Ca²⁺-sensor region of IP₃ receptor controls intracellular Ca²⁺ signaling

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Many important cell functions are controlled by Ca²⁺ release from intracellular stores via the inositol 1,4,5-trisphosphate receptor (IP₃R), which requires both IP₃ and Ca²⁺ for its activity. Due to the Ca²⁺ requirement, the IP₃R and the cytoplasmic Ca²⁺ concentration form a positive feedback loop, which has been assumed to confer regenerativity on the IP₃-induced Ca²⁺ release and to play an important role in the generation of spatiotemporal patterns of Ca²⁺ signals such as Ca²⁺ waves and oscillations. Here we show that glutamate 2100 of rat type 1 IP₃R (IP₃R1) is a key residue for the Ca²⁺ requirement. Substitution of this residue by aspartate (E2100D) results in a 10-fold decrease in the Ca²⁺ sensitivity without other effects on the properties of the IP₃R1. Agonist-induced Ca²⁺ responses are greatly diminished in cells expressing the E2100D mutant IP₃R1, particularly the rate of rise of initial Ca²⁺ spike is markedly reduced and the subsequent Ca²⁺ oscillations are abolished. These results demonstrate that the Ca²⁺ sensitivity of the IP₃R is functionally indispensable for the determination of Ca²⁺ signaling patterns.

Keywords: calcium/calcium signaling/inositol 1,4,5-trisphosphate/IP₃ receptor/point mutation

Introduction

Inositol 1,4,5-trisphosphate (IP₃)-mediated release of Ca²⁺ from intracellular stores regulates various cell functions, including contraction, secretion, immunity, development, differentiation and synaptic plasticity. It is fascinating that a molecule as simple as Ca²⁺ can regulate such a vast array of important cell functions. The versatility of Ca²⁺ signals has been attributed to its ability to assume huge variations in spatiotemporal patterns of the intracellular Ca²⁺ concentration changes (Berridge *et al.*, 2000). Thus, Ca²⁺ signals may take the form of Ca²⁺ waves so that the Ca²⁺ signals spread from one point to another within a cell, making a time difference in the Ca²⁺ signals at different

regions of the cell (Kasai and Augustine, 1990; Kasai and Petersen, 1994). Ca²⁺ signals may also take the form of oscillations, i.e. periodic spiking of the intracellular Ca²⁺ concentration. Indeed, certain molecular mechanisms, such as those involving transcription factors (Dolmetsch *et al.*, 1998; Li *et al.*, 1998) and a protein kinase (De Koninck and Schulman, 1998), have been shown to depend on the characteristic frequency of Ca²⁺ oscillations, thus enabling differential activation of Ca²⁺-dependent reactions. Therefore, it has been a challenge to determine how these spatiotemporal patterns of Ca²⁺ signals are generated.

Binding of IP₃ to the IP₃ receptor (IP₃R) is necessary but not sufficient to open the Ca²⁺ release channel, and Ca²⁺ is required as a coagonist of the IP₃R (Iino, 1990; Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991). Thus, IP₃-induced Ca²⁺ release is under the feedback control of Ca²⁺ concentration. Although the molecular mechanism of the Ca²⁺ sensitivity of IP₃R has not been clarified, the feedback mechanism is thought to be important for the generation of Ca²⁺ spikes and Ca²⁺ waves (Iino and Endo, 1992; Lechleiter and Clapham, 1992; Iino *et al.*, 1993; Bootman *et al.*, 1997; Horne and Meyer, 1997). It is also recognized that the dual-agonist requirement of the IP₃R confers on this molecule the role of a coincidence detector, i.e. IP₃Rs can detect the concomitant increase in Ca²⁺ and IP₃ concentrations (Berridge, 1993). Indeed, it has been suggested that the coincidence of two synaptic inputs may be required to activate the IP₃R in hippocampal neurons and in Purkinje cells in the cerebellum, and the resulting Ca²⁺ signal has been implicated in the synaptic plasticity in these neurons (Nakamura *et al.*, 1999; Wang *et al.*, 2000).

