## Review

# Spatiotemporal dynamics of $Ca^{2+}$ signaling and its physiological roles

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**Abstract:** Changes in the intracellular $Ca^{2+}$ concentration regulate numerous cell functions and display diverse spatiotemporal dynamics, which underlie the versatility of $Ca^{2+}$ in cell signaling. In many cell types, an increase in the intracellular $Ca^{2+}$ concentration starts locally, propagates within the cell ($Ca^{2+}$ wave) and makes oscillatory changes ($Ca^{2+}$ oscillation). Studies of the intracellular $Ca^{2+}$ release mechanism from the endoplasmic reticulum (ER) showed that the $Ca^{2+}$ release mechanism has inherent regenerative properties, which is essential for the generation of $Ca^{2+}$ waves and oscillations. $Ca^{2+}$ may shuttle between the ER and mitochondria, and this appears to be important for pacemaking of $Ca^{2+}$ oscillations. Importantly, $Ca^{2+}$ oscillations are an efficient mechanism in regulating cell functions, having effects supra-proportional to the sum of duration of $Ca^{2+}$ increase. Furthermore, $Ca^{2+}$ signaling mechanism studies have led to the development of a method for specific inhibition of $Ca^{2+}$ signaling, which has been used to identify hitherto unrecognized functions of $Ca^{2+}$ signals.

**Keywords:** $Ca^{2+}$, $IP_3$, NFAT, imaging, smooth muscle, synapse

## Introduction

Intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) is kept extremely low at resting conditions with approximately a 1:10,000 ratio compared to the extracellular $Ca^{2+}$ concentration. Upon various stimuli to the cells, $[Ca^{2+}]_i$ increases due to $Ca^{2+}$ influx *via* the plasma membrane and/or release of $Ca^{2+}$ from the intracellular store to regulate various cell functions. Thus, $[Ca^{2+}]_i$ functions as a cellular switch. This notion was first established in skeletal muscle cells.[1),2)] Subsequently, a long list of cell functions that are switched on by $Ca^{2+}$ signals has been compiled, and the switching time and switching distance within the cell are extremely diverse (Fig. 1). The versatility of $Ca^{2+}$ signals has fascinated many investigators and has been studied extensively. Furthermore, there should be many hitherto unrecognized functions that are regulated by $Ca^{2+}$ signals. In this review, I would like to summarize how the studies on the switching mechanisms of $Ca^{2+}$ signals evolved, referring to our work centering on the intracellular $Ca^{2+}$ release mechanism. There have been extensive studies on $Ca^{2+}$ signaling in striated muscle cells; that is, excitation-contraction coupling. Readers are referred to numerous excellent reviews on this subject.[3)–6)] Here, I would like to concentrate on $Ca^{2+}$ signaling in non-striated-muscle cells. The $Ca^{2+}$ influx pathways are also important for the generation of $Ca^{2+}$ signals, but a full account of the trans-plasmalemmal pathways is out of the scope of this review.

## 1. $Ca^{2+}$ signal and cell functions

Recent studies on $Ca^{2+}$ signaling have been greatly influenced by imaging methods owing to the advent of fluorescent $Ca^{2+}$ indicators.[7)] Indeed, it can be said that the importance of spatiotemporal distribution of intracellular signaling molecules was first systematically recognized in $Ca^{2+}$ signaling studies. Although fluorescence $Ca^{2+}$ imaging was initially carried out in cultured cells or isolated single

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Abbreviations: ER: endoplasmic reticulum; $IP_3$: inositol 1,4,5-trisphosphate; $IP_3R$: $IP_3$ receptor; CICR: $Ca^{2+}$-induced $Ca^{2+}$ release; PLC: phospholipase C; GFP-PHD: GFP-tagged Pleckstrin homology domain; NFAT: nuclear factor of activated T cells; PC: Purkinje cell; PF: parallel fiber; BDNF: brain-derived neurotrophic factor.

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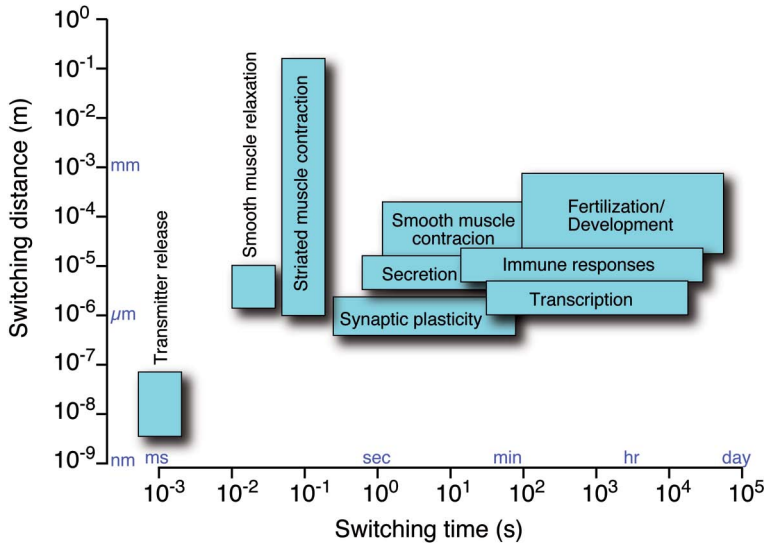


Fig. 1. Switching time and distance of $Ca^{2+}$ signals in various cell functions. Boxes, representing each cell function, indicate approximate period of time and distance regulated by $Ca^{2+}$ signals.

cells, this method was gradually applied to tissue preparations. We first imaged $Ca^{2+}$ signals in vascular smooth muscle cells within the intact arterial tissue, which consists of endothelial, smooth muscle and sympathetic nerve layers from the inside to the outside of arterial walls.[8] Electrical stimulation of the perivascular sympathetic nerve network results in the contraction of smooth muscle cells, mimicking the vascular response to the sympathetic nerve activity.

