Encoding of Ca\textsuperscript{2+} signals by differential expression of IP\textsubscript{3} receptor subtypes

Tomoyuki Miyakawa, Akito Maeda\textsuperscript{1}, Toshiko Yamazawa, Kenzo Hirose, Tomohiro Kurosaki\textsuperscript{1} and Masamitsu Iino\textsuperscript{2}

Department of Pharmacology, Faculty of Medicine, The University of Tokyo, CREST, Japan Science and Technology Corporation, Bunkyo-ku, Tokyo 113 and \textsuperscript{1}Akito Maeda, Tomohiro Kurosaki, Department of Molecular Genetics, Institute for Liver Research, Kansai Medical University, Moriguchi 570, Japan

\textsuperscript{2}Corresponding author
e-mail: iino@m.u-tokyo.ac.jp

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

\textit{Keywords}: calcium/calium imaging/gene targeting/inositol 1,4,5-trisphosphate/IP\textsubscript{3} receptor

Introduction

Inositol 1,4,5-trisphosphate (IP\textsubscript{3})-mediated Ca\textsuperscript{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\textsuperscript{2+} can control a multitude of cellular functions with specificity, and the variety of spatio-temporal patterns of Ca\textsuperscript{2+} signalling has been implicated in its versatility. Indeed, the frequency of periodic increases in intracellular Ca\textsuperscript{2+} concentration (Ca\textsuperscript{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\textsuperscript{2+}-signalling patterns. Multiple subtypes of IP\textsubscript{3} receptors (IP\textsubscript{3}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; Monka et al., 1995). Therefore, the complex expression pattern of IP\textsubscript{3}R subtypes may be responsible for the generation of cell type-specific Ca\textsuperscript{2+} signalling. Recent studies of individual IP\textsubscript{3}R subtypes incorporated into lipid bilayers have suggested functional differences (Hagar et al., 1998; Ramos-Franco et al., 1998). However, the properties of IP\textsubscript{3}R subtypes have not been systematically compared under equivalent cellular conditions and it has not been demonstrated whether or not there are IP\textsubscript{3}R subtype-specific Ca\textsuperscript{2+}-signalling patterns. We have studied the physiological significance of the differential expression of IP\textsubscript{3}R subtypes in conjunction with Ca\textsuperscript{2+}-signalling patterns, using genetically engineered B cells that express either a single or a combination of IP\textsubscript{3}R subtypes. Our results show that temporal patterns of Ca\textsuperscript{2+} signals depend critically on the expressed set of IP\textsubscript{3}R subtypes, which differ significantly in their response to intracellular agonists, i.e. IP\textsubscript{3}, Ca\textsuperscript{2+} and ATP. Furthermore, we present the first evidence that coexpressed IP\textsubscript{3}R subtypes make functional interactions. Thus, Ca\textsuperscript{2+}-signalling patterns can be encoded by differential expression of IP\textsubscript{3}R subtypes.

Results and discussion

Ca\textsuperscript{2+} signalling in cells expressing a single IP\textsubscript{3}R subtype

Three subtypes of IP\textsubscript{3}R are expressed in DT40 B cells (Sugawara et al., 1997). Using an homologous recombination technique, we disrupted either one, two or all three IP\textsubscript{3}R subtype genes (Sugawara et al., 1997) (Figure 1A). The loss of protein expression of IP\textsubscript{3}R-1 and IP\textsubscript{3}R-2 was confirmed in the cells with the respective gene disruption using antibodies specific to each IP\textsubscript{3}R subtype (Figure 1B).

Ligation of the B-cell receptor (BCR) with an anti-BCR antibody induces the production of IP\textsubscript{3} via phospholipidation of phospholipase C\textsubscript{yt}, resulting in mobilization of the Ca\textsuperscript{2+} stores in DT40 B cells (Kurosaki, 1997). We studied the changes in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) upon activation of BCR in Fura-2-loaded DT40 cells with various combinations of IP\textsubscript{3}R subtype expression. Ca\textsuperscript{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 IP\textsubscript{3}R-2 showed Ca\textsuperscript{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 IP\textsubscript{3}R-1 or IP\textsubscript{3}R-3 showed only monophasic Ca\textsuperscript{2+} transient or very rapidly damped Ca\textsuperscript{2+} oscillations (Figure 2B, D and E). In ~75% of the mutant

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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 lino, 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 lino, 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 (t₁/₂). 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

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**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 Ca\(^{2+}\) monitoring and genetically engineered cells provided us with a unique system to study quantitatively the properties of IP\(_3\)R subtypes expressed individually or along with other subtypes in an identical cellular context.