Although functional roles of the Ca²⁺-mediated activation mechanism of the IP₃R have been postulated in many cellular Ca²⁺ signaling events as mentioned above, other feedback regulation mechanisms of IP₃-induced Ca²⁺ release have also been implicated in the generation of spatiotemporal patterns of Ca²⁺ signaling (Meyer and Stryer, 1991). Our recent results suggest that IP₃ production catalyzed by phospholipase C is also under the feedback control of cytoplasmic Ca²⁺ concentration (Hirose *et al.*, 1999). Therefore, it has been difficult to determine unequivocally the physiological significance of the Ca²⁺ requirement of IP₃R for Ca²⁺ signaling. In this report we show that a point mutation of the IP₃R1 at glutamate residue 2100 results in a 10-fold decrease in the Ca²⁺ sensitivity of the IP₃R without changes in other properties of the Ca²⁺ release channel, including the IP₃ sensitivity. Therefore, the coagonist effect of Ca²⁺ is greatly diminished in the mutant IP₃R1. Furthermore, we show that the B-cell receptor-mediated Ca²⁺ signaling in B cells expressing the mutant IP₃R1 is greatly affected, with a marked reduction in the rate of increase in Ca²⁺ concentration and a loss of Ca²⁺ oscillations. Thus, we now

present definitive experimental proof that the coagonist action of Ca²⁺ on the IP₃R is essential for IP₃-mediated Ca²⁺ signaling.

Results

Design and expression of IP₃R1 mutants

The ryanodine receptor (RyR) is a family of intracellular Ca²⁺ release channels with a close structural and functional relationship with the IP₃R (Pozzan *et al.*, 1994; Berridge *et al.*, 2000; Iino, 2000). Thus, the two classes of Ca²⁺ release channels have partial amino acid sequence identities, and at the same time both channels are activated by (sub)micromolar Ca²⁺ concentrations, causing Ca²⁺-induced Ca²⁺ release from the Ca²⁺ stores. It has been shown in the rabbit type 3 RyR (RyR3) that glutamate at position 3885 is important for the Ca²⁺ sensitivity of the channel activity, because substitution of the glutamate residue by alanine resulted in a 10 000-fold decrease in the Ca²⁺ sensitivity of the channel activity (Chen *et al.*, 1998). Results that are in general agreement with this work have been obtained for glutamate 4032 in rabbit RyR1 (Du and MacLennan, 1998). Based on the sequence alignment between IP₃R and RyR (Du and MacLennan, 1998), the glutamate residues at positions 4032 and 3885 of the RyR1 and RyR3, respectively, correspond to glutamate at position 2100 (E2100) of the IP₃R1 (Figure 1). Therefore, we reasoned that E2100 may also be important for the Ca²⁺ sensitivity of the IP₃R1, considering the kinship between the two classes of Ca²⁺ release channels. Thus, we generated expression vectors carrying a mutant IP₃R1 cDNA with substitution of E2100 by alanine (E2100A), glutamine (E2100Q) or aspartate (E2100D).

We used plasmid transfection or retrovirus infection systems to introduce stably either wild-type or mutant cDNAs into mutant (IP₃R-null) DT40 cells, in which all three intrinsic IP₃R genes had been disrupted by homologous recombination (Sugawara *et al.*, 1997). Heterologous expression of the IP₃R1 was confirmed by immunocytochemistry (Figure 2A). The levels of IP₃R1 expression in DT40 cells were estimated by flow cytometry of the cells stained with an antibody against the IP₃R1 (Figure 2B), and were correlated with the level of the Ca²⁺ release activities (see below).

Luminal Ca²⁺ concentration monitoring to analyze mutant IP₃R1 functions

A method of continuously monitoring the Ca²⁺ concentration in the lumen of the Ca²⁺ store has been developed in our previous studies and was used for the analysis of the IP₃R functions (Hirose and Iino, 1994; Miyakawa *et al.*, 1999). Figure 3 shows the real-time monitoring record of the luminal Ca²⁺ concentration in permeabilized single cells expressing IP₃R1 with or without a mutation at E2100. The IP₃R-null DT40 cells not heterologously expressing IP₃R1 showed no response to Ca²⁺ loading or to IP₃ application (Figure 3, upper thin black trace). Indeed, the luminal Ca²⁺ concentration was already high prior to Ca²⁺ loading, suggesting that the luminal Ca²⁺ concentration is constantly high, which is most likely to be due to the lack of Ca²⁺ release channels in these mutant cells. It has also been shown that the IP₃R-null DT40 cells do not respond to caffeine, an activator of the RyR, indicating the

