of the other GFP-tagged IP₃R1 proteins used in this study (results not shown) co-localized very well with CRT in most of the cells. However, occasionally, the GFP-tagged IP₃R1 proteins expressed formed large aggregates that did not co-localize with CRT (Figure 1J). These aggregates were observed more frequently in cells that expressed GFP–IP₃R1-casp or GFP–IP₃R1-ES than in those that expressed GFP–IP₃R1-N, -C, or -D610, and we assume that the high-molecular-mass bands seen in lanes 5 and 6 of Figures 1(B) and 1(C) are derived from these aggregates. No cells containing such aggregates were used in the subsequent Ca²⁺-imaging experiments.

Expression of GFP–IP₃R1-casp or GFP–IP₃R1-ES suppressed both IP₃-induced Ca²⁺ release and Ca²⁺ leak

We performed single-cell Ca²⁺ imaging of HeLa cells that expressed GFP-tagged IP₃R1 proteins. HeLa cells express purinergic receptors that are coupled with phospholipase C, and thus ATP stimulation causes IICR in the cells [21]. As shown in Figure 2(A), expression of GFP–IP₃R1-N had little effect on the signalling pattern of [Ca²⁺]_i evoked by 100 μ M ATP, a concentration that elicits a full response in most of the cells. Thapsigargin (TG)-induced Ca²⁺ leak in the GFP–IP₃R1-N-expressing cells was also roughly the same as in the control cells (Figure 2A). As GFP–IP₃R1-N has essentially the same channel activity as the endogenous IP₃R1 (see Figure 5), exogenous expression of this construct will increase the total number of functional IP₃Rs. Nonetheless, the result in Figure 2(A) indicates that the increase has little effect on the dynamics of [Ca²⁺]_i that is evoked by supra-threshold agonist concentrations and on the total amount of releasable Ca²⁺ content of the internal store.

When stimulated with 100 μ M ATP or 0.3 μ M TG, HeLa cells expressing GFP–IP₃R1-D610 exhibited [Ca²⁺]_i signalling patterns that were indistinguishable from those of non-transfected cells (Figure 2B). These findings indicated that GFP–IP₃R1-D610, which does not bind IP₃ and thus should not have IICR activity, does not have inhibitory effect on the dynamics of [Ca²⁺]_i (shaped by the endogenous IP₃Rs) under the present conditions. Expression of GFP–IP₃R1-casp, on the other hand, suppressed the ATP- and TG-induced increase in [Ca²⁺]_i (Figure 2C). The peak amplitudes of the responses evoked by ATP and TG in cells that moderately expressed GFP–IP₃R1-casp (Figure 2C, arrowheads) were typically half those of the control cells (Figure 2C, bold line). In cells that abundantly expressed GFP–IP₃R1-casp (Figure 2C, arrows), neither ATP nor TG induced an increase in [Ca²⁺]_i (Figure 2C, broken line). Similar results were obtained for GFP–IP₃R1-ES (Figure 2D). The results are summarized in Figure 2(E). In summary, expression of GFP–IP₃R1-casp or GFP–IP₃R1-ES decreased the amplitude of [Ca²⁺]_i elevation that was elicited by ATP or TG. Essentially the same phenomenon was observed in COS-7 cells as well as in HeLa cells (results not shown).

Low Ca²⁺ content of the internal Ca²⁺ store of cells expressing GFP–IP₃R1-casp or GFP–IP₃R1-ES

There were two possible explanations for the results shown in Figures 2(C) and 2(D). One was that GFP–IP₃R1-casp and

Figure 1 Schematic representation and expression of GFP-tagged IP₃R1 constructs

(A) Molecular domain structure of GFP-tagged IP₃R1 constructs. LBD, ligand-binding domain. The epitope recognized by anti-IP₃R1 monoclonal antibody 18A10 is indicated at the top. (B) and (C) Western blot analysis of whole cell extracts of HeLa cells transfected with a control vector (lane 1) and the expression plasmids of GFP-IP₃R1-N (lane 2), GFP-IP₃R1-C (lane 3), GFP-IP₃R1-D610 (lane 4), GFP-IP₃R1-casp (lane 5) or GFP-IP₃R1-ES (lane 6). Proteins were separated by SDS/PAGE (5% gels) and transferred on to a PVDF membrane. The antibodies used are stated at the bottom of each panel. The asterisk indicates the position of endogenous IP₃R1. (D)–(I) GFP-IP₃R1-N co-localized with CRT. HeLa cells (D)–(F) and COS-7 cells (G)–(I) were transfected with the expression plasmid for GFP-IP₃R1-N. Cells were fixed and stained with anti-CRT (E) and (H). Merged images (F)–(I) show co-localization of GFP-IP₃R1-N with CRT. (J) An example of a HeLa cell in which GFP-IP₃R1-ES formed aggregates and did not exhibit reticular pattern. Scale bars in (D)–(J), 10 μ m.