When we imaged the $Ca^{2+}$ signaling in $Ca^{2+}$ indicator-loaded vascular smooth muscle cells receiving input from the perivascular sympathetic nerves, we found that the spatiotemporal characteristics of the $Ca^{2+}$ response of vascular smooth muscle cells were extremely dynamic (Fig. 2A).[8] In the initial $Ca^{2+}$ response shown in Fig. 2Ab, all the cells responded rather uniformly. This $Ca^{2+}$ response was insensitive to α-adrenergic antagonists, and is due to purinergic P2X receptor stimulation by the sympathetic co-transmitter ATP released from the nerve endings. However, the purinergic response faded away rapidly due to receptor desensitization. In the following responses (Fig. 2Ac–f), wave-like increases in $[Ca^{2+}]_i$ ($Ca^{2+}$ waves) traversed within the spindle-shaped smooth muscle cells along their longitudinal axis. $Ca^{2+}$ waves occurred repeatedly during stimulation with intervals to generate oscillatory changes in $[Ca^{2+}]_i$ ($Ca^{2+}$ oscillations, Fig. 2B). These $Ca^{2+}$ waves and oscillations were blocked by α-adrenergic antagonists. Similar $Ca^{2+}$ dynamics were also observed when noradrenaline, the sympathetic nerve transmitter, was directly applied to the arterial tissue. Interestingly, the frequency of $Ca^{2+}$ oscillations, rather than their amplitude, increased with increasing concentration of noradrenaline (Fig. 2C). In other words, vascular smooth muscle contraction seems to be regulated by the frequency of $Ca^{2+}$ oscillations (frequency modulation).

It has been shown that many other cell functions are regulated by $Ca^{2+}$ oscillations. Indeed, $Ca^{2+}$ oscillation is one of the most ubiquitous forms of $Ca^{2+}$ signaling.[9] Following from this, a couple of fundamental questions arise; primarily, how are $Ca^{2+}$ oscillations generated? And is there any advantage to regulating cell functions through $Ca^{2+}$ oscillations?

## 2. Potentiation of intracellular $Ca^{2+}$ release by $Ca^{2+}$

Agonist-induced breakdown of phosphatidyl inositol 4,5-bisphosphate generates inositol 1,4,5-trisphosphate ($IP_3$), which releases $Ca^{2+}$ from the intracellular stores.[10] Today, $IP_3$-induced $Ca^{2+}$ release is known to underlie $Ca^{2+}$ oscillations in many cell types, including vascular smooth muscle cells. However, a simple signaling mechanism involving receptor activation, $IP_3$ generation, and $Ca^{2+}$ release is not sufficient to cause oscillatory changes in $[Ca^{2+}]_i$. There must be a complex mechanism to explain

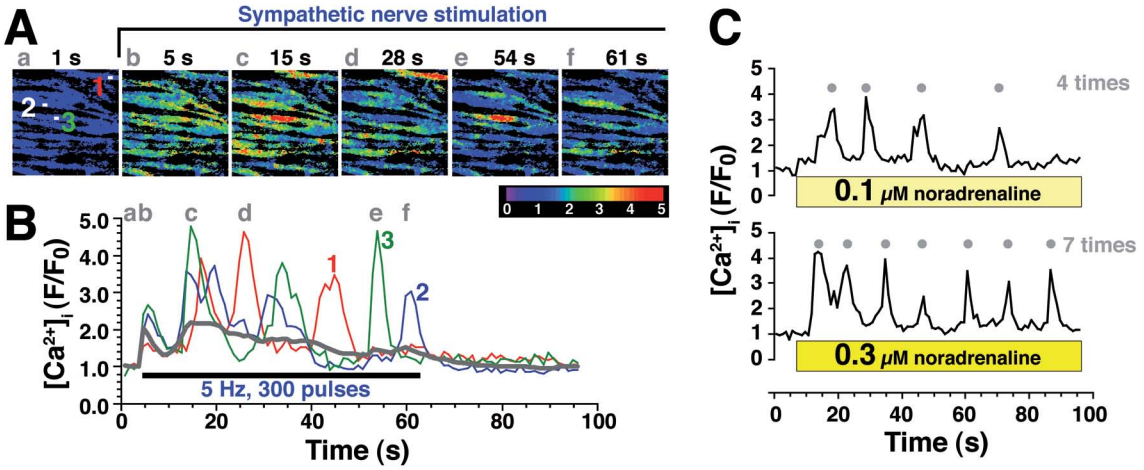


Fig. 2. Spatiotemporal dynamics of $Ca^{2+}$ signaling in vascular smooth muscle cells. (A) Confocal images of the $Ca^{2+}$ concentration in the smooth muscle layer of rat tail artery before (a) and during (b–f) the electrical stimulation of sympathetic nerves. The fluorescence intensity of the $Ca^{2+}$ indicator Fluo-3 was normalized by the initial fluorescence intensity ($F/F_0$) and pseudo-color-coded. Each frame was taken every 1 s and has a dimension of 115 × 115 μm. The frame numbers are shown above each panel. Electrical stimulation (5 Hz, 300 pulses) was applied between frames 5 and 64. (B) Fluorescence intensity changes of Fluo-3 (red, blue and green lines) in the three cells (white boxes in A) and the averaged value of all the cells in the field (thick black line). (C) Dependence of $Ca^{2+}$ oscillation on noradrenaline concentration. Modified from M. Iino *et al.*, Ref. 8.

how $IP_3$-induced $Ca^{2+}$ release becomes oscillatory. Studies on the basic properties of $IP_3$-induced $Ca^{2+}$ release have provided clues to this question.