		2100	
		→ A, Q, D	
IP ₃ R1	KRMDLVLELKNNASKLLLAIME	SRHDS	ENAEIRILYNMRPK 2079-2118
IP ₃ R2	YRMDLVQLKNNASKLLLAIME	SRHDS	ENAEIRILFNMRPK 2032-2071
IP ₃ R3	YRMDLVQLKDNASKLLLAIME	SRHDS	ENAEIRILISLRPQ 1983-2022
RyR1	ELLKELLDLQKDMVVM	LLSLLEG	NVNGMIARQMVMDLVE 4011-4050
RyR2	ELLKELMDLQKDMVVM	LLSMLLEG	NVNGTIGKQMVMDLVE 3967-4006
RyR3	ELLKQLLDLQKDMVVM	LLSLLEG	NVNGTIGKQMVDTLVE 3864-3903

Fig. 1. Alignment of the putative cytosolic Ca²⁺-sensor region of the rabbit ryanodine receptor (RyR) and the rat inositol 1,4,5-trisphosphate receptor (IP₃R) subtypes. Amino acid residue numbers are shown on the right hand side.

Fig. 2. Heterologous expression of wild-type and mutant IP₃R1 in DT40 B cells. (A) Immunolocalization of stably expressed IP₃R (pMXΔ vector) in DT40 B cells. IP₃R-null mutant cells and cells expressing the wild-type, E2100D, E2100Q or E2100A IP₃R1 were incubated with the rabbit anti-rat IP₃R1 polyclonal antibody and then with the secondary Alexa Fluor 488-labeled anti-rabbit IgG antibody. The fluorescence images were acquired with a confocal microscope. The central black circle is occupied by a nucleus. Scale bar, 5 μm. (B) Flow cytometry of immunostained DT40 cells. Cell counts of IP₃R-null cells and cells expressing the wild-type IP₃R1 (pcDNA3 vector) are plotted against the secondary antibody fluorescence intensity. (C) Six independent clones expressing the wild-type IP₃R1 (pcDNA3 vector) and IP₃R-null mutant cells were immunostained with the anti-IP₃R1 antibody and subjected to flow cytometry. The same clones were subjected to luminal Ca²⁺ measurement, and the rate of IP₃-induced Ca²⁺ release was measured at 10 μM IP₃ and 0.3 μM Ca²⁺ (filled circles). For comparison, results obtained from the cells infected with the wild-type and mutant pMXΔ-IP₃R1-IRES-GFP are also shown (open symbols). Symbols for E2100A and E2100Q are overlapping. Mean ± SEM, n = 4.

absence of RyR expression (Miyakawa *et al.*, 1999). In the cell heterologously expressing the wild-type IP₃R1, the luminal Ca²⁺ concentration increased during Ca²⁺ loading, and upon application of 10 μM IP₃ and 0.3 μM Ca²⁺, a rapid decline in luminal Ca²⁺ concentration due to the Ca²⁺ release via the IP₃R1 was observed (Figure 3, thick black trace).

We obtained several clones of DT40 cells stably expressing IP₃R1 after transfection with plasmids carrying the IP₃R1 cDNA. We measured the rate of Ca²⁺ release upon application of 10 μM IP₃ in the different clones, and compared them with the level of IP₃R1 expression in each

clone determined by flow cytometry. There was a good correlation between the two parameters (Figure 2C, filled circles). The levels of IP₃R1 expression in retrovirally

