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Identification of new functions of Ca^{2+} release from intracellular stores in central nervous system

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Abstract

Ca^{2+} release from intracellular stores regulates muscle contraction and a vast array of cell functions, but its role in the central nervous system (CNS) has not been completely elucidated. A new method of blocking IP_3 signaling by artificially expressing IP_3 5-phosphatase has been used to clarify the functions of intracellular Ca^{2+} mobilization in CNS. Here I review two of such functions: the activity-dependent synaptic maintenance mechanism and the regulation of neuronal growth by spontaneous Ca^{2+} oscillations in astrocytes. These findings add new bases for better understanding CNS functions and suggest the presence of as yet unidentified neuronal and glial functions that are regulated by Ca^{2+} store-dependent Ca^{2+} signaling.

Keywords: Inositol 1,4,5-trisphosphate; IP_3 receptor; Ryanodine receptor; Synapse; Neuronal growth; Glia; Calcium signaling

The pioneering work of Setsuro Ebashi on the regulation of muscle contraction [1] led to the recognition of the physiological roles of Ca^{2+} release from the intracellular store in not only muscle contraction but also many other cell functions. The mechanism of Ca^{2+} release from the intracellular store in muscle cells (sarcoplasmic reticulum) was initially identified as the "Ca^{2+}-induced Ca^{2+} release" (CICR) mechanism, in which Ca^{2+} at micromolar concentrations activates the release of Ca^{2+} from the sarcoplasmic reticulum [2]. The molecule responsible for the CICR mechanism was subsequently identified as the ryanodine receptor (RyR). Three subtypes of RyR are expressed in a tissue-specific manner in mammals. Another type of Ca^{2+} release mechanism that is sensitive to inositol 1,4,5-trisphosphate (IP_3) was then discovered and its molecular basis was identified as the IP_3 receptor, which consists of three subtypes in mammals [3]. The IP_3-induced Ca^{2+} release mechanism was also found to be sensitive to Ca^{2+}, so that not only RyR but also IP_3R functions as the CICR mechanism [4,5]. Indeed, RyR and IP_3R are homologous molecules [6,7]. Thus, Ca^{2+} release from the intracellular store is mediated by a family of CICR channels consisting of three subtypes of RyR and three subtypes of IP_3R in mammals.

The intracellular Ca^{2+} release channels IP_3Rs and RyRs are also expressed in the brain in both neurons and glia. Although Ca^{2+} release mechanisms have been implicated in CNS functions such as certain forms of synaptic plasticity [8], their roles in the regulation of cell functions in the CNS remains to be elucidated In this review, I will discuss newly identified CNS functions that are regulated by Ca^{2+} mobilization from the intracellular store, by referring to our recent results [9,10].

IP_3 and Ca^{2+} signaling in cerebellum

Purkinje cells are the sole output neurons from the cerebellar cortex receiving two types of excitatory glutamatergic input: numerous inputs from parallel fibers or granule cell axons and a strong input from usually single climbing fibers originating from the inferior olive. Purkinje cells

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express both IP_3R and RyR. In particular, they have an extremely high type 1 IP_3R expression level. Indeed, the properties and roles of Ca^{2+} release mechanisms in neurons have been most extensively studied in parallel fiber-to-Purkinje cell (PF→PC) synapses.

The primary electrical transmission in PF→PC synapses is mediated by the postsynaptic ionotropic glutamate receptor, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor. Thus, the PF input generates excitatory postsynaptic current (EPSC) in PCs. Ca^{2+} imaging studies in PF→PC synapses have shown that physiologically plausible repetitive PF inputs generate two types of local Ca^{2+} signal in spines and dendrites receiving activated PF inputs [11,12]. The first one is generated by an influx of Ca^{2+} via voltage-dependent ion channels in response to AMPA-receptor-dependent depolarization. This is followed by a delayed Ca^{2+} signal that is dependent on the type 1 metabotropic glutamate receptor (mGluR1), which is coupled to phospholipase C via the G-protein to generate IP_3. The delayed Ca^{2+} response is blocked by the intracellular application of heparin, an inhibitor of IP_3R [11,12]. Thus, the mGluR1-dependent Ca^{2+} signal seems to be mediated by IP_3-induced Ca^{2+} release. We imaged IP_3 signals in fine dendrites of PCs using GFP-PHD, an IP_3 indicator [13]. PF stimulation at 50 Hz induced dendritic IP_3 signaling, and the magnitude of IP_3 signals was dependent on the number of pulses delivered to PFs up to about 20 pulses [14].

