Encoding of Ca^{2+} signals by differential expression of IP_3 receptor subtypes

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Inositol 1,4,5-trisphosphate (IP_3) plays a key role in Ca^{2+} signalling, which exhibits a variety of spatio-temporal patterns that control important cell functions. Multiple subtypes of IP_3 receptors ($\text{IP}_3\text{R-1}$, -2 and -3) are expressed in a tissue- and development-specific manner and form heterotetrameric channels through which stored Ca^{2+} is released, but the physiological significance of the differential expression of IP_3R subtypes is not known. We have studied the Ca^{2+} -signalling mechanism in genetically engineered B cells that express either a single or a combination of IP_3R subtypes, and show that Ca^{2+} -signalling patterns depend on the IP_3R subtypes, which differ significantly in their response to agonists, i.e. IP_3 , Ca^{2+} and ATP. $\text{IP}_3\text{R-2}$ is the most sensitive to IP_3 and is required for the long lasting, regular Ca^{2+} oscillations that occur upon activation of B-cell receptors. $\text{IP}_3\text{R-1}$ is highly sensitive to ATP and mediates less regular Ca^{2+} oscillations. $\text{IP}_3\text{R-3}$ is the least sensitive to IP_3 and Ca^{2+} , and tends to generate monophasic Ca^{2+} transients. Furthermore, we show for the first time functional interactions between coexpressed subtypes. Our results demonstrate that differential expression of IP_3R subtypes helps to encode IP_3 -mediated Ca^{2+} signalling.

Keywords: calcium/calcium imaging/gene targeting/inositol 1,4,5-trisphosphate/ IP_3 receptor

Introduction

Inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} signalling controls important cell functions, such as contraction, secretion, gene expression and synaptic plasticity (Berridge, 1993). It is remarkable that a molecule as simple as Ca^{2+} can control a multitude of cellular functions with specificity, and the variety of spatio-temporal patterns of Ca^{2+} signalling has been implicated in its versatility. Indeed, the frequency of periodic increases in intracellular Ca^{2+} concentration (Ca^{2+} oscillation) has been shown to be important for the efficiency and specificity of gene expression (Dolmetsch *et al.*, 1998; Li *et al.*, 1998), and protein kinase activation (De Koninck and Schulman, 1998). However, it is not known how cells generate

specific Ca^{2+} -signalling patterns. Multiple subtypes of IP_3 receptors ($\text{IP}_3\text{R-1}$, -2 and -3) are expressed in a tissue- and development-specific manner (Newton *et al.*, 1994; Wojcikiewicz, 1995; Dent *et al.*, 1996) and form heterotetrameric channels (Joseph *et al.*, 1995; Monkawa *et al.*, 1995). Therefore, the complex expression pattern of IP_3R subtypes may be responsible for the generation of cell type-specific Ca^{2+} signalling. Recent studies of individual IP_3R subtypes incorporated into lipid bilayers have suggested functional differences (Hagar *et al.*, 1998; Ramos-Franco *et al.*, 1998). However, the properties of IP_3R subtypes have not been systematically compared under equivalent cellular conditions and it has not been demonstrated whether or not there are IP_3R subtype-specific Ca^{2+} -signalling patterns. We have studied the physiological significance of the differential expression of IP_3R subtypes in conjunction with Ca^{2+} -signalling patterns, using genetically engineered B cells that express either a single or a combination of IP_3R subtypes. Our results show that temporal patterns of Ca^{2+} signals depend critically on the expressed set of IP_3R subtypes, which differ significantly in their response to intracellular agonists, i.e. IP_3 , Ca^{2+} and ATP. Furthermore, we present the first evidence that coexpressed IP_3R subtypes make functional interactions. Thus, Ca^{2+} -signalling patterns can be encoded by differential expression of IP_3R subtypes.

Results and discussion

Ca^{2+} signalling in cells expressing a single IP_3R subtype

Three subtypes of IP_3Rs are expressed in DT40 B cells (Sugawara *et al.*, 1997). Using an homologous recombination technique, we disrupted either one, two or all three IP_3R subtype genes (Sugawara *et al.*, 1997) (Figure 1A). The loss of protein expression of $\text{IP}_3\text{R-1}$ and $\text{IP}_3\text{R-2}$ was confirmed in the cells with the respective gene disruption using antibodies specific to each IP_3R subtype (Figure 1B).