GFP-IP₃R1-ES inhibited both IICR and all the Ca²⁺-leak mechanisms. The other was that the Ca²⁺ stores of the cells expressing GFP-IP₃R1-casp or GFP-IP₃R1-ES were already empty. We therefore estimated the Ca²⁺ content of the Ca²⁺ stores of cells expressing GFP-IP₃R1 constructs by measuring TG-induced Ca²⁺ leak in the absence of extracellular Ca²⁺. Its subsequent Ca²⁺ entry [store-operated (-like) Ca²⁺ entry] was also measured as an indication of store emptiness. Expression of GFP-IP₃R1-N or GFP-IP₃R1-D610 had little effect on TG-induced Ca²⁺ leak (Figures 3A and 3B). On the other hand, the TG-induced increase in [Ca²⁺]_i was quite small in cells that expressed high amounts of GFP-IP₃R1-casp (Figure 3C) or GFP-IP₃R1-ES (Figure 3D). The peak amplitude, on average, was $36.0 \pm 21.4\%$ and $17.1 \pm 12.0\%$ in cells that expressed GFP-IP₃R1-casp and GFP-IP₃R1-ES, respectively, compared with cells that did not express these proteins in the same dish (Figure 3E). On the

other hand, Ca²⁺ influx was clearly observed in the cells expressing GFP-IP₃R1-casp or GFP-IP₃R1-ES (Figures 3C and 3D respectively), although its initial rates were smaller than in the control cells (Figure 3F). Altogether, these results strongly suggested that the expression of GFP-IP₃R1-casp or GFP-IP₃R1-ES makes the Ca²⁺ content within the store considerably low. If this were true, then TG pre-treatment would be unnecessary to observe Ca²⁺ entry in cells expressing GFP-IP₃R1-casp or GFP-IP₃R1-ES. We therefore simply changed the extracellular solution (BSS) containing 2 mM Ca²⁺ to BSS without (nominally free of) Ca²⁺, and then switched back to BSS containing 2 mM Ca²⁺ 12 min later. [Ca²⁺]_i was stable throughout this procedure in control cells and cells expressing GFP-IP₃R1-N or GFP-IP₃R1-D610 as shown in Figures 4(A) and 4(B) respectively. In most cells that expressed GFP-IP₃R1-casp or GFP-IP₃R1-ES, however, [Ca²⁺]_i decreased slightly

Figure 2 Responses to ATP and TG by HeLa cells expressing various GFP-tagged IP₃R1 proteins

HeLa cells that express GFP-IP₃R1-N (**A**), GFP-IP₃R1-D610 (**B**), GFP-IP₃R1-casp (**C**) or GFP-IP₃R1-ES (**D**) were stimulated with 100 μ M ATP and then with 0.3 μ M TG. GFP (left-hand panel) and fura-2 (right-hand panel) images are shown at the top of each panel. In (**A**) and (**B**), the thin and thick lines represent the responses of cells that expressed no exogenous proteins and GFP-tagged IP₃R1 proteins respectively. The responses to ATP and TG of cells that expressed GFP-IP₃R1-N and GFP-IP₃R1-D610 were not significantly different from those of control cells. In (**C**) and (**D**), the thin, thick and broken lines represent the responses of cells that expressed no exogenous proteins, low levels of GFP-tagged IP₃R1 proteins and high levels of GFP-tagged IP₃R1 proteins respectively. The 'low-expression' and 'high-expression' cells in the GFP images are indicated by arrowheads and arrows respectively. The asterisks indicate cells that have mislocalized or aggregated GFP-tagged IP₃R1 proteins. Representative traces from at least four independent experiments are shown. (**E**) Summary of the results. 'No response' and 'weak response' mean that the peak amplitudes evoked by both ATP and TG were less than 10% and 50%, respectively, of the normal response. Numbers are sums of GFP-positive cells from at least four independent experiments and the bars indicate the percentages of cells that gave a particular response.