The CF input to PCs is also mediated by AMPA receptors and induces action potentials called complex spikes. Complex spikes generate Ca^{2+} influx via voltage-dependent Ca^{2+} channels and induce an increase in intracellular Ca^{2+} concentration in the entire dendritic tree of PCs. Although single complex spikes are not associated with IP_3 signals, repetitive CF stimulation at 1 Hz induces a slow increase in IP_3 concentration within PCs [15]. The physiological role of the CF-induced IP_3 signal remains to be identified.

The synaptic strength of PF→PC synapses undergoes long-term depression (LTD) when PF inputs are coupled with CF inputs for about 5 min [16]. Cerebellar LTD is considered to underlie certain forms of motor learning, such as vestibulo-ocular reflex [16]. It has been shown that Ca^{2+} release via IP_3R is essential for LTD induction, and that no LTD is observed in mutant mice in which PC spines are free of intracellular stores so that Ca^{2+} release within PC spines is absent, although the uncaging of caged Ca^{2+} induces LTD [17]. Because IP_3Rs require IP_3 and Ca^{2+} simultaneously for their activation [4,5], IP_3Rs may function as a coincidence detector of Ca^{2+} and IP_3 signals. Indeed, the pairing of the CF input (generating a Ca^{2+} signal throughout dendritic arborization in PCs) with the PF input (generating a local IP_3 signal) enhances Ca^{2+} release via IP_3Rs [18]. The coincidence detector property of IP_3Rs is considered to underlie the requirement of conjunctive PF and CF stimulations in cerebellar LTD.

Ca^{2+}-dependent maintenance of synaptic strength in cerebellum

The above results indicate that mGluR1-mediated IP_3-induced Ca^{2+} release is important for LTD induction. We then asked whether its role in LTD is the only one played by intracellular Ca^{2+} release in PF→PC synapses. To answer this question, IP_3-Ca^{2+} signaling in PCs was inhibited and the PF→PC synapse was examined to determine if there are any changes in synaptic functions. Because there is no specific drug that inhibits IP_3-induced Ca^{2+} release, IP_3 5-phosphatase (5ppase), which specifically hydrolyzes IP_3 to generate inositol 1,4-bisphosphate, was used [13,14].

5ppase was expressed in PCs by injecting the Sindbis virus encoding 5ppase into the mouse cerebellum, and cerebellar slices were prepared the following day [9]. The magnitude of EPSC (output) recorded in PCs in response to varying PF stimulus (input) intensity was measured to assess the synaptic strength of the PF→PC synapse. There was a significant reduction in the slope of the input–output relationship for the PF→PC synapse. On the other hand, the expression of mutant 5ppase, in which one of the arginine residues within the active center was replaced with alanine (R343A) to reduce the enzyme's activity [14], had no significant effect on synaptic strength. These observations indicate that synaptic strength decreases when IP_3-Ca^{2+} signaling is inhibited in PCs.

The mechanism underlying the reduced synaptic strength at the PF→PC synapse could be either reduced transmitter release from the presynaptic terminal or reduced postsynaptic glutamate sensitivity. The amplitude of quantal EPSC, which was generated by asynchronous vesicular release following PF stimulation in a bath solution in which Ca^{2+} was replaced with Sr^{2+}, was not altered. This result suggests that postsynaptic glutamate sensitivity remained constant. Paired-pulse ratio (PPR), which is often used in assessing presynaptic transmitter release probability in PF→PC synapses, increased, suggesting that the presynaptic function was reduced. The coefficient of variation of EPSCs, which is another measure of transmitter release probability, also increased in agreement with the notion that transmitter release probability decreases when IP_3-Ca^{2+} signal is blocked in PCs.