Ligation of the B-cell receptor (BCR) with an anti-BCR antibody induces the production of IP_3 via phosphorylation of phospholipase $\text{C}\gamma$, resulting in mobilization of the Ca^{2+} stores in DT40 B cells (Kurosaki, 1997). We studied the changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) upon activation of BCR in Fura-2-loaded DT40 cells with various combinations of IP_3R subtype expression. Ca^{2+} oscillations were observed in wild-type cells, which decayed in ~2–3 min and resumed thereafter, lasting for 1 h or longer (Figure 2A). Strikingly, mutant cells expressing only $\text{IP}_3\text{R-2}$ showed Ca^{2+} oscillations which were more regular and robust than those observed in wild-type cells (Figure 2C). On the other hand, mutant cells expressing either $\text{IP}_3\text{R-1}$ or $\text{IP}_3\text{R-3}$ showed only monophasic Ca^{2+} transient or very rapidly damped Ca^{2+} oscillations (Figure 2B, D and E). In ~75% of the mutant

cells expressing IP₃R-1, irregular Ca²⁺ oscillations with attenuated amplitude resumed subsequently (Figure 2B, lower panel). A delayed Ca²⁺ response was rarely observed (<1/h) in cells expressing IP₃R-3. Mutant cells expressing IP₃R-2 along with either IP₃R-1 or -3 also showed robust Ca²⁺ oscillations, while those expressing both IP₃R-1 and -3 but not IP₃R-2 showed a Ca²⁺ response similar to that of cells expressing only IP₃R-1 (data not shown). Thus, expression of IP₃R-2 is required for efficient generation of Ca²⁺ oscillations in DT40 cells. Disruption of IP₃R genes did not significantly affect cell-surface expression of the BCR (Sugawara *et al.*, 1997). Furthermore, the subtype-

specific Ca²⁺-signalling patterns were not altered when the antibody concentration was varied between 0.01 and 10 µg/ml, although the fraction of cells responding changed (data not shown). These results indicate that the characteristic Ca²⁺-signalling patterns were determined not by the stimulus intensity but by the expressed subtypes of IP₃Rs.

Luminal Ca²⁺ monitoring to study IP₃R functions

To investigate further the subtype-specific Ca²⁺-signalling mechanism, we examined the functional differences between the subtypes using luminal Ca²⁺ monitoring (Hofer and Machen, 1993; Hirose and Iino, 1994; Hajnóczky and Thomas, 1997). Cells were loaded with Fura-2, a low-affinity fluorescent Ca²⁺ indicator (Raju *et al.*, 1989) (Figure 3A and B). The fluorescence remaining after permeabilization of the cell membrane with β-escin was distributed in the extranuclear region in accordance with its localization within the endoplasmic reticulum (Figure 3C). Indeed, the luminal Ca²⁺ concentration increased with activation of the Ca²⁺ pump and declined upon application of IP₃ (Figure 3D). Thus, we measured the unidirectional flux of Ca²⁺ through the IP₃Rs by continuous monitoring of luminal Ca²⁺ concentration after withdrawing Mg²⁺-ATP to disable Ca²⁺-pump activity. No Ca²⁺ release was observed upon application of caffeine, an activator of the ryanodine receptor (Figure 3E); nor was IP₃-induced Ca²⁺ release observed in cells in which all the three IP₃R genes were disrupted (Sugawara *et al.*, 1997) (Figure 3F). Therefore, the three IP₃R subtypes account for all the Ca²⁺-release channels expressed in this cell line.

The time course of Ca²⁺ release did not follow a single exponential as reported in many other cell types (Figure 3G). However, as was the case in smooth muscle cells (Hirose and Iino, 1994), the time course of Ca²⁺ release at lower IP₃ concentrations became superimposable with that at 10 µM IP₃ if normalized to the half time (*t*_{1/2}). This indicates that the level of activation of the IP₃Rs can be quantitatively compared by the initial rate of Ca²⁺ release, which we estimated by fitting an exponential curve to the initial part of the Ca²⁺ decay signal. Thus, the

Fig. 1. Generation of DT40 cells expressing a single IP₃R subtype. (A) Northern analysis of wild-type (WT) and mutant DT40 cells with disruption of two of the three IP₃R genes (e.g. IP₃R-2 and -3 genes were disrupted in cells marked with 2/3⁻). (B) Western blot analysis after immunoprecipitation confirming the loss of expression of IP₃R-1 and -2 when the respective genes were disrupted using a polyclonal antibody against either IP₃R-1 or IP₃R-2 (see Materials and Methods). In cells marked with 1/2/3⁻, all three IP₃R subtype genes were disrupted.