Fig. 3. Luminal Ca²⁺ concentration measurement in single DT40 cells. Cells were subjected to Ca²⁺ loading (0.4 μM Ca²⁺ plus 0.5 mM Mg-ATP), washout of Ca²⁺/Mg-ATP and then Ca²⁺ release with 10 or 30 μM IP₃ at 0.3 or 3 μM Ca²⁺. No change in the luminal Ca²⁺ concentration was observed in the IP₃R-null cell or in the cell expressing the E2100A mutant IP₃R1 (thin black traces, 30 μM IP₃, 3 μM Ca²⁺). Rapid IP₃-induced Ca²⁺ release was observed in the cell expressing the wild-type IP₃R1 (thick black trace, 10 μM IP₃, 0.3 μM Ca²⁺). The cell expressing the E2100D IP₃R1 showed very slow Ca²⁺ release at 0.3 μM Ca²⁺, but upon increasing the Ca²⁺ concentration to 3 μM an immediate increase in the Ca²⁺ release rate was observed (red trace, 10 μM IP₃).

transduced cells were also determined by flow cytometry. We used cells expressing the wild-type or mutant IP₃R1 at equivalent levels in the following experiments (Figure 2C, open symbols).

The luminal Ca²⁺ concentration was constantly high and no IP₃ (30 μM)-induced Ca²⁺ release was observed in cells expressing the E2100A mutant IP₃R1, as was the case in the IP₃R-null DT40 cells (Figure 3, thin black traces). Neither did we find 30 μM IP₃-induced Ca²⁺ release in cells expressing the E2100Q IP₃R1. However, in about half of cells expressing the E2100Q mutants, the initial Ca²⁺ concentration level was not as high as that in the IP₃R-null mutant cells. In those cells we observed an increase in the luminal Ca²⁺ concentration during the Ca²⁺ loading, albeit smaller in size than that in cells expressing the wild-type IP₃R1 (not shown), which suggests that the E2100Q mutant IP₃R was functional under certain conditions. Analysis of the E2100D IP₃R1 revealed a unique property of this mutant Ca²⁺ release channel. The luminal Ca²⁺ concentration increased during Ca²⁺ loading, as was the case in cells expressing the wild-type IP₃R1. Although the rate of IP₃-induced Ca²⁺ release was extremely low at 10 μM IP₃ and 0.3 μM Ca²⁺, it was dramatically increased when the ambient Ca²⁺ concentration was increased to 3 μM even at the same IP₃ concentration (Figure 3, red trace). Therefore, the E2100D mutant IP₃R1 requires much higher Ca²⁺ concentrations for its channel activity.

Ca²⁺, IP₃ and ATP dependence of the mutant IP₃R1

We measured the rate of 10 μM IP₃-induced Ca²⁺ release at various Ca²⁺ concentrations (Figure 4A). In the wild-type IP₃R1, its Ca²⁺ dependence showed a characteristic biphasic pattern with a peak at 0.3 μM Ca²⁺, as has been reported in previous studies (Iino, 1990; Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991; Miyakawa *et al.*, 1999). With regard to the E2100D mutant IP₃R1, the Ca²⁺ release rate was extremely low at 0.3 μM Ca²⁺ concentration, and the biphasic Ca²⁺ dependence was shifted toward the

Fig. 4. IP₃- and Ca²⁺-concentration dependence of Ca²⁺ release. (A) Ca²⁺-concentration dependence of Ca²⁺ release via the wild-type and E2100D IP₃R1. IP₃ concentration was 10 μM. (B) IP₃-concentration dependence of Ca²⁺ release via the wild-type and E2100D IP₃R1. The Ca²⁺ release rate at 30 μM IP₃ is also plotted for the E2100A and E2100Q IP₃R1. The Ca²⁺ concentration was 0.3 μM for wild-type and 3 μM for mutant IP₃R1. ATP concentration was 1 mM. Mean ± SEM ($n = 4-5$).

higher Ca²⁺ concentration by a factor of ~10 with the peak at ~3 μ M Ca²⁺. These results clearly show that E2100 is critically involved in the Ca²⁺ sensor of the IP₃R1.