Taken together, the above results indicate that postsynaptic IP_3-Ca^{2+} signaling in PCs is necessary for the maintenance of presynaptic functions in terms of glutamate release. Therefore, it is likely that a retrograde messenger that maintains presynaptic functions is released from PCs. What then is the retrograde signal? The brain-derived neurotrophic factor (BDNF) is a neurotrophin that is strongly expressed in PCs [19–21]. Furthermore, TrkB, the BDNF receptor, is expressed in granule cells [22,23]. Thus, BDNF can be a potential retrograde messenger at the PF→PC synapse. Indeed, after chronic application of an anti-BDNF antibody to the cerebellar cortex *in vivo*, the PPR subsequently measured in cerebellar slices

increased, suggesting a decrease in the transmitter release probability. To determine whether IP_3-Ca^{2+} signals in PCs and BDNF signals share the same mechanism for maintaining presynaptic functions, both signals were blocked at the same time. Interestingly, the anti-BDNF antibody had no inhibitory effect when IP_3-Ca^{2+} signaling was inhibited by 5ppase. Furthermore, the application of extrinsic BDNF reversed the effect of 5ppase. These results suggest that there is a sequence of events from the PF input to the maintenance of synaptic functions: mGluR1 activation, IP_3 generation, Ca^{2+} release, BDNF production and presynaptic maintenance (Fig. 1).

mGluR1s are located at the periphery of PF→PC synapses, and stronger inputs are required to activate mGluR1 than the AMPA receptor. Indeed, multiple pulses of PF inputs efficiently generate IP_3 signals in PCs [14]. Although PFs generate simple spikes in PCs, which consist of single action potentials, sensory inputs to the cerebellum via mossy fibers generate a burst of action potentials (several pulses on the order of 100 Hz) in granule cells, the somata of parallel fibers [24]. Thus, sensory inputs to the cerebellum are suitable for activating IP_3-Ca^{2+} signaling. In other words, IP_3-Ca^{2+} signaling in PCs may function as an activity sensor of PF→PC synapses. Therefore, the BDNF-mediated presynaptic maintenance mechanism may be an activity-dependent synaptic maintenance mechanism. Through this mechanism, the connection of synapses that are rarely used is expected to become weaker.

Regulation of neurite growth by spontaneous Ca^{2+} oscillations in astrocytes

Glial cells are the major cell type in the brain; they outnumber neurons by a factor of about ten in the mammalian brain. Although glial cells have been traditionally assumed to play mere supporting roles helping neurons with their functions, recent studies clearly showed that glial cells play active roles in the regulation of brain functions [25,26]. Interestingly, astrocytes, the most abundant type of glial cell, generate spontaneous Ca^{2+} oscillations both *in vivo* and *in vitro* [27–30]. However, the physiological significance of the spontaneous Ca^{2+} oscillations remains to be elucidated. Because glial cells play an important role in neuronal growth, the potential role of glial spontaneous Ca^{2+} oscillations in the regulation of neurite growth was examined by inhibiting IP_3-Ca^{2+} signaling [10].