Inspired by the first report on $IP_3$-induced $Ca^{2+}$ release in pancreatic acinar cells,[11] I examined whether $IP_3$ had the same $Ca^{2+}$ releasing activity in smooth muscle cells. The initial results were quite disappointing, and very little $Ca^{2+}$ releasing activity of $IP_3$ was observed despite extensive efforts. I was about to conclude that $IP_3$-induced $Ca^{2+}$ release plays no major role in smooth muscle cells, when I realized that I had always applied $IP_3$ in the virtual absence of $Ca^{2+}$. A breakthrough was brought about when $IP_3$ was applied with a sub-micromolar concentration of $Ca^{2+}$, which markedly enhanced $IP_3$-induced $Ca^{2+}$ release (Fig. 3A).[12),13)] As shown in Fig. 3B, the application of either $IP_3$ or $Ca^{2+}$ alone resulted in almost no $Ca^{2+}$ releasing activity. Only when both $IP_3$ and $Ca^{2+}$ were applied simultaneously was there an activation of $Ca^{2+}$ release.[12)–14)] Following further increase in the $Ca^{2+}$ concentration, there was an inhibition of $Ca^{2+}$ release around 1 μM (Fig. 3C). These results made it clear that there is a bell-shaped dependence of $IP_3$-induced $Ca^{2+}$ release on the cytoplasmic $Ca^{2+}$ concentration.[13)] This original finding in smooth muscle cells was then reproduced in various cell types.[15)–19)]

The bell-shaped $Ca^{2+}$ dependence is the steady-state property of $IP_3$-induced $Ca^{2+}$ release. It is also important to study the time-dependence of the effect of $Ca^{2+}$. Thus, the kinetic property of the $Ca^{2+}$ dependence of $IP_3$-induced $Ca^{2+}$ release was studied using caged $Ca^{2+}$, which can generate a step increase in $Ca^{2+}$ concentration upon a brief ultraviolet light flash. The results indicated that $Ca^{2+}$ had instantaneous potentiating and inhibitory effects on the $IP_3$-induced $Ca^{2+}$ release depending on the magnitude of the step increase in $Ca^{2+}$ concentration (potentiation $<\sim$0.3 μM, inhibition $>\sim$1 μM).[20)] These results indicate that the $IP_3$-induced $Ca^{2+}$ release mechanism has the inherent property of becoming *regenerative* at submicromolar $Ca^{2+}$ concentrations.

The $Ca^{2+}$ sensitivity of $IP_3$-induced $Ca^{2+}$ release bears a resemblance to the $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) mechanism that was initially observed in skeletal muscle cells.[21),22)] The CICR mechanism is now attributed to the ryanodine receptor, which binds ryanodine, a plant alkaloid, in an essentially irreversible manner.[23)] There are three subtypes of ryanodine receptors in mammals, and they function as the $Ca^{2+}$ release channel as homotetramers. Each subunit of the RyR $Ca^{2+}$ release channel consists of about 5,000 amino acids.[23)–25)] On the other hand, the $IP_3$-induced $Ca^{2+}$ release mechanism is mediated by

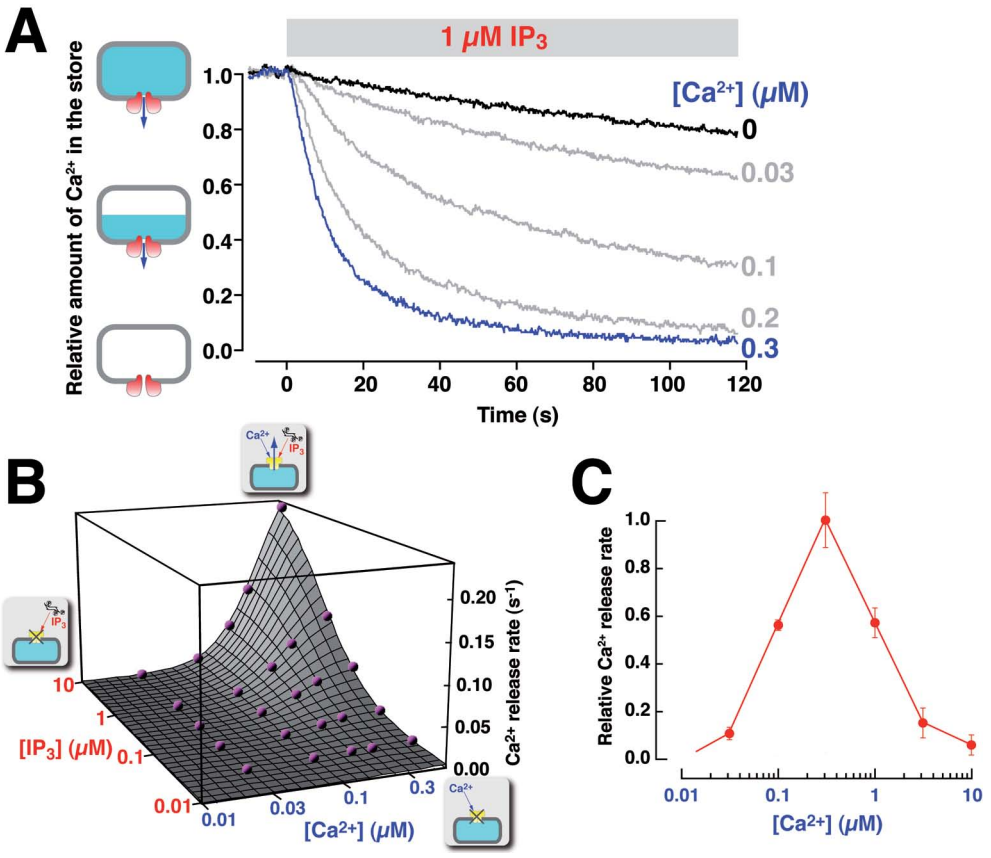


Fig. 3. $Ca^{2+}$ dependence of $IP_3$-induced $Ca^{2+}$ release. (A) Time courses of $IP_3$-induced $Ca^{2+}$ release at different $Ca^{2+}$ concentrations. The luminal $Ca^{2+}$ concentration changes within the ER are shown. The experiments were carried out in the absence of ATP so that there was no uptake of $Ca^{2+}$. (B) Dependence of the rate of $IP_3$-induced $Ca^{2+}$ release on $IP_3$ and $Ca^{2+}$ concentrations. (C) Bell-shaped dependence of $IP_3$-induced $Ca^{2+}$ release on $Ca^{2+}$ concentration. Modified from K. Hirose *et al.*, Ref. 14 (A and B), and from M. Iino, Ref. 13 (C).