In order to study whether or not E2100 is also involved in the IP₃ sensitivity, we compared the IP₃ concentration–Ca²⁺ release rate relationship of both the wild-type and E2100D mutant IP₃R1s at their respective optimal Ca²⁺ concentrations. We found no significant difference between the two relationships (Figure 4B). Thus, the E2100D mutation has no effect on the IP₃ sensitivity of the Ca²⁺ release channel. Furthermore, no major difference was observed between the wild-type and E2100D IP₃R1s with regard to the maximum Ca²⁺ release rates. This suggests that the E2100D mutation does not affect the maximal channel activity. No Ca²⁺ release was observed via the E2100Q or E2100A mutant IP₃R1 even at 30 μ M IP₃ and 3 μ M Ca²⁺.

IP₃R1 requires millimolar ATP for its full activation (Iino, 1991; Bezprozvanny and Ehrlich, 1993; Miyakawa *et al.*, 1999). We studied the IP₃ (10 μ M)-induced Ca²⁺ release rates in the presence and absence of 1 mM ATP. In the wild-type IP₃R1, the Ca²⁺ release rates were 0.110 ± 0.009 s⁻¹ (no ATP) and 0.165 ± 0.016 s⁻¹ (1 mM ATP) at 0.3 μ M Ca²⁺ ($n = 4$). In the E2100D mutant IP₃R1, they were 0.089 ± 0.002 s⁻¹ (no ATP) and 0.145 ± 0.006 s⁻¹ (1 mM ATP) at 3 μ M Ca²⁺ ($n = 4$). Therefore, in both types of IP₃R1s the Ca²⁺ release rate was lower in the absence of ATP than in the presence of ATP to a similar extent (~67% in the wild type and ~62% in E2100D).

Cell surface receptor-induced Ca²⁺ signaling in cells expressing mutant IP₃R1

The above results show that the E2100D mutant IP₃R1 has the same sensitivities to IP₃ and ATP, as well as the same maximum rates of Ca²⁺ release, but the Ca²⁺ sensitivity is reduced by a factor of 10. Therefore, examination of Ca²⁺ signaling in intact cells expressing the mutant IP₃R should provide an insight into the physiological significance of the coagonist role of Ca²⁺ in IP₃R-mediated Ca²⁺ signaling. Thus, we studied Ca²⁺ mobilization upon crosslinking of the B-cell receptor (BCR) in single DT40 cells (Kurosaki, 1999) expressing exogenous IP₃R1 (Figure 5).

Most single DT40 cells (82.9%, $n = 689$ cells) expressing the wild-type IP₃R1 responded with an initial Ca²⁺ spike with or without damped oscillations within a few minutes of BCR activation (1 μ g/ml anti-BCR antibody). This initial response was followed by a quiescent period lasting for 10–20 min. Thereafter, Ca²⁺ oscillations with lower amplitudes than that of the initial Ca²⁺ spike resumed and these continued for 60 min or longer (Figure 5A). This Ca²⁺ signaling pattern is similar to that of single DT40 cells expressing the intrinsic chicken IP₃Rs (Miyakawa *et al.*, 1999). On the other hand, single DT40 cells expressing the E2100D mutant IP₃R1 produced markedly different Ca²⁺ signals. Although there was an initial Ca²⁺ transient in 26.3% of cells ($n = 657$ cells), no Ca²⁺ oscillations followed it (Figure 5B). Both IP₃R-null DT40 cells and cells expressing the E2100A mutant IP₃R1 did not respond to BCR stimulation at all ($n > 600$ cells in each cell type, Figure 5D). Although the majority of cells expressing the E2100Q mutant IP₃R1 did not respond to BCR stimulation, a small population of cells produced a

Fig. 5. Ca²⁺ signaling in single DT40 cells upon BCR stimulation (long time course, 60 min). Ca²⁺ responses of single DT40 cells expressing wild-type (A), E2100D (B), E2100Q (C) and E2100A (D) IP₃R1. The anti-BCR antibody (1 μ g/ml) was applied as indicated by the horizontal bars below the traces. A representative trace of >600 cells in each panel.

Ca²⁺ response (6.4%, $n = 645$ cells). However, the average peak amplitude of the Ca²⁺ response in those cells was extremely low (18.4 ± 1.2 nM, $n = 41$, Figure 5C).