when the extracellular solution was switched to Ca²⁺-free buffer, and a sharp Ca²⁺ influx was observed upon switching back to BSS containing 2 mM Ca²⁺ (Figures 4C and 4D). This [Ca²⁺]_i pattern was observed in 65.6 $\pm$ 8.3% and 62.1 $\pm$

9.8% of cells expressing GFP-IP₃R1-casp and GFP-IP₃R1-ES respectively ($n = 5$ and 4 respectively), but only 4.6 $\pm$ 3.0% and 5.5 $\pm$ 2.2%, respectively, of the GFP-negative cells in the same dishes showed this pattern. These findings indicated that

Figure 3 Responses to TG and subsequent Ca²⁺ entry of HeLa cells expressing various GFP-tagged IP₃R1 proteins

HeLa cells that express GFP-IP₃R1-N (A), GFP-IP₃R1-D610 (B), GFP-IP₃R1-casp (C) or GFP-IP₃R1-ES (D) were treated with 0.3 μM TG in nominally Ca²⁺-free BSS for 10 min. The medium was then changed to BSS containing 2 mM Ca²⁺ without TG. The thin and thick lines represent the mean ($n=12-14$) responses of cells in the same dish that expressed no exogenous proteins and GFP-tagged IP₃R1 proteins respectively. The data are representatives of at least three independent experiments. (E) Means of peak amplitude induced by TG in cell expressing GFP-IP₃R1 proteins. N, GFP-IP₃R1-N; D610, GFP-IP₃R1-D610; casp, GFP-IP₃R1-casp; ES, GFP-IP₃R1-ES. Results are normalized peak amplitudes (compared with GFP-negative cells in the same optical field) $\pm$ S.D. (F) Initial rates of Ca²⁺ influx. Results are normalized means (compared with GFP-negative cells in the same optical field) $\pm$ S.D. In (E) and (F), at least 50 traces from at least three independent preparations for each construct were used for analysis.

GFP-IP₃R1-casp and GFP-IP₃R1-ES continuously leaked Ca²⁺ from the intracellular Ca²⁺ stores and thus that store-operated Ca²⁺ entry was constitutively active.

GFP-IP₃R1-N has Ca²⁺-releasing activity comparable with that of wild-type IP₃R1

To confirm that GFP tagging to the N-terminus of IP₃R1 had no effect on channel activity, we established a stable cell line that expressed either wild-type IP₃R1 [15] or GFP-IP₃R1-N (Figure 5A, inset). DT40 chicken B-lymphoma cells from which all the IP₃R genes had been genetically removed (IP₃R-TKO cells) [10] were chosen as the parent cells to exclude any contribution of endogenous IP₃R. Microsome fractions were prepared from the stable cell lines and their IICR activity was measured (Figure 5A). Microsomes from IP₃R-TKO cells released no measurable Ca²⁺ in response to IP₃ up to 5 μM (Figure 5B, thin line). In contrast, microsomes from IP₃R-TKO cells that stably expressed GFP-IP₃R1-N manifested IICR activity (Figure 5B, thick line), and the dose-response curve was very similar to that of microsomes from IP₃R-TKO cells expressing untagged IP₃R1 (Figure 5B, broken line). The response was comparable with that of native mouse

Figure 4 Effect of changing the extracellular Ca²⁺ concentration on [Ca²⁺]_i of HeLa cells expressing various GFP-tagged IP₃R1 proteins

The medium of HeLa cells that expressed GFP-IP₃R1-N (A), GFP-IP₃R1-D610 (B), GFP-IP₃R1-casp (C) or GFP-IP₃R1-ES (D) was exchanged from BSS containing 2 mM Ca²⁺ to nominally Ca²⁺-free BSS. After 12 min, the medium was changed back to BSS containing 2 mM Ca²⁺. The thin and thick lines represent the mean ($n=10-14$) responses of cells in the same dish that expressed no exogenous proteins and GFP-tagged IP₃R1 proteins respectively. The data are representative of at least three independent experiments.

IP₃R1 from mouse cerebellum [16], indicating that tagging with GFP at the N-terminus had little effect on IICR activity of IP₃R1. We also verified that GFP-IP₃R1-C possessed IICR activity by using microsomes from IP₃R-TKO cells that transiently expressed GFP-IP₃R1-C, although its expression was too low to perform a dose-response analysis (results not shown).