Fig. 2. Ca²⁺ signalling in DT40 cells expressing a single IP₃R subtype upon BCR stimulation. (A–D) Ca²⁺ response in single cells upon ligation of BCR with anti-BCR antibody (1 µg/ml). Wild-type cells (A) and mutant cells expressing either IP₃R-1 (B), -2 (C) or -3 (D). At the antibody concentration used, 95% of the cells exhibited at least one Ca²⁺ transient within 220 s in all cell types. Upper and lower traces show representative traces of the early and late responses, respectively. Antibody was applied as indicated by the horizontal bars below the traces. (E) Histogram for the number of Ca²⁺ oscillations within 220 s of BCR stimulation (100 cells from three cultures in each cell type).

combined use of luminal Ca²⁺ monitoring and genetically engineered cells provided us with a unique system to study quantitatively the properties of IP₃R subtypes expressed individually or along with other subtypes in an identical cellular context.

Agonist sensitivities of individual IP₃R subtypes

We first analysed the IP₃-induced Ca²⁺ release in cells expressing a single IP₃R subtype. Although IP₃ (10 μ M) induced rapid Ca²⁺ release in wild-type cells in the absence of ATP, the rate of Ca²⁺ release was extremely low in IP₃R-1-expressing cells (Figure 4A). It has been shown that IP₃R activity is enhanced by ATP in a hydrolysis-independent manner in smooth muscle and cerebellar preparations (Iino, 1991; Bezprozvanny and Ehrlich, 1993; Missiaen *et al.*, 1998) in which the dominant IP₃R subtype is IP₃R-1 (Newton *et al.*, 1994; Wojcikiewicz, 1995). Indeed, the rate of Ca²⁺ release was enhanced by ATP (in the absence of Mg²⁺) in IP₃R-1-

Fig. 3. Luminal Ca²⁺ measurement in single cells. Single DT40 cells under transmitted light (A), fluorescence intensity at 380 nm after loading with Fura-2 (B) and after subsequent permeabilization with β -escin (C). The central region of the cells is occupied by the nucleus. Scale bar, 10 μ m. (D) Change in fluorescence intensity ratio (R/R_0) normalized by R_0 in the Ca²⁺-depleted state during Ca²⁺ loading (400 nM Ca²⁺ plus 0.5 mM Mg²⁺-ATP), washout of Mg²⁺-ATP and Ca²⁺ release (3 μ M IP₃ at 100 nM Ca²⁺). The Ca²⁺ loading-release cycles could be repeated several times in the same cells. (E) Caffeine (Caf; 20 mM) failed to induce Ca²⁺ release, but subsequent application of IP₃ (10 μ M) released Ca²⁺. (F) IP₃ (10 μ M) failed to induce Ca²⁺ release in cells in which all three IP₃R subtype genes were disrupted, although subsequent application of ionomycin (IO; 1 μ M) induced Ca²⁺ release. (G) Normalized time course of Ca²⁺ release at different IP₃ concentrations. Continuous lines, time courses of Ca²⁺ release at 0.1, 1 and 10 μ M IP₃ (lower abscissa). Time course at 0.1 μ M ($\square$), 1 μ M ($\circ$) and 10 μ M (continuous line) was normalized to the half-time ($t_{1/2}$) of Ca²⁺ release for each IP₃ concentration (upper abscissa).

expressing cells with an EC₅₀ of 0.39 mM, while no effect was observed in wild-type cells (Figure 4A and B). The effect of ATP on IP₃R-1 was mimicked by β , γ -methylene ATP, a nonhydrolysable ATP analogue (data not shown). A less significant effect of ATP on IP₃R-3 was observed, whereas Ca²⁺ release via IP₃R-2 was insensitive to ATP (Figure 4C).

We then studied the IP₃ concentration dependence of Ca²⁺ release (Figure 5A). The IP₃ sensitivity was in the order of IP₃R-2 > IP₃R-1 > IP₃R-3; the EC₅₀ obtained by hyperbolic fitting (see the legend to Figure 5) being 0.35, 4.7 and 18.6 μ M, respectively. The extrapolated values of the maximal rate of Ca²⁺ release ($r_{\max}$) were 0.063, 0.129 and 0.108 s⁻¹ in cells expressing IP₃R-1, -2 and -3, respectively. Thus $r_{\max}$ was comparable within a factor of two among DT40 clones expressing different IP₃R subtypes.