The Ca²⁺ signal patterns of the initial spike also showed a marked difference between single cells expressing the wild-type IP₃R1 and those expressing the E2100D mutant IP₃R1 (Figure 6). In the E2100D mutant single cells as compared with the cells expressing the wild-type IP₃R1, the peak Ca²⁺ response size was lower and the Ca²⁺ response was considerably delayed (Figure 6A and B). In particular, the rate of increase in Ca²⁺ concentration was as much as ~80-fold lower in the E2100D mutant cells (Figure 6A and C). These differences could not be overcome even when the BCR stimulation was increased 10-fold (10 μ g/ml anti-BCR antibody, Figure 6A thick traces, B and C), although the fractional population of the cells showing a Ca²⁺ response was greater (87.3%, $n = 614$ in wild type; 48.7%, $n = 624$ in E2100D) than that at 1 μ g/ml antibody.

Discussion

In this work we have identified the glutamate residue at position 2100 to be a critical amino acid residue for the Ca²⁺ sensor of the rat IP₃R1. Substitution of the glutamate by aspartate resulted in a 10-fold decrease in the Ca²⁺ sensitivity without affecting the IP₃ sensitivity, maximal rate of Ca²⁺ release or ATP sensitivity. Substitution of E2100 by alanine or glutamine seems to decrease further the Ca²⁺ sensitivity (>300-fold) beyond the range of our measurement.

The rat IP₃R1 contains 2749 amino acid residues and its tetramer forms the Ca²⁺ release channel. E2100 is located between the IP₃ binding region near the N-terminus (Mignery and Sudhof, 1990; Miyawaki *et al.*, 1991) and

Fig. 6. Initial Ca^{2+} spike in single DT40 cells upon BCR stimulation. (A) Representative Ca^{2+} responses in cells expressing the wild-type IP_3R (black traces) and E2100D IP_3R (red traces) are shown. The anti-BCR antibody (1 $\mu\text{g/ml}$, thin traces; 10 $\mu\text{g/ml}$, thick traces) was applied as indicated by a horizontal bar below the traces. (B) Peak amplitude (peak Ca^{2+} concentration minus resting Ca^{2+} concentration) of the Ca^{2+} spike in cells expressing wild-type and E2100D IP_3R at two different anti-BCR antibody concentrations as indicated. (C) Maximum rates of Ca^{2+} increase. Mean $\pm$ SEM ($n = 80$ for each category).

the channel region close to the C-terminal end (Michikawa *et al.*, 1994). It is possible that E2100 is located within the Ca^{2+} -binding site that regulates the channel activity, considering that the elimination of the negative charge at this residue results in the virtual loss of Ca^{2+} sensitivity. Although the Ca^{2+} -binding capacity of a series of partial recombinant peptides of the IP_3R has been analyzed using Ca^{2+} overlay methods, E2100 has not been included in the previous studies (Sienaert *et al.*, 1996, 1997). A peptide spanning positions 2124 and 2146 of the IP_3R is located close to E2100 and is reported to bind Ca^{2+} with a dissociation constant of 0.8 μM (Sienaert *et al.*, 1996). We substituted one of the glutamate residues in this region (E2139), which is conserved among the IP_3R subtypes, for alanine, but we did not find any effect on the IP_3 -induced Ca^{2+} release (our unpublished data). Our results, however, do not completely exclude the possibility that E2100 is an allosteric site that changes the Ca^{2+} -binding affinity of a regulatory site.

Cells expressing the E2100D mutant IP_3R provided us with a unique opportunity to study the physiological significance of the coagonist role of Ca^{2+} in IP_3 -mediated

Ca^{2+} signaling. The rate of rise of Ca^{2+} spike was significantly reduced and no subsequent Ca^{2+} oscillations were observed in cells expressing the E2100D mutant IP_3R . The difference could not be overcome even at the 10-fold higher BCR stimulation level. These results clearly show that the Ca^{2+} -mediated feedback control of the channel activity is of critical importance for the generation of agonist-induced Ca^{2+} spikes and for Ca^{2+} oscillations.