the $IP_3$ receptor ($IP_3R$) with about 2,700 amino acid residues.[26] There are three subtypes of $IP_3R$,[26–28] which form $Ca^{2+}$ release channels as homo- or hetero-tetramers. Interestingly, the primary structure of $IP_3R$ has similarity to that of RyR.[26] Hence, RyR and $IP_3R$ are homologous proteins, and both function as a $Ca^{2+}$-dependent $Ca^{2+}$ release channel on the endoplasmic reticulum (ER) membrane. The major difference between the two types of $Ca^{2+}$ release channels is that while RyR can be activated by $Ca^{2+}$ alone, $IP_3R$ requires both $IP_3$ and $Ca^{2+}$ simultaneously for activation. There is another notable difference between $IP_3R$ and RyR. $Mg^{2+}$ is a strong inhibitor of the CICR mechanism of RyR.[6] However, $Mg^{2+}$ has very little, if any, effect on the $Ca^{2+}$ dependence of $IP_3R$ activity.[13,29]

## 3. Regenerative $Ca^{2+}$ release and $Ca^{2+}$ wave/oscillation

The regenerativity of $Ca^{2+}$ release *via* the $Ca^{2+}$ release channels (Fig. 4A) may play an important role in the shaping of spatiotemporal patterns of $Ca^{2+}$ signals. When $Ca^{2+}$ is released locally in one part of the cell, this will further enhance $Ca^{2+}$ release from the adjacent sites, and this will generate a $Ca^{2+}$ wave like 'toppling dominos' (Fig. 4B). $IP_3$-dependent $Ca^{2+}$ waves have been observed in many cell types including smooth muscle cells,[30] oocytes,[31,32] neurons[33] and exocrine cells.[34] In most cases, the velocity of $Ca^{2+}$ waves is about 10–40 μm $s^{-1}$.[35] What may be the physiological significance of $Ca^{2+}$ waves? Diffusion of $Ca^{2+}$ within cells is limited due

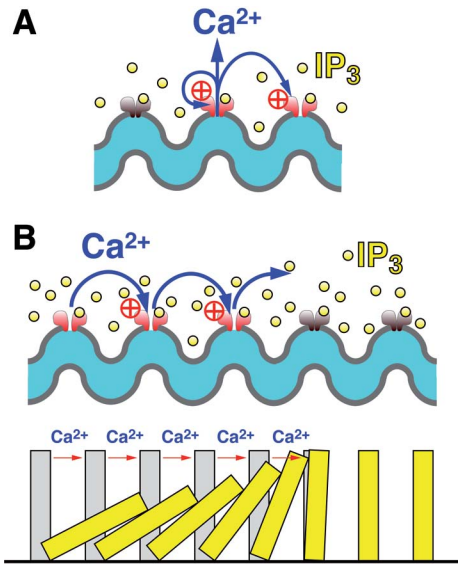


Fig. 4. Regenerative $Ca^{2+}$ release and $Ca^{2+}$ waves. (A) Due to the $Ca^{2+}$ dependence of $IP_3R$, $IP_3$-induced $Ca^{2+}$ release has the inherent property of becoming regenerative. (B) The regenerative $Ca^{2+}$ release may result in the generation of a $Ca^{2+}$ wave (upper panel) like 'toppling dominos' (lower panel).

to the presence of high concentrations of $Ca^{2+}$ binding proteins. Indeed, the diffusion coefficient of $Ca^{2+}$ in cytoplasm is only ~1/30 of that in water and is even ~1/20 of the diffusion coefficient of $IP_3$ in cytoplasm.[36] Thus, for rapid and uniform increase in the intracellular $Ca^{2+}$ concentration, a mechanism to overcome the diffusion delay is required. $Ca^{2+}$ waves can be regarded as a system for transmitting $Ca^{2+}$ signals from one site of the cell to another, and is essentially the same mechanism as the action potentials for the propagation of information in nerve axons. The regenerative activation of sodium channels underlies the propagation of action potentials in neurons. Similarly, the regenerative activation of $Ca^{2+}$ release channels underlies the propagation of $Ca^{2+}$ waves in many types of cells. There have been many theoretical studies to simulate $Ca^{2+}$ waves using mathematical models of the intracellular $Ca^{2+}$ dynamics.[37),38] The regenerative $Ca^{2+}$ release mechanism is the key factor of these models. An additional physiological significance has been attached to the $Ca^{2+}$ wave in pancreatic acinar cells, in which $Ca^{2+}$ waves always initiate from the apical side of the cell and spread toward the basolateral side. In this way, there is a time delay in the increase in $[Ca^{2+}]_i$ between the apical and basolateral membranes. The time difference in $[Ca^{2+}]_i$ is proposed to be important for the unilateral movement of solutes in the acinar cells for excretion.[34]

The importance of regenerative $Ca^{2+}$ release in the generation of $Ca^{2+}$ oscillations has been also postulated in theoretical studies of $Ca^{2+}$ oscillations.[37)–40] This notion was subsequently tested experimentally. Based on the aforementioned structural similarity between $IP_3R$ and RyR, the glutamate residue at position 2100 (E2100) was identified to be a critical amino acid residue of the type 1 $IP_3R$ ($IP_3R1$) for the $Ca^{2+}$ dependence of channel function (Fig. 5A).[41] When E2100 was replaced with aspartate (E2100D), there was a marked reduction in the $Ca^{2+}$ sensitivity without significant change in the $IP_3$ dependence (Fig. 5C). This provided a unique opportunity to test the role of regenerative $Ca^{2+}$ release in the generation of $Ca^{2+}$ oscillations. Indeed, $Ca^{2+}$ oscillation was suppressed in cells expressing E2100D $IP_3R1$ (Fig. 5B). These results established experimentally that the regenerative $Ca^{2+}$ release is essential for the generation of $Ca^{2+}$ oscillations.