**Agonist sensitivities of individual IP\(_3\)R subtypes**

We first analysed the IP\(_3\)-induced Ca\(^{2+}\) release in cells expressing a single IP\(_3\)R subtype. Although IP\(_3\) (10 \(\mu\)M) induced rapid Ca\(^{2+}\) release in wild-type cells in the absence of ATP, the rate of Ca\(^{2+}\) release was extremely low in IP\(_3\)R-1-expressing cells (Figure 4A). It has been shown that IP\(_3\)R 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 IP\(_3\)R subtype is IP\(_3\)R-1 (Newton et al., 1994; Wojcikiewicz, 1995). Indeed, the rate of Ca\(^{2+}\) release was enhanced by ATP (in the absence of Mg\(^{2+}\)) in IP\(_3\)R-1-expressing cells with an EC\(_{50}\) of 0.39 mM, while no effect was observed in wild-type cells (Figure 4A and B). The effect of ATP on IP\(_3\)R-1 was mimicked by \(\beta,\gamma\)-methylene ATP, a nonhydrolysable ATP analogue (data not shown). A less significant effect of ATP on IP\(_3\)R-3 was observed, whereas Ca\(^{2+}\) release via IP\(_3\)R-2 was insensitive to ATP (Figure 4C).

We then studied the IP\(_3\) concentration dependence of Ca\(^{2+}\) release (Figure 5A). The IP\(_3\) sensitivity was in the order of IP\(_3\)R-2 > IP\(_3\)R-1 > IP\(_3\)R-3; the EC\(_{50}\) obtained by hyperbolic fitting (see the legend to Figure 5) being 0.35, 4.7 and 18.6 \(\mu\)M, respectively. The extrapolated values of the maximal rate of Ca\(^{2+}\) release (\(r_{\text{max}}\)) were 0.063, 0.129 and 0.108 s\(^{-1}\) in cells expressing IP\(_3\)R-1, -2 and -3, respectively. Thus \(r_{\text{max}}\) was comparable within a factor of two among DT40 clones expressing different IP\(_3\)R subtypes.

IP\(_3\)R activity has been shown to be dependent on the cytoplasmic Ca\(^{2+}\) 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. 3.** Luminal Ca\(^{2+}\) measurement in single cells. Single DT40 cells under transmitted light (A), fluorescence intensity at 380 nm after loading with Furaptra (B) and after subsequent permeabilization with \(\beta\)-escin (C). The central region of the cells is occupied by the nucleus. Scale bar, 10 \(\mu\)m. (D) Change in fluorescence intensity ratio (\(R\)) normalized by \(R_0\) in the Ca\(^{2+}\)-depleted state during Ca\(^{2+}\) loading (400 nM Ca\(^{2+}\) plus 0.5 mM Mg\(^{2+}\)-ATP), washout of Mg\(^{2+}\)-ATP and Ca\(^{2+}\) release (3 \(\mu\)M IP\(_3\) at 100 nM Ca\(^{2+}\)). The Ca\(^{2+}\) loading–release cycles could be repeated several times in the same cells. (E) Caffeine (Caf; 20 mM) failed to induce Ca\(^{2+}\) release, but subsequent application of IP\(_3\) (10 \(\mu\)M) released Ca\(^{2+}\). (F) IP\(_3\) (10 \(\mu\)M) failed to induce Ca\(^{2+}\) release in cells in which all three IP\(_3\)R subtype genes were disrupted, although subsequent application of ionomycin (IO; 1 \(\mu\)M) induced Ca\(^{2+}\) release. (G) Normalized time course of Ca\(^{2+}\) release at different IP\(_3\) concentrations. Continuous lines, time courses of Ca\(^{2+}\) release at 0.1, 1 and 10 \(\mu\)M IP\(_3\) (lower abscissa). Time course at 0.1 \(\mu\)M (□), 1 \(\mu\)M (○) and 10 \(\mu\)M (continuous line) was normalized to the half-time (\(t_{1/2}\)) of Ca\(^{2+}\) release for each IP\(_3\) concentration (upper abscissa).

**Fig. 4.** ATP dependence of IP\(_3\)-induced Ca\(^{2+}\) release. (A) ATP (5 mM) had virtually no effect in wild-type cells, but greatly enhanced IP\(_3\)-induced Ca\(^{2+}\) release in IP\(_3\)R-1 expressing cells. (B) ATP dependence of the rate of IP\(_3\)-induced Ca\(^{2+}\) release in IP\(_3\)R-1 expressing cells. (C) Percentage of the rate of Ca\(^{2+}\) release (10 \(\mu\)M IP\(_3\), 300 nM Ca\(^{2+}\)) in the absence of ATP compared with that in the presence of 10 mM ATP in cells expressing various IP\(_3\)R subtypes. Results are the mean ± SEM for four experiments.
underlie the regenerative Ca\(^{2+}\) release during the Ca\(^{2+}\) wave and rapid upstroke of [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\(_3\)R-1 and IP\(_3\)R-2 exhibited clear biphasic Ca\(^{2+}\) dependence with a peak rate obtained near 300 nM (Figure 6A), whereas IP\(_3\)R-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\(_3\)R subtypes.