IP₃R activity has been shown to be dependent on the cytoplasmic Ca²⁺ concentration in a biphasic manner in many cell types (Iino, 1990; Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991). This property has been thought to

Fig. 4. ATP dependence of IP₃-induced Ca²⁺ release. (A) ATP (5 mM) had virtually no effect in wild-type cells, but greatly enhanced IP₃-induced Ca²⁺ release in IP₃R-1 expressing cells. (B) ATP dependence of the rate of IP₃-induced Ca²⁺ release in IP₃R-1-expressing cells. (C) Percentage of the rate of Ca²⁺ release (10 μ M IP₃, 300 nM Ca²⁺) in the absence of ATP compared with that in the presence of 10 mM ATP in cells expressing various IP₃R subtypes. Results are the mean $\pm$ SEM for four experiments.

Fig. 5. IP_3 -concentration dependence of Ca^{2+} release. (A) Ca^{2+} release in cells expressing a single IP_3R subtype. Continuous curves represent the best fit hyperbolic equations, $r_{\max,i}/(1 + \text{EC}_{50,i}/[\text{IP}_3])$ for subtype i . For IP_3R -1 and -3, the fitted curves were scaled upward (dashed curves) so that $r_{\max}$ was the same as that of IP_3R -2 for the ease of comparison of EC_{50} s. (B) Ca^{2+} release rate in cells expressing multiple IP_3R subtypes. Dashed curve shows the fitted curve for IP_3R -2 in (A). Dotted curves show summation of the fitted equations, $\Sigma\{f_i \cdot r_{\max,i}/(1 + \text{EC}_{50,i}/[\text{IP}_3])\}$, for $i = 1, 2, 3$. When subtype i was not expressed f_i was set to zero. Sets of (f_1, f_2, f_3) obtained by a least-squares method were as follows: (0.32, 0.60, 0), (0, 0.57, 0.82), (0.71, 0, 0.51), (0.37, 0.70, 0.31) for cells expressing IP_3R subtypes 1/2, 2/3, 1/3 and 1/2/3 (wild type), respectively. Ca^{2+} concentration was 100 nM. Ca^{2+} release via IP_3R -1 was studied in the presence of 5 mM ATP. Results are the mean $\pm$ SEM for 4–5 experiments.

Fig. 6. Ca^{2+} -concentration dependence of IP_3 -induced Ca^{2+} release. (A) Ca^{2+} release in cells expressing a single IP_3R subtype. (B) Ca^{2+} release in cells expressing multiple IP_3R subtypes. (C) Ca^{2+} -mediated activation of IP_3 -induced Ca^{2+} release. Percentage increase of the Ca^{2+} -release rate at 300 nM Ca^{2+} compared with that at 10 nM Ca^{2+} . IP_3 concentrations were 0.3 μM for cells expressing subtype(s) 2, 1/2, 2/3, 1/2/3; 6 μM for subtype(s) 1, 1/3; and 20 μM for subtype 3. Ca^{2+} release via IP_3R -1 was studied in the presence of 5 mM ATP. Results are the mean $\pm$ SEM for 3–5 experiments.

underlie the regenerative Ca^{2+} release during the Ca^{2+} wave and rapid upstroke of $[\text{Ca}^{2+}]_i$ increase (Iino and Endo, 1992; Lechleiter and Clapham, 1992; Iino *et al.*, 1993; Bootman *et al.*, 1997; Horne and Meyer, 1997). Ca^{2+} release via IP_3R -1 and IP_3R -2 exhibited clear biphasic Ca^{2+} dependence with a peak rate obtained near 300 nM (Figure 6A), whereas IP_3R -3-mediated Ca^{2+} release exhibited flatter Ca^{2+} dependence. Figure 6C plots the extent of Ca^{2+} -induced activation, or the percentage activation of the rate of Ca^{2+} release, when the cytoplasmic Ca^{2+} concentration was increased from 10 to 300 nM in cells containing the various IP_3R subtypes.