DISCUSSION

Effect of tagging IP₃R1 with GFP

In the present study, we utilized GFP tagging of various IP₃R1 constructs to identify cells that transiently expressed these proteins and performed single-cell Ca²⁺ imaging. This method also made it possible to ignore cells in which exogenous protein clearly mislocalized (Figure 1J). Tagging with GFP at the N-terminus of IP₃R1 (GFP-IP₃R1-N) has virtually no effect on localization (Figure 1) or the channel property (Figure 5). Thus expressing GFP-IP₃R1-N will increase the total number of functional IP₃Rs. However, this manipulation in HeLa cells had little effect on cellular Ca²⁺ regulation under the conditions used in this study (Figures 2-4). In preliminary experiments, however, we found that expressing GFP-IP₃R1-N affected the [Ca²⁺]_i pattern in both HeLa and COS-7 cells elicited by lower concentrations of ATP than used in the present study (results not shown). Stable over-expression of IP₃R has been reported to have a rather complex effect on [Ca²⁺]_i signalling patterns, depending on the cells and

Figure 5 IP_3 -induced Ca^{2+} release activity of GFP- $\text{IP}_3\text{R1-N}$ is comparable with that of wild-type $\text{IP}_3\text{R1}$

(A) Representative measurement of the extramicrosomal F340/F380 ratio by monitoring fura-2 fluorescence. Concentration of solution added: ATP, 2 mM; IP_3 , 5 μM ; BrA23187, 2 μM ; CaCl_2 , 2 μM ; EGTA, 10 mM. Inset, confocal GFP image of $\text{IP}_3\text{R-TKO}$ cells stably expressing GFP- $\text{IP}_3\text{R1-N}$. (B) Dose-response relationship between IP_3 concentration and Ca^{2+} release activity. Various concentrations of IP_3 were added to the microsomes and the Ca^{2+} released was measured. Values are shown as percentages of the Ca^{2+} concentration induced by BrA23187. Microsomes from the parent $\text{IP}_3\text{R-TKO}$ cells did not show any Ca^{2+} release (closed squares, thin line). Microsomes from $\text{IP}_3\text{R-TKO}$ cells stably expressing GFP- $\text{IP}_3\text{R1-N}$ (open circles, thick line) and wild-type $\text{IP}_3\text{R1}$ (open squares, broken line) showed very similar patterns of Ca^{2+} release.

systems used [22–25]. To the best of our knowledge, detailed analysis of transient overexpression of IP_3R on $[\text{Ca}^{2+}]_i$ signalling patterns has never been reported. Thus the next challenge would be to analyse the relationship between the amount of IP_3R and cellular Ca^{2+} regulations by utilizing GFP-tagged IP_3R proteins.

$\text{IP}_3\text{R1}$ tagged with GFP at its C-terminus (GFP- $\text{IP}_3\text{R1-C}$) was expressed less than GFP- $\text{IP}_3\text{R1-N}$ in all of the cell types tested, and IICR activity of microsomes from GFP- $\text{IP}_3\text{R1-C}$ -expressing $\text{IP}_3\text{R-TKO}$ cells was barely detectable (results not shown). We showed recently that small mutations in the C-terminal cytosolic domain completely abolished the IICR activity of $\text{IP}_3\text{R1}$ [15], and it is interesting to note that tagging the RyR with GFP at its C-terminus has been found to abolish its sensitivity to caffeine [26]. Since RyRs and IP_3Rs share a high degree of similarity in this region, tagging at the C-terminus presumably has a malignant effect on the overall structure of $\text{IP}_3\text{R1}$, leading to low expression and activity. Accordingly, GFP- $\text{IP}_3\text{R1-N}$, but not GFP- $\text{IP}_3\text{R1-C}$, would be needed to investigate the effects of $\text{IP}_3\text{R1}$ overexpression or to monitor the intracellular dynamics of $\text{IP}_3\text{R1}$.