**Functional interaction between IP\(_3\)R subtypes**

It has been shown that the IP\(_3\)R 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\(_3\)R function. However, functional interactions between the IP\(_3\)R subtypes have not previously been investigated. We studied IP\(_3\)-induced Ca\(^{2+}\) release in cells expressing multiple IP\(_3\)R subtypes. The IP\(_3\) dependence of the Ca\(^{2+}\)-release rate in cells with multiple IP\(_3\)R 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\(_3\)Rs 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\(_3\)R-1 and IP\(_3\)R-3 were coexpressed, the ATP dependence was similar.
to that of IP$_3$R-3 but the Ca$^{2+}$ dependence was similar to that of IP$_3$R-1. IP$_3$-induced Ca$^{2+}$ release in cells expressing both IP$_3$R-1 and IP$_3$R-2 lost the prominent ATP dependence that was characteristic of IP$_3$R-1. Cells expressing both IP$_3$R-2 and IP$_3$R-3 showed ATP and Ca$^{2+}$ sensitivities similar to those of IP$_3$R-2-expressing cells. In wild-type cells, the ATP sensitivity was similar to that of IP$_3$R-2 expressing cells. Taken together, coexpression of multiple subtypes resulted in simple additive IP$_3$ sensitivity, whereas the property of either one of the subtypes became dominant in terms of ATP and Ca$^{2+}$ sensitivity. The latter result indicates that there is indeed a molecular interaction between the different subtypes, probably within the hetero-tetrameric structure of the Ca$^{2+}$ release channels.

**Ca$^{2+}$ signalling and IP$_3$R subtypes**

The present results highlight the functional variations among IP$_3$R subtypes. IP$_3$R-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. IP$_3$R-3 has the lowest IP$_3$ and Ca$^{2+}$ sensitivities, while IP$_3$R-2 has the highest sensitivity to IP$_3$. The order of IP$_3$ sensitivity is in general agreement with that of the IP$_3$-binding affinity of the IP$_3$R subtypes (Newton et al., 1994). Furthermore, BCR-mediated Ca$^{2+}$-signalling patterns differed significantly among cells expressing different IP$_3$R subtypes: Ca$^{2+}$ oscillations were found in IP$_3$R-1 and -2 expressing cells, while monophasic Ca$^{2+}$ transients were observed in IP$_3$R-3-expressing cells. Although difference in the level of expression of various IP$_3$R subtypes may affect the Ca$^{2+}$-signalling patterns, the following considerations make it unlikely to be a major determinant in the present study. The expression levels of IP$_3$R subtypes can be estimated from $r_{\text{max}}$, i.e. the maximum rate of Ca$^{2+}$ release, and the values were comparable within a factor of two among DT40 clones expressing different IP$_3$R subtypes (Figure 5A). If we take ATP dependence of IP$_3$R-3 into consideration (see Figure 4C), difference in $r_{\text{max}}$ values between IP$_3$R-2- and IP$_3$R-3-expressing cells is probably minimal under physiological intracellular conditions, where ATP is present at millimolar levels. Moreover, the $r_{\text{max}}$ value of IP$_3$R-3-expressing cells was greater than that of IP$_3$R-1-expressing cells even in the presence of ATP. Thus there is no correlation between the Ca$^{2+}$-oscillation pattern in intact cells and the level of functional expression of IP$_3$Rs.

We have obtained evidence for the first time that coexpressed IP$_3$R subtypes interact functionally. Coexpression of subtypes with different affinities for IP$_3$ widens the range of IP$_3$ sensitivity of intracellular Ca$^{2+}$ stores. Furthermore, expression of IP$_3$R-2, with or without other subtypes, facilitates Ca$^{2+}$ oscillations in DT40 cells. It is of interest to note that IP$_3$R-2 is the dominantly expressed IP$_3$R in hepatocytes (Wojcikiewicz et al., 1994), in which Ca$^{2+}$ oscillations were first observed (Woods et al., 1986). The high IP$_3$ sensitivity of IP$_3$R-2 may underlie the long-lasting Ca$^{2+}$ oscillations, but other possibilities, such as preferential modification of IP$_3$R-2 during Ca$^{2+}$ oscillations, cannot be excluded. The expression patterns of IP$_3$R 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 IP$_3$R subtypes in cell-type-specific encoding of Ca$^{2+}$ signalling.

**Materials and methods**

**Cell culture and generation of DT40 B cells expressing only IP$_3$R-1 or IP$_3$R-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 IP$_3$R-3 gene, two targeting vectors, pIpR type 3-bleo and pIpR type 3-bsr, were used. The latter was constructed by replacing the bleo cassette of pIpR type 3-bleo (Sugawara et al., 1997) with the bsr cassette. For disruption of both alleles of the IP$_3$R-3 gene, these two targeting vectors were transfected sequentially into DT40 cells expressing a combination of IP$_3$R-1/Ip$_3$R-3 or IP$_3$R-2/Ip$_3$R-3, thereby resulting in the generation of DT40 cells expressing only IP$_3$R-1 or IP$_3$R-2, respectively. Other IP$_3$R gene-targeted DT40 cells had been established previously (Sugawara et al., 1997).

**Immunoprecipitation and immunoblotting**

RNA was prepared from wild-type and mutant DT40 cells using the guanidium thiocyanate method. Total RNA (20 μg) 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 IP$_3$R. The expression levels of IP$_3$R subtypes can be probed with 32P-labelled cDNA fragments specific encoding of Ca$^{2+}$ release channels.
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, $e^{-rt}$. The rate constant, $r$ (s$^{-1}$), thus estimated was used as an index of the IP$_3$R activity.

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