The three subtypes of the $IP_3R$ have functional differences[42)–44] and are differentially expressed in various tissues[45)–50] (Table 1). The order of $IP_3$ sensitivity is $IP_3R2 > IP_3R1 > IP_3R3$.[42),43] When multiple subtypes are expressed in the same cell, the $IP_3$ sensitivity becomes roughly the weighted average of the single subtypes.[42] The $Ca^{2+}$ sensitivity has a subtle difference between the three subtypes, and the $Ca^{2+}$ concentration for half maximal activation ($EC_{50}$) is 30–100 nM.[42),44] These $EC_{50}$ values are similar to the resting intracellular $Ca^{2+}$ concentrations, thus are ideal for the regenerative activation of $Ca^{2+}$ release *via* the $IP_3R$. Another similarity between $IP_3R$ and RyR is that both types of $Ca^{2+}$ release channel are activated by ATP and other adenine nucleotides,[6),51] which enhance channel activity without altering the $Ca^{2+}$ dependence. The ATP dependence is observed in $IP_3R1$ and $IP_3R3$, but is absent in $IP_3R2$.[42),43] Interestingly, when multiple $IP_3R$ subtypes are coexpressed in the same cell, the property of $IP_3R2$ in terms of the ATP dependence becomes dominant.[42] This suggests that heterotetramers containing $IP_3R2$ subtypes have no ATP dependence, indicating strong inter-subunit functional interaction. Together these functional differences are reflected in the patterns of $Ca^{2+}$ oscillations, and $IP_3R2$ seems to be the most efficient subtype in gen-

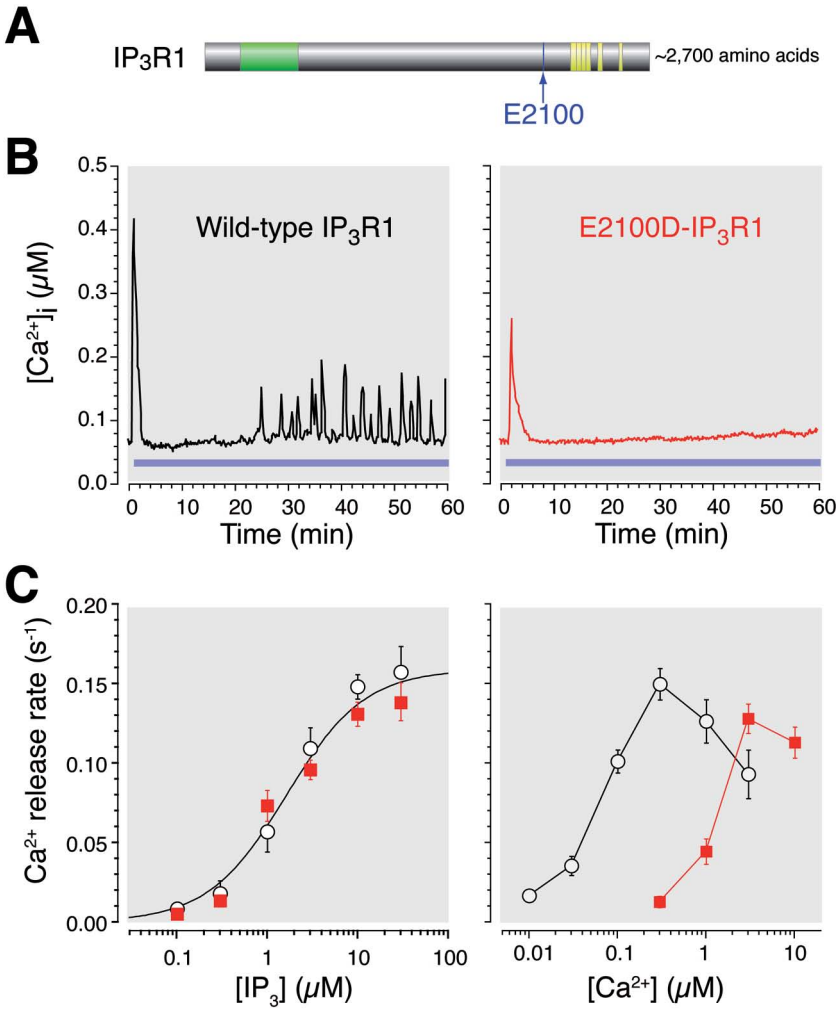


Fig. 5. Requirement of regenerative $Ca^{2+}$ release in $Ca^{2+}$ oscillation. (A) Schematic representation of the primary structure of $IP_3R1$. $IP_3$ binding region (green) and membrane spanning region (yellow) are shown. Position of the $Ca^{2+}$ sensor amino acid (E2100) is also indicated. (B) $Ca^{2+}$ responses of DT40 B cells expressing wild-type (left panel) and E2100D-mutant (right panel) $IP_3R1$ upon activation of B cell receptor (BCR). The time course of $IP_3$ concentration after BCR stimulation measured using $IP_3$ binding protein assay had two phases (Miyakawa, T. and Iino, M., unpublished observation). An initial transient increase after the start of BCR stimulation was followed by a decrease in the $IP_3$ concentration for 10–20 min. Then, a secondary prolonged increase in the $IP_3$ concentration was observed. The biphasic $IP_3$ concentration change seems to underlie the ~20-min silent period after the initial $Ca^{2+}$ response in the left panel. (C) $IP_3$ and $Ca^{2+}$ dependence of $Ca^{2+}$ release *via* wild-type (open symbols) and E2100D (red symbols) $IP_3R1$. Modified from T. Miyakawa *et al.*, Ref. 41.

erating $Ca^{2+}$ oscillations.[42] These findings provide a clear functional basis for the physiological significance of the differential expression of $IP_3R$ subtypes in cell-type specific encoding of $Ca^{2+}$ signaling.