Expression of GFP- $\text{IP}_3\text{R1-casp}$ or GFP- $\text{IP}_3\text{R1-ES}$ leads to a decrease in releasable Ca^{2+} within the ER

The results in Figures 2–4 clearly show that the amount of releasable Ca^{2+} in the ER is in inverse proportion to the expression level of GFP- $\text{IP}_3\text{R1-casp}$ or GFP- $\text{IP}_3\text{R1-ES}$. This can be explained either by inhibition of Ca^{2+} uptake into the ER or by continuous Ca^{2+} leak from the ER. We favour the latter explanation for two reasons. First, interaction between IP_3R and sarcoendoplasmic reticulum Ca^{2+} ATPase, the major Ca^{2+} pump of the ER, has never been documented. In fact, we were unable to detect any association between GFP- $\text{IP}_3\text{R1-casp}$ or GFP- $\text{IP}_3\text{R1-ES}$ and sarcoendoplasmic reticulum Ca^{2+} ATPase 2B in HeLa or COS-7 cells (T. Nakayama, M. Hattori, K. Uchida, Y. Tateishi, M. Iwai, T. Michikawa, T. Inoue and K. Mikoshiba, unpublished work), making it unlikely that these proteins inhibit Ca^{2+} uptake. Secondly, Ramos-Franco et al. [27] showed that an $\text{IP}_3\text{R1}$ mutant lacking the first four transmembrane domains constitutes an IP_3 -

independent, constitutively open channel. It is conceivable that the regulatory domain, which is adjacent to the first transmembrane domains, is also involved in keeping the channel closed.

Another interesting point is that Ca^{2+} entry after TG pretreatment was smaller in cells expressing GFP- $\text{IP}_3\text{R1-casp}$ or GFP- $\text{IP}_3\text{R1-ES}$ than in the control cells (Figure 3F). This may be due to 'rundown' of store-operated Ca^{2+} channels, since the intracellular Ca^{2+} store is already almost empty, and thus the store-operated Ca^{2+} channels have been open for long time. Alternatively, some unknown mechanism that conveys the store-depletion information to store-operated Ca^{2+} channels may be down-regulated (or adapted). In any case, using GFP- $\text{IP}_3\text{R1-casp}$ or GFP- $\text{IP}_3\text{R1-ES}$ to prolong depletion may help identify the molecular mechanism of the regulation of store-operated Ca^{2+} channels.

How does the regulatory domain keep the channel domain closed? The three-dimensional structure of $\text{IP}_3\text{R1}$ revealed that this domain undergoes a large conformational change by binding to Ca^{2+} [6]. Although direct interaction between the regulatory domain and the channel domain is unlikely [28,29], this conformational change may influence the overall structure of the channel domain. Finally, it should be mentioned that, although most of the $\text{IP}_3\text{R1}$ protein remains assembled and functional after mild trypsin digestion [16], caspase 3 cleavage leads to loss of its function [7]. In fact, we found that, after treatment of cerebellar microsomes with caspase 3 (as in [7]), a significant amount (35–65%) of the cytosolic fragment of $\text{IP}_3\text{R1}$ was released from the membrane fraction (T. Nakayama, M. Hattori, K. Uchida, Y. Tateishi, M. Iwai, T. Michikawa, T. Inoue and K. Mikoshiba, unpublished work). Therefore it is likely that cleavage by caspase 3 of $\text{IP}_3\text{R1}$ indeed makes the leaky, channel-domain-only form $\text{IP}_3\text{R1}$ *in vivo*.

What happens in cells that express constitutively leaky IP_3R proteins?

The results in Figure 4 can be explained by Figure 6, although this is by no means the only possible explanation. In control cells (and cells that express GFP- $\text{IP}_3\text{R1-N}$ or GFP- $\text{IP}_3\text{R1-D610}$;

Figure 6 Schematic representation of Ca^{2+} dynamics expressing leaky IP₃R1

(A) Control situation. The Ca^{2+} uptake is predominant over the Ca^{2+} leak and Ca^{2+} flow across the plasma membrane is balanced. (B) When leaky IP₃R1 is present in the presence of Ca^{2+} , the Ca^{2+} concentration within the ER decreases and thus store-operated Ca^{2+} influx is activated. Meanwhile, a certain mechanism that pumps Ca^{2+} out of the plasma membrane is activated to maintain $[\text{Ca}^{2+}]$ at basal level. (C) When leaky IP₃R1 is present and extracellular Ca^{2+} is removed, the Ca^{2+} entry is terminated and $[\text{Ca}^{2+}]$ decreases. See the text for more detail.

