Review

Ca²⁺-Dependent Inositol 1,4,5-Trisphosphate and Nitric Oxide Signaling in Cerebellar Neurons

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Received March 13, 2006

Abstract. Intracellular Ca²⁺ signals are important for the regulation of synaptic functions in the central nervous system. In this review, I summarize findings of our recent studies on upstream and downstream Ca²⁺ signaling mechanisms in cerebellar synapses using novel molecular imaging methods. Inositol 1,4,5-trisphosphate (IP₃)-induced Ca²⁺ release plays a pivotal role in central synapses. The visualization of IP₃ at fine dendrites of Purkinje cells (PCs) using a fluorescent IP₃ indicator showed that intracellular Ca²⁺ concentration has a stimulatory effect on phospholipase C activity, which catalyzes IP₃ production. This indicates that metabotropic and ionotropic glutamate receptors collaborate to generate IP₃ signals. Using a novel nitric oxide (NO) indicator, the spatial distribution of NO signals originating from parallel fiber (PF) terminals was visualized. Our results show that the NO signal decays steeply with distance