Mechanisms other than the regenerative activation of $IP_3R$ may be involved in the generation of $Ca^{2+}$ oscillations. There are positive and negative feedback mechanisms that regulate intracellular $IP_3$ production. First, the enzyme activity of phospholipase C (PLC) is positively regulated by the intracellular $Ca^{2+}$ concentration at >100 nM concentrations.[52),53] Second, PLC activation induces protein kinase C activation, which in turn inhibits $IP_3$ generation *via* the inhibition of agonist receptor or PLC activity.[54] These positive and negative feedback loops may cause oscillatory changes in the in-

Table 1. Comparison of $IP_3R$ subtypes

| | Type 1 | Type 2 | Type 3 |
|---|---|---|---|
| $IP_3$ sensitivity | Medium | High | Low |
| permeabilized cell (chicken) ($EC_{50}$) | 4.7 μM | 0.35 μM | 18.6 μM |
| planar lipid bilayer (rat) ($EC_{50}$) | 0.27 μM | 0.10 μM | 0.40 μM |
| $Ca^{2+}$ sensitivity (activation)* | Yes | Yes | Yes |
| permeabilized cell (chicken) ($EC_{50}$) | 50 nM | 100 nM | 100 nM |
| planar lipid bilayer (rat) ($EC_{50}$) | 30 nM | 60 nM | 60 nM |
| ATP sensitivity | Yes | No | Yes |
| permeabilized cell (chicken) [Fold increase ($EC_{50}$)] | 5.7-fold (0.4 mM) | 1-fold | 1.7-fold (n.a.) |
| planar lipid bilayer (rat) [Fold increase ($EC_{50}$)] | 5.6-fold (0.13 mM) | 1-fold | 5.9-fold (2 mM) |
| Tissue expression | neuron (enriched in cerebellum) | | neuron (enriched in olfactory bulb) |
| | vascular smooth muscle | | |
| | pancreatic islet | | |
| | endothelium | endothelium | endothelium |
| | liver | liver | |
| | | parotid gland | parotid gland |
| | | glia | |
| | | cardiac myocytes | |

* $Ca^{2+}$ sensitivity of the ascending limb of the bell-shaped $Ca^{2+}$ dependence (see Fig. 3C). See text for references.

tracellular $IP_3$ concentration. Indeed, $IP_3$ oscillation has been observed using the $IP_3$-dependent translocation of GFP-tagged pleckstrin homology domain of PLC$\delta$1 (GFP-PHD).[53),55),56)] Such $IP_3$ oscillations may assist oscillatory release of $Ca^{2+}$ from the ER.

## 4. Mitochondria as another key player in $Ca^{2+}$ oscillation

Although regenerative $Ca^{2+}$ release is essential for $Ca^{2+}$ oscillations, it remains to be clarified how the regenerative $Ca^{2+}$ release is initiated at the onset of each $Ca^{2+}$ oscillation. Using an ER-targeted $Ca^{2+}$ indicator, we observed intraluminal $Ca^{2+}$ concentration during $Ca^{2+}$ oscillations.[57)] Indeed, the ER $Ca^{2+}$ concentration represented a mirror image of the cytoplasmic $Ca^{2+}$ concentration: a decrease in the ER $Ca^{2+}$ concentration was observed during an increase in the cytoplasmic $Ca^{2+}$ concentration. However, there were important deviations from the perfect mirror image. In the first $Ca^{2+}$ oscillation, the cytoplasmic $Ca^{2+}$ concentration reached its peak even though the ER is still releasing $Ca^{2+}$. This suggests that a considerable fraction of $Ca^{2+}$ released from the ER enters non-ER $Ca^{2+}$ stores during the first $Ca^{2+}$ oscillation. In the second and subsequent $Ca^{2+}$ oscillations, on the other hand, the $Ca^{2+}$ release from the ER lagged behind the increase in the cytoplasmic $Ca^{2+}$ concentration. These results indicate that there is a non-ER $Ca^{2+}$ compartment that takes up $Ca^{2+}$ in the first $Ca^{2+}$ oscillation and supplies $Ca^{2+}$ before the initiation of the second and subsequent ER $Ca^{2+}$ release. Since mitochondria have been shown to function as $Ca^{2+}$ buffering organelles,[58)–61)] a mitochondria-targeted GFP-based $Ca^{2+}$ indicator was used to image the intra-mitochondrial $Ca^{2+}$ concentration.[57)] The results showed that there is a preferential loading of mitochondria with $Ca^{2+}$ from the ER during the $Ca^{2+}$ release phase in the first $Ca^{2+}$ oscillation (Fig. 6A). After that, $Ca^{2+}$ was released from the mitochondria, when the ER was taking up $Ca^{2+}$.

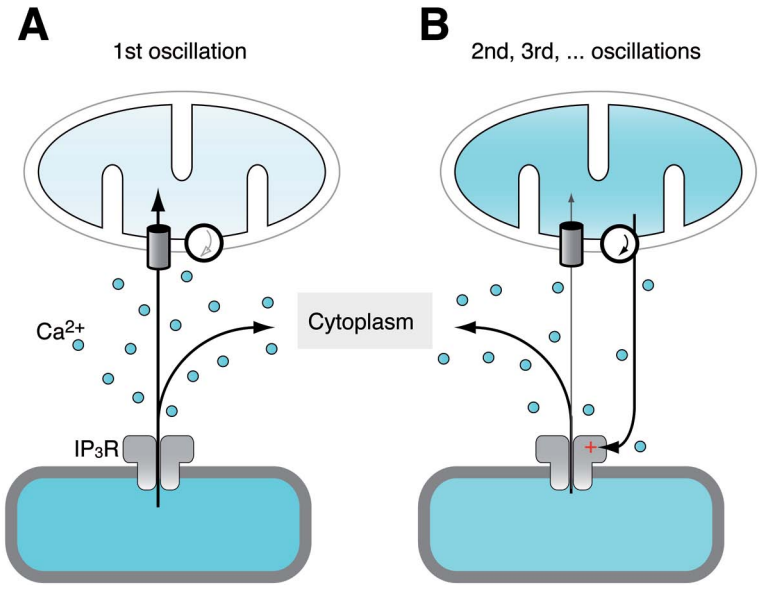


Fig. 6. $Ca^{2+}$ regulation by endoplasmic reticulum and mitochondria during $Ca^{2+}$ oscillations. (A) The first $Ca^{2+}$ oscillation is generated by $Ca^{2+}$ release from the ER *via* $IP_3R$. A considerable fraction of $Ca^{2+}$ released from the ER enters mitochondria. (B) The second and subsequent $Ca^{2+}$ oscillations are initiated by the $Ca^{2+}$ release from mitochondria, which then triggers regenerative $Ca^{2+}$ release *via* $IP_3R$ from the ER. Mitochondrial $Ca^{2+}$ is partially reloaded. The sequence is repeated until mitochondrial $Ca^{2+}$ is depleted. From K. Ishii *et al.*, Ref. 57.