Retroviruses encoding 5ppase were used to inhibit IP_3-Ca^{2+} signaling in astrocytes. Ca^{2+} oscillations were completely abolished in cultured astrocytes transduced with 5ppase. Interestingly, neurons cultured on Ca^{2+} signal-deficient astrocytes had much shorter neurites than those cultured on control astrocytes (Fig. 2). Thus, astrocytic Ca^{2+} oscillations are necessary for supporting neuronal growth. In titrating spontaneous Ca^{2+} oscillations, two types of mutant 5ppase were generated: R343A and R343A/R350A. The double-mutant R343A/R350A 5ppase had a very low enzymatic activity and had no effect on either Ca^{2+} oscillations or neurite growth (Fig. 2). R343A 5ppase had a low enzymatic activity, and inhibited spontaneous Ca^{2+} oscillations but not agonist-evoked Ca^{2+} signals. Thus, when cocultured with neurons, astrocytes expressing R343A 5ppase generated low-frequency Ca^{2+} oscillations that were generated by neuronal activities. Interestingly, neurons cultured on astrocytes expressing R343A 5ppase had significantly shorter neurites than those cultured on control astrocytes (Fig. 2), indicating that spontaneous Ca^{2+} oscillations play a pivotal role in the regulation of neurite growth.

In line with the effect of 5ppase on neurite lengths, the speed of growth cone advancement, as determined by time-lapse imaging, was markedly reduced on Ca^{2+} signal-deficient astrocytes. We then asked whether a diffusible or non-diffusible factor is involved in astrocyte-dependent growth cone advancement. To answer this question, astrocytes were transduced with the 5ppase retrovirus at a lower

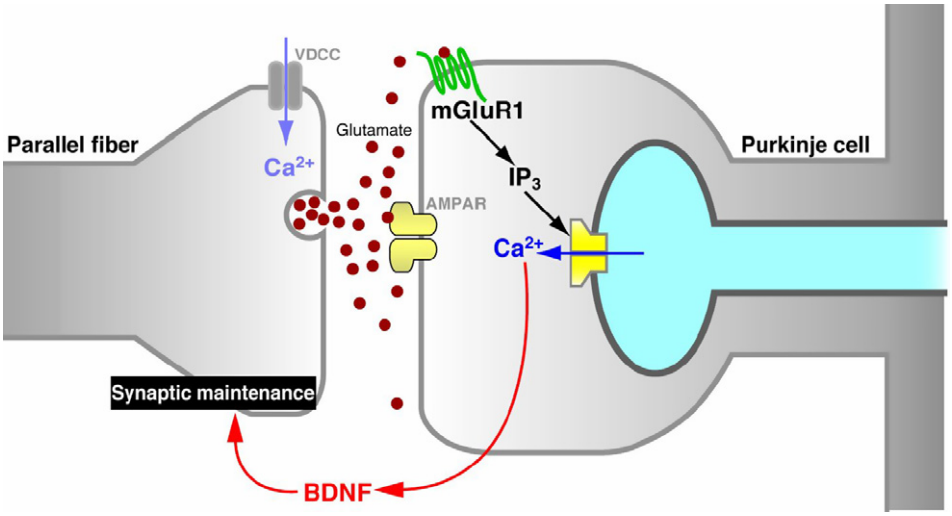

Fig. 1. Schematic of mechanism of synaptic maintenance at PF→PC synapse. VDCC, voltage-dependent Ca^{2+} channel; AMPAR, AMPA receptor.