In the E2100D mutant IP_3R , not only the activation phase of the biphasic Ca^{2+} dependence of the IP_3 -induced Ca^{2+} release but also the inhibition phase seems to be shifted toward the higher Ca^{2+} concentration (Figure 4). This suggests that E2100 may also be involved in the inhibition phase. Since the high Ca^{2+} -mediated inhibitory effect on the IP_3R should also be important for the time course of agonist-induced Ca^{2+} release (Montero *et al.*, 1997; Mogami *et al.*, 1998; Park *et al.*, 2000), it will be interesting to study further the kinetic properties of the mutant IP_3R s using the bilayer measurement on recombinant proteins.

Ca^{2+} waves and oscillations have been shown to be important for the regulation of cell functions, and multiple feedback mechanisms including the Ca^{2+} requirement of the IP_3R have been postulated to generate a variety of spatiotemporal patterns of Ca^{2+} signals (Berridge *et al.*, 2000). The molecular mechanism of generating spatiotemporal patterns of Ca^{2+} signals can now be addressed from an entirely novel standpoint. Although it has been difficult to observe Ca^{2+} waves in DT40 cells due to their small size ($\sim 10 \mu\text{m}$ in diameter), the role of the Ca^{2+} requirement of the IP_3R in the Ca^{2+} wave formation can be studied in cells with a greater size and expressing the E2100D mutant IP_3R . The role of coincidence detection has been attributed to the IP_3R in the cerebellar Purkinje cells during the induction of long-term depression (Wang *et al.*, 2000). However, alternative mechanisms have been postulated for the coincidence detection (Linden and Connor, 1995; Maeda *et al.*, 1999). This important issue can now be addressed in a direct manner when genetically engineered mice carrying the E2100D mutant IP_3R are generated. Our results, therefore, established the importance of the Ca^{2+} -mediated feedback mechanism in intracellular Ca^{2+} mobilization and will also provide a novel tool to advance our understanding of Ca^{2+} signaling mechanisms.

Materials and methods

Cell culture and generation of DT40 B cells expressing IP_3R

DT40 chicken B lymphoma cells were cultured in RPMI1640 supplemented with 10% fetal calf serum (FCS), 1% chicken serum, penicillin (100 U/ml), streptomycin (100 U/ml) and 2 mM glutamine.

Mutant DT40 cells with all three of their IP_3R genes disrupted (Sugawara *et al.*, 1997) were transfected with the linearized rat IP_3R -pcDNA3 plasmid (Kaznatcheyeva *et al.*, 1998) by electroporation (330 V, 250 μF). Several stably expressing clones were isolated in the presence of 2 mg/ml G418 (Geneticin, Gibco-BRL).

For production of DT40 cells expressing the mutant IP_3R , vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped retrovirus was used. A pantropic packaging cell line, GP293, (Clontech) was cotransfected with 12 μg of pMXA- IP_3R -IRES (internal ribosomal entry site)-green fluorescent protein (GFP) and 1 μg of pVSVG (Clontech) using LipofectAmine reagent (Gibco-BRL). Two days later, retroviral particles were collected from the medium and were used to infect IP_3R -null DT40

cells after concentration by centrifugation (6000 g for 16 h). Infected cells were subjected to cell sorting using FACS Vantage SE (Becton Dickinson) for collecting GFP-expressing cells. The IP₃R1 expression level in the DT40 cells was determined by flow cytometry analysis (see below). The range of variation in expression levels among the sorted cells was similar to that among the cloned cells expressing IP₃R1 after transformation using the IP₃R1-pcDNA3 vector. Thus, we used the retrovirally transduced cells after cell sorting to analyze Ca²⁺ responses without further cloning. Percentages of GFP-positive cells were 96.2, 84.1, 85.9 and 90.8% for cells expressing the wild-type, E2100D, E2100Q and E2100A IP₃R1s, respectively.

Construction of the expression vector and mutagenesis

The 5'-EcoRI and 3'-NotI sites were generated by PCR in the rat IP₃R1 cDNA, and cloned into the pIRES2-EGFP vector (Clontech). The resulting IP₃R-IRES-GFP fragment was then excised and subcloned into the pMXΔ vector, a derivative of retroviral vector pMX (a generous gift from Dr Kitamura, The University of Tokyo). The pMXΔ vector was generated by replacing the *SpeI*-*HindIII* fragment (1.2 kb) of the pMX vector with the *SpeI*-*HindIII* fragment (0.5 kb) of the pDON-AI vector (Takara).