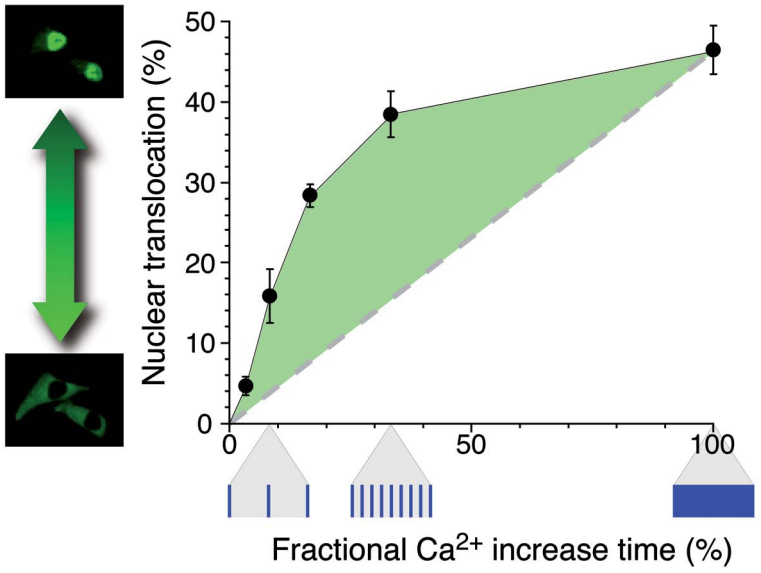


Fig. 7. Nuclear translocation of GFP-tagged NFAT in response to $Ca^{2+}$ oscillations. The steady state value of the NFAT in the nucleus during continuous or oscillatory changes in $[Ca^{2+}]_i$ were plotted against the fractional $Ca^{2+}$ increase time ([sum of the duration of $Ca^{2+}$ increase] ÷ [total duration] × 100%). The observed values lie above the proportional dependence (dashed line). Modified from T. Tomida *et al.*, Ref. 65.

Thus, $Ca^{2+}$ shuttles between the ER and mitochondria during each $Ca^{2+}$ oscillation. As the ER $Ca^{2+}$ loading increased, there was a decline in the ER $Ca^{2+}$ uptake rate, which then allowed mitochondrial $Ca^{2+}$ release to increase the cytoplasmic $Ca^{2+}$ concentration. The increase in the cytoplasmic $Ca^{2+}$ concentration was followed by regenerative $Ca^{2+}$ release from the ER *via* the $IP_3R$ to generate the second and subsequent $Ca^{2+}$ oscillations (Fig. 6B). These findings suggest that mitochondria play a role in the pacemaking of $Ca^{2+}$ oscillations, and that $Ca^{2+}$ efflux from the mitochondria underlies the pacemaker $Ca^{2+}$ increase before the regenerative $Ca^{2+}$ release from the ER.[57)]

## 5. Physiological significance of $Ca^{2+}$ oscillations

$Ca^{2+}$ oscillation regulates many important cell functions. One such function is T-cell activation. Introduction of foreign tissues to the host is detected by T cells, in which $Ca^{2+}$ oscillations are generated.[62)] The $Ca^{2+}$ oscillation then activates the $Ca^{2+}$-calmodulin-dependent phosphatase calcineurin. Calcineurin then dephosphorylates nuclear factor of activated T cells (NFAT), which in turn translocates to the nucleus to enhance transcription of a set of genes.[63)] Because $Ca^{2+}$ oscillation frequency regulates NFAT-dependent transcription,[64)] the dependence of nuclear translocation of NFAT on $Ca^{2+}$ oscillations is a physiologically important mechanism.

We studied the relationship between GFP-tagged NFAT translocation to the nucleus and the frequency of $Ca^{2+}$ oscillation.[65)] Indeed, there was a frequency-dependent translocation of NFAT to the nucleus. In $Ca^{2+}$ oscillations, $[Ca^{2+}]_i$ increases only a fraction of time, i.e., the fractional $Ca^{2+}$ increase time ([sum of the duration of $Ca^{2+}$ increase] $\div$ [total duration] $\times$ 100%) is less than 100%. When the extent of nuclear translocation of NFAT was plotted against the fractional $Ca^{2+}$ increase time, it showed a convex upward dependence rather than a proportional dependence (Fig. 7). This means that the extent of NFAT translocation induced by $Ca^{2+}$ oscillation is supra-proportional to the duration of $Ca^{2+}$ increase. In other words, $Ca^{2+}$ oscillation is an 'energy-saving' signaling mechanism. Since prolonged increase in $[Ca^{2+}]_i$ may have adverse effects on cell survival,[9)] $Ca^{2+}$ oscillation is an efficient system to regulate cell functions.

The major reason why NFAT nuclear translocation utilizes the $Ca^{2+}$ increase efficiently in an energy-saving manner lies with the ability of NFAT to 'memorize' $Ca^{2+}$ increase for a certain amount of time after termination of the $Ca^{2+}$ signal. Dephosphorylation of NFAT by calcineurin is a rapid process and proceeds within a short period of time. However, rephosphorylation of NFAT is rather a slow process taking several minutes. Thus, dephosphorylated NFAT, which is ready for translocation to the nucleus, has a life time of $\sim$7 min.[65)] Therefore, $Ca^{2+}$ oscillations with an interval shorter than the life time of dephosphorylated NFAT are nearly as effective as a continuous $Ca^{2+}$ increase. This property associated with $Ca^{2+}$ oscillations can be generalized, and any molecule that can sustain the effect of $Ca^{2+}$ for a certain length of time should be able to utilize $Ca^{2+}$ oscillations efficiently. For example, phosphorylation of smooth muscle myosin by the myosin light chain kinase is a rapid process, whereas dephosphorylation by the myosin light chain phosphatase is a relatively slow process.[66)] Therefore, phosphorylated myosin can memorize transient increases in $Ca^{2+}$ concentration, and $Ca^{2+}$ oscillation is expected to be an efficient signal to regulate contractions of smooth muscle cells.