Figure 6A), Ca^{2+} uptake into the ER predominates over the Ca^{2+} leak from the ER, and, as a result, the Ca^{2+} concentration within the ER is high and $[\text{Ca}^{2+}]$ is at its basal level. Under these conditions, Ca^{2+} influx and efflux across the plasma membrane are well balanced. When constitutively open (or leaky) IP₃R proteins are expressed in the presence of extracellular Ca^{2+} , the Ca^{2+} concentration within the ER would be decreased and store-operated Ca^{2+} entry would be activated. Since both Ca^{2+} efflux from the stores and Ca^{2+} entry serve to increase $[\text{Ca}^{2+}]$, some mechanism would be activated to maintain $[\text{Ca}^{2+}]$ at its basal level (Figure 6B). The mechanisms may possibly include up-regulation of the function of plasma membrane ATPases and/or other ion-exchange pumps. When extracellular Ca^{2+} is suddenly removed under these conditions (Figure 6C), the hypothetically up-regulated mechanism that pumps out Ca^{2+} , in addition to the increased function of sarcoendoplasmic reticulum Ca^{2+} ATPase (because of a lower thermodynamic gradient of Ca^{2+} across the ER membrane) would work to decrease $[\text{Ca}^{2+}]$. Then, finally, when the extracellular Ca^{2+} is replenished, $[\text{Ca}^{2+}]$ would rise sharply, mainly by means of the store-operated Ca^{2+} channels. These models are, admittedly, speculative and further experiments will be necessary to solve all the pathways.

Possible involvement of IP₃R1 truncation in elevated $[\text{Ca}^{2+}]$ in apoptotic cells

Caspase 3 is considered to be the main effector caspase [30], and substrates of caspase 3 include several Ca^{2+} -related proteins, such as plasma membrane Ca^{2+} ATPase 4b [31] and Ca^{2+} /calmodulin-dependent protein kinase IV [32]. Our results suggest strongly that specific cleavage of IP₃R1 by caspase 3 leads both to Ca^{2+} leak from the internal stores and to continuous Ca^{2+} entry (probably via store-operated Ca^{2+} channels). Several studies have reported slow and gradual Ca^{2+} release following apoptotic stimuli, and, recently, Tombal et al. [33] continuously measured $[\text{Ca}^{2+}]$ for 120 h following various apoptotic stimuli and found that the stimuli induced a delayed $[\text{Ca}^{2+}]$ rise that was required for the execution phase of apoptosis. It should be mentioned that IP₃R1 alone, not IP₃R2 or IP₃R3, is a substrate of caspases during apoptosis [7]. Thus different repertoires of IP₃R expression may at least partly explain why the same stimuli generate distinct long-term $[\text{Ca}^{2+}]$ patterns in different cell types [1]. Finally, Ghribi

et al. [34] recently demonstrated that the active caspase 3 in hippocampus of a rabbit neurotoxicity model is mainly localized in the ER. Since IP₃R1 is predominantly expressed in the hippocampus [35–37] and dysregulation of $[\text{Ca}^{2+}]$ (especially Ca^{2+} release from the ER) is known to be involved in neuronal cell death [38,39], it would be interesting to see if cleavage of IP₃R1 by caspase 3 plays a role in neurodegenerative diseases.

It is noteworthy that several mutations in the cytoplasmic region of RyR1 found in patients with central core disease (myopathy resulting from Ca^{2+} dysregulation in muscle) make this channel protein leaky [40,41]. Thus one of the main functions of the cytosolic region of both Ca^{2+} -release channel families appears to be keep the channel domain tightly closed in the resting state.

Truncated, GFP-tagged IP₃R as a new tool for emptying the intracellular Ca^{2+} store

Although inhibition of Ca^{2+} release from the internal store is often indispensable to analysing not only $[\text{Ca}^{2+}]$ pattern formation, but also various signal transduction pathways, no ideal method of Ca^{2+} release inhibition or specific inhibitor of IP₃R has been available [3]. TG is frequently used to deplete the internal Ca^{2+} store (thus preventing all the subsequent Ca^{2+} release), but because its effect is acute and practically irreversible [42], its use is inevitably accompanied by a rise in $[\text{Ca}^{2+}]$, which could activate a myriad signalling pathways and makes the contribution of Ca^{2+} release unclear. Expressing the constitutively open (or leaky) IP₃R described in this study provides a new, though not ideal, tool for inhibiting Ca^{2+} release: it decreases releasable Ca^{2+} content within the store without significant rise in $[\text{Ca}^{2+}]$. In combination with other methods, this technique will greatly facilitate dissecting out the molecular mechanisms of $[\text{Ca}^{2+}]$ regulation.

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