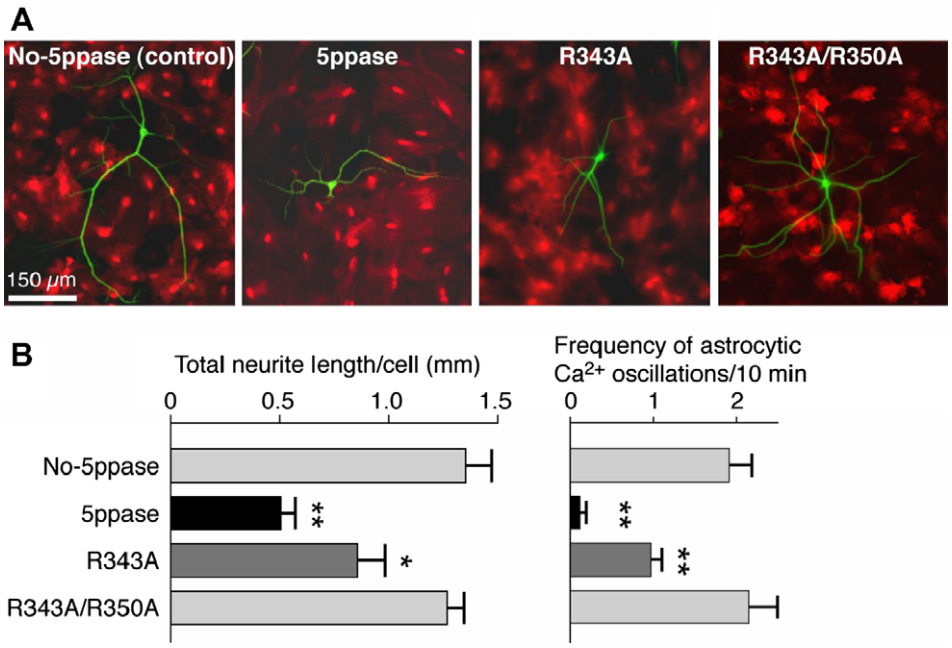

Fig. 2. Inhibition of neurite growth on astrocytes in which spontaneous Ca^{2+} oscillations were suppressed to various extents by wild-type and mutant IP_3 5-phosphatases. (A) Representative images of neuron–glia coculture. Red, astrocytes expressing infection marker DsRed2. Green, neurons immunostained with MAP2. (B) Compiled data of effect of 5ppase on neurite length and frequency of astrocytic Ca^{2+} oscillations. Modified from Ref. [10] (Copyright 2007 by the Society for Neuroscience).

infection rate that will express 5ppase in only about half of the cells in a mosaic pattern. If a diffusible factor is responsible, growth cone advancement will be evenly reduced on the culture; if a non-diffusible factor is involved, growth cones will advance faster on uninfected astrocytes than on infected (Ca^{2+} signal-deficient) astrocytes. The obtained results clearly support the latter, indicating the involvement of a non-diffusible factor.

Astrocytes express on their surface various molecules that either promote or inhibit neuronal growth, such as N-cadherin, laminin and proteoglycans. The above results suggest that the inhibition of Ca^{2+} oscillations in astrocytes down- or up-regulates these molecules. The surface expression of these molecules was examined using immunocytochemistry. Results showed no clear changes in all the tested molecules except N-cadherin. Results of the immunocytochemistry indicate that N-cadherin is down-regulated in Ca^{2+} signal-deficient astrocytes. This was confirmed by western blot and real-time PCR analyses. Thus, spontaneous Ca^{2+} oscillations seem to be necessary for the maintenance of N-cadherin expression.

N-cadherin is an extracellular Ca^{2+}-dependent cell–cell adhesion molecule expressed in neurons and glia, and has an important role in neuronal growth [31–34]. Indeed, when a function-blocking antibody against N-cadherin was added to the culture medium, neurite extension was inhibited. Furthermore, the artificial expression of N-cadherin in Ca^{2+} signal-deficient astrocytes partially rescued neuronal growth. Therefore, N-cadherin down-regulation is at least partly responsible for the inhibition of neuronal growth.

The above results show that spontaneous Ca^{2+} oscillations in astrocytes maintain N-cadherin expression, which is necessary for the regulation of neuronal growth [10]. However, unanswered questions remain. How do Ca^{2+} oscillations control N-cadherin expression? Does the Ca^{2+} oscillation-dependent regulation of neurite growth involve other molecules? What is the *in vivo* role of the Ca^{2+} oscillation-dependent regulation of neurite growth? By answering these questions, we expect to obtain a clearer picture of how glial cells support neuronal growth.

Perspective

Using 5ppase as a new tool for studying the physiological roles of intracellular Ca^{2+} release, we have identified two new roles of intracellular Ca^{2+} release in the CNS. We expect that there are as yet unidentified functions of intracellular Ca^{2+} release that could be identified using the 5ppase method. The identification of such mechanisms will add new bases for better understanding neuronal and glial functions in the CNS.

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