## 6. Search for the new functions of $Ca^{2+}$ signaling

As shown in Fig. 1, $Ca^{2+}$ has been shown to regulate numerous cell functions. It seems likely that there are still many unknown cell functions that are regulated by $Ca^{2+}$ signals. Identification of such cell functions will not only further clarify the physiological significance of $Ca^{2+}$ signals but also shed new light on various cell functions. For the search of hitherto unrecognized functions of $Ca^{2+}$ signals, a new and specific method to inhibit $Ca^{2+}$ signaling was developed.

$IP_3$ 5-phosphatase hydrolyzes $IP_3$ with a high specificity.[67)] Therefore, overexpression of the enzyme is expected to block $Ca^{2+}$ signaling by preventing the increase in $IP_3$ concentration.[53),68)] Indeed, when cerebellar Purkinje cells (PCs) were transduced with $IP_3$ 5-phosphatase by Sindbis viral infection, synaptic input-dependent $IP_3$ and $Ca^{2+}$ signaling were effectively inhibited.[69)] PCs are the principal neurons in the cerebellar cortex and receive numerous inputs from the axons (parallel fibers, PFs) of

granule cells to their dendrites. The PF-PC synapses are considered to play a major role in such functions as motor learning and motor coordination.[70] This method clarified a new role of $IP_3$ signaling in the PF-PC synapse. In the spines of the PCs, that is, the postsynaptic side of the PF-PC synapse, both ionotropic and metabotropic glutamate receptors are expressed. When PFs are stimulated with burst stimulation consisting of several pulses at 50–100 Hz, which mimics the physiological stimulation pattern,[71] metabotropic glutamate receptor-dependent $IP_3$ signal is generated in the PCs.[69] The $IP_3$ signal in turn induces a local and transient $Ca^{2+}$ signal in the spines and dendrites of PCs receiving the PF inputs.[72),73] In other words, the postsynaptic $IP_3$-$Ca^{2+}$ signaling functions as a detector of the presynaptic activity. When the $IP_3$ signaling was blocked in PCs by $IP_3$ 5-phosphatase, the synaptic strength of the PF-PC synapse was inhibited. Further analyses showed that postsynaptic $IP_3$ signaling drives a brain-derived neurotrophic factor (BDNF) signaling from the postsynaptic PCs to the presynaptic PF terminals and that the retrograde signaling maintains the presynaptic function.[74] Therefore, the postsynaptic $IP_3$-$Ca^{2+}$ signaling mechanism plays an important role in the activity-dependent synaptic maintenance mechanism.

The $IP_3$ 5-phosphatase method clarified another mechanism that is regulated by $IP_3$-$Ca^{2+}$ signaling. Astrocytes, the major glial cells in mammalian brain, generate spontaneous $Ca^{2+}$ oscillations *in vivo* as well as *in vitro*.[75)–78] The astrocytic $Ca^{2+}$ oscillations were blocked by $IP_3$ 5-phosphatase, and neurite growth over the $Ca^{2+}$ signal-deficient astrocytes was inhibited. The analysis clarified that $Ca^{2+}$ oscillations regulate the expression of N-cadherin on the surface of astrocytes, and that N-cadherin is important for the maintenance of neurite growth.[79]

## 7. Perspectives

Studies on the basic principle of $Ca^{2+}$ signaling led to the clarification of the critical role of the regenerative nature of the $IP_3$-induced $Ca^{2+}$ release mechanism in the spatiotemporal generation pattern of $Ca^{2+}$ signals, such as $Ca^{2+}$ waves and oscillations. $Ca^{2+}$ oscillations provide an efficient way to drive cell functions with a temporally-distributed short duration of $Ca^{2+}$ increase, thus avoiding the adverse effects of continuous $Ca^{2+}$ increases. In parallel with such studies, a new specific method to inhibit $Ca^{2+}$ signaling was discovered and helped us to identify new cellular functions, i.e., an activity-dependent synaptic maintenance mechanism and a neuron-glial cell interaction. Further clarification of the new roles of $Ca^{2+}$ signaling is expected to shed new light on our understanding of many important biological functions.

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## Profile

Masamitsu Iino was born in 1950 in Yamagata, Japan. After receiving MD in 1976 from School of Medicine, Tohoku University, he started his research carrier in Department of Pharmacology headed by Professor Makoto Endo in the same university. After obtaining PhD from Graduate School of Medicine, Tohoku University in 1980, he carried out research in Department of Physiology, University College London as a postdoctoral fellow. After two years of study in the United Kingdom, he returned to Tohoku University in 1982. He then moved to The University of Tokyo in 1984. He was promoted to lecturer in 1991, and then became professor of Department of Pharmacology, Graduate School of Medicine, The University of Tokyo in 1995. Since then he led a research group strong in innovative generation of new imaging methods of signaling molecules. In the early years of his research, he studied $Ca^{2+}$ signaling mechanism in muscle cells. He found a regenerative property of the intracellular $Ca^{2+}$ release mechanism. His following research proved that the mechanism is physiologically essential to generate spatiotemporal patterns of $Ca^{2+}$ signaling, which forms a basis for the versatility of $Ca^{2+}$ as a cellular switch. He then changed his field to neuroscience, and is now extending his research to find novel functions of $Ca^{2+}$ signaling in the brain to shed new light on the mystery of our brain. For his achievements he was awarded Uehara Prize in 2009. His laboratory has produced many talented young investigators. He is an editorial board member of The Journal of Physiology, and is serving as vice dean of the Graduate School of Medicine, The University of Tokyo since 2007.