Functional interaction between IP_3R subtypes

It has been shown that the IP_3R subtypes form heterotetramers (Joseph *et al.*, 1995; Monkawa *et al.*, 1995), which were thought to create an additional degree of

variability with regard to the IP_3R function. However, functional interactions between the IP_3R subtypes have not previously been investigated. We studied IP_3 -induced Ca^{2+} release in cells expressing multiple IP_3R subtypes. The IP_3 dependence of the Ca^{2+} release rate in cells with multiple IP_3R subtypes was closely fitted by the summation of the fits to the corresponding individual subtypes after reduction of the maximal rates to 31–82% of those in cells expressing a single subtype (Figure 5B, dotted curves). The downward scaling of the maximal release rate may reflect compensatory upregulation of IP_3Rs in cells expressing only a single subtype. This is consistent with the increased mRNA or protein levels observed in mutant cells (Figure 1). We also studied the ATP and Ca^{2+} dependence of Ca^{2+} release in cells expressing multiple subtypes (Figures 4C and 6B). When IP_3R -1 and IP_3R -3 were coexpressed, the ATP dependence was similar

to that of IP₃R-3 but the Ca²⁺ dependence was similar to that of IP₃R-1. IP₃-induced Ca²⁺ release in cells expressing both IP₃R-1 and IP₃R-2 lost the prominent ATP dependence that was characteristic of IP₃R-1. Cells expressing both IP₃R-2 and IP₃R-3 showed ATP and Ca²⁺ sensitivities similar to those of IP₃R-2-expressing cells. In wild-type cells, the ATP sensitivity was similar to that of IP₃R-2-expressing cells. Taken together, coexpression of multiple subtypes resulted in simple additive IP₃ sensitivity, whereas the property of either one of the subtypes became dominant in terms of ATP and Ca²⁺ sensitivity. The latter result indicates that there is indeed a molecular interaction between the different subtypes, probably within the heterotetrameric structure of the Ca²⁺ release channels.

Ca²⁺ signalling and IP₃R subtypes

The present results highlight the functional variations among IP₃R subtypes. IP₃R-1, when expressed singly, exhibited prominent ATP dependence and the potential to function as an ATP sensor just like the ATP-sensitive K⁺ channels in pancreatic β cells. IP₃R-3 has the lowest IP₃ and Ca²⁺ sensitivities, while IP₃R-2 has the highest sensitivity to IP₃. The order of IP₃ sensitivity is in general agreement with that of the IP₃-binding affinity of the IP₃R subtypes (Newton *et al.*, 1994). Furthermore, BCR-mediated Ca²⁺-signalling patterns differed significantly among cells expressing different IP₃R subtypes: Ca²⁺ oscillations were found in IP₃R-1 and -2 expressing cells, while monophasic Ca²⁺ transients were observed in IP₃R-3-expressing cells. Although difference in the level of expression of various IP₃R subtypes may affect the Ca²⁺-signalling patterns, the following considerations make it unlikely to be a major determinant in the present study. The expression levels of IP₃R subtypes can be estimated from $r_{\max}$, i.e. the maximum rate of Ca²⁺ release, and the values were comparable within a factor of two among DT40 clones expressing different IP₃R subtypes (Figure 5A). If we take ATP dependence of IP₃R-3 into consideration (see Figure 4C), difference in $r_{\max}$ values between IP₃R-2- and IP₃R-3-expressing cells is probably minimal under physiological intracellular conditions, where ATP is present at millimolar levels. Moreover, the $r_{\max}$ value of IP₃R-3-expressing cells was greater than that of IP₃R-1-expressing cells even in the presence of ATP. Thus there is no correlation between the Ca²⁺-oscillation pattern in intact cells and the level of functional expression of IP₃Rs.

We have obtained evidence for the first time that coexpressed IP₃R subtypes interact functionally. Coexpression of subtypes with different affinities for IP₃ widens the range of IP₃ sensitivity of intracellular Ca²⁺ stores. Furthermore, expression of IP₃R-2, with or without other subtypes, facilitates Ca²⁺ oscillations in DT40 cells. It is of interest to note that IP₃R-2 is the dominantly expressed IP₃R in hepatocytes (Wojcikiewicz *et al.*, 1994), in which Ca²⁺ oscillations were first observed (Woods *et al.*, 1986). The high IP₃ sensitivity of IP₃R-2 may underlie the long-lasting Ca²⁺ oscillations, but other possibilities, such as preferential modification of IP₃R-2 during Ca²⁺ oscillations, cannot be excluded. The expression patterns of IP₃R subtypes differ among different tissues (Newton *et al.*, 1994; Wojcikiewicz *et al.*, 1994), during development (Dent *et al.*, 1996), and in subcellular

localization (Lee *et al.*, 1997). Our results provide a clear functional basis for the physiological significance of the differential expression of IP₃R subtypes in cell-type-specific encoding of Ca²⁺ signalling.