To generate the mutant IP₃R1s (E2100D, E2100Q and E2100A), mutagenesis was accomplished by two separate PCRs. The first PCR used a forward primer flanking an endogenous *NarI* site (Nar-F: 5'-GGAATTCGGCGCCTCCAATCTGGTCATC-3') and a reverse primer (NarDQ-R: 5'-GATGGCCAGGAGTAGCTTTGAAGCATTG-3' for aspartate or glutamine; NarA-R: 5'-TCTCTAGAACGCTAGCCATGATGGCCAGGAGTAGC-3' for alanine). The second amplification was performed using a forward primer encoding the mutation (E2100D-F: 5'-ATGGACAGCAGACGATAGTG-3', E2100Q-F: 5'-ATGCAGAGCAGACGATAGTG-3' or E2100A-F: 5'-GGAATTCGCTAGCAGACGATAGTGAAATG-3' for aspartate, glutamine or alanine, respectively) and a reverse primer flanking a *BstBI* site (Bst-R: 5'-GCTCTAGAACTTCGAAGTTTCGATTCCTTAG-3'). After combining the two PCR fragments, the original *NarI*-*BstBI* fragment was replaced to generate full-length mutant IP₃R1 cDNAs. The mutations were confirmed by sequencing.

Immunocytochemistry and flow cytometry

The rabbit anti-rat IP₃R1 polyclonal antibody was raised against a synthetic peptide within the C-terminal cytoplasmic region, GHPPHNMVNPPQQA. DT40 cells were fixed with ice-cold methanol, permeabilized with 0.2% Triton X-100, and incubated with the affinity-purified polyclonal antibody. The secondary antibody was Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes), and the fluorescence images were acquired with a confocal laser scanning microscope (Olympus Fluoview). The immunostained cells were suspended in phosphate-buffered saline, and subjected to EPICS XL flow cytometry (Beckman Coulter).

Ca²⁺ imaging

Ca²⁺ imaging was performed as described previously (Miyakawa *et al.*, 1999). Briefly, cells on poly-L-lysine and collagen-coated coverslips were loaded with either 1 μM Fura-2AM or 20 μM Fura2prAM. The fluorescence images were captured with an Olympus IX70 inverted microscope, equipped with a cooled CCD camera (Photometrics) and a polychromatic illumination system (T.I.L.L. Photonics), at a rate of one pair of frames with excitation at 345 and 380 nm every 10, 1 or 0.25 s. Intracellular Ca²⁺ concentrations of the Fura-2-loaded cells were calculated using the equation reported previously (Grynkiewicz *et al.*, 1985).

The Fura2prAM-loaded cells were permeabilized with β-escin to remove Fura2prAM from the cytoplasm, and the Ca²⁺ concentration within the Ca²⁺ store was measured. Solutions were applied to the cells through an electrically controlled puffing pipette. The solutions containing various concentrations of Ca²⁺ were prepared by mixing CaEGTA and EGTA solutions at appropriate ratios (Hirose *et al.*, 1998). All the Ca²⁺-releasing solutions contained an appropriate concentration of IP₃ and 1 mM ATP unless otherwise mentioned.

Evaluation of IP₃R activity

The IP₃R activity was evaluated in terms of the initial Ca²⁺ release rate, as described previously (Hirose and Iino, 1994; Miyakawa *et al.*, 1999). Briefly, the observed change in the ratio of fluorescence intensity of luminal Fura2prAM was normalized so that 1 and 0 corresponded, respectively, to the values just before the test application of IP₃ and after complete depletion of the store following application of 10 μM IP₃

at either 0.3 μM Ca²⁺ (for wild-type IP₃R1) or 3 μM Ca²⁺ (for mutant IP₃R1s). To average the cell-to-cell variations, fluorescence intensities of 30–40 cells within a field of view were summed for analysis. The initial 10 s period of the normalized time course was fitted using a single exponential function, e^{-rt} . The rate constant, r (s⁻¹), thus estimated was used as an index of the IP₃R activity.

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