Materials and methods

Cell culture and generation of DT40 B cells expressing only IP₃R-1 or IP₃R-2

DT40 chicken B lymphoma cells were cultured in RPMI1640 supplemented with 10% fetal calf serum (FCS), 1% chicken serum, penicillin, streptomycin and glutamine. To inactivate the IP₃R-3 gene, two targeting vectors, pIP₃R type 3-*bleo* and pIP₃R type 3-*bsr*, were used. The latter was constructed by replacing the *bleo* cassette of pIP₃R type 3-*bleo* (Sugawara *et al.*, 1997) with the *bsr* cassette. For disruption of both alleles of the IP₃R-3 gene, these two targeting vectors were transfected sequentially into DT40 cells expressing a combination of IP₃R-1/IP₃R-3 or IP₃R-2/IP₃R-3, thereby resulting in the generation of DT40 cells expressing only IP₃R-1 or IP₃R-2, respectively. Other IP₃R gene-targeted DT40 cells had been established previously (Sugawara *et al.*, 1997).

Northern analysis

RNA was prepared from wild-type and mutant DT40 cells using the guanidium thiocyanate method. Total RNA (20 μ g) was separated in a 1.2% formaldehyde gel, transferred onto a Hybond-N membrane and probed with ³²P-labelled cDNA fragments specific for each type of chicken IP₃R gene (Sugawara *et al.*, 1997) and the chicken β -actin gene (Kost *et al.*, 1983).

Immunoprecipitation and immunoblotting

Cells were lysed in a solution containing 30 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 1 mM Na₃VO₄, 30 mM NaF, 1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 0.1% bovine serum albumen (BSA) (lysis buffer). Lysates were clarified by centrifugation at 15 000 g for 10 min at 4°C. Immunoprecipitation was performed at 4°C using a rabbit polyclonal anti-IP₃R antibody (raised against a synthetic peptide within the C-terminal cytoplasmic domain of either chicken IP₃R-1, LGHPPPMNVQPQA, or IP₃R-2, LGSHTPHVNHMPPPP) for >1 h, and then with addition of protein G-Sepharose for >1 h. The immune complexes were washed and heated at 100°C in SDS sample buffer. Solubilized proteins were separated by SDS-PAGE and transferred electrophoretically to a PVDF membrane. After blocking in 5% fatty acid-free BSA, the blot was incubated with the same anti-IP₃R antibody and then incubated with [¹²⁵I]protein G. The associated radioactivity was visualized using a Fuji BAS2000 Bio Imaging Analyzer.

Ca²⁺ imaging

Cells were attached to poly-L-lysine and collagen-coated coverslips, and loaded with either 1 μ M Fura-2AM for 20–30 min or 20 μ M Fura2prAM for 60 min in a physiological salt solution: 150 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, 5.6 mM glucose, pH 7.4. The Fura2prAM-loaded cells were then permeabilized by incubation with 40 μ M β -escin for 2–4 min in an internal solution (Hirose *et al.*, 1998) to wash out the Fura2prAM in the cytoplasm, which enabled measurement of the Ca²⁺ concentration within the organelles. An Olympus IX70 inverted microscope, equipped with a cooled CCD camera (Photometrics, USA) and a polychromatic illumination system (T.I.L.L. Photonics, Germany), was used to capture the fluorescence images at a rate of one pair of frames with excitations at 340 and 380 nm per 1, 2, 5 or 10 s. The ratio of the fluorescence intensity between the pair of frames was then calculated after subtraction of the baseline fluorescence. Cells were viewed under an oil-immersion objective (100 $\times$, NA 1.35). Experiments were carried out at room temperature (22–24°C), and solutions were applied to the cells through an electrically controlled puffing pipette. Solutions containing various concentrations of Ca²⁺ were prepared by mixing CaEGTA and EGTA solutions at appropriate ratios (Hirose *et al.*, 1998).

Evaluation of IP₃R activity

The IP₃R activity was evaluated in terms of the initial Ca²⁺ release rate as described previously (Hirose and Iino, 1994). Briefly, the observed change in the ratio of fluorescence intensities of 30–40 cells within a frame were normalized so that 1 and 0 corresponded, respectively, to the values just before the application of IP₃ and after complete depletion

by 10 μM IP_3 at 300 nM Ca^{2+} . The initial 20 s period of the normalized time course was fitted by a single exponential function, $e^{-t/\tau}$. The rate constant, τ (s^{-1}), thus estimated was used as an index of the IP_3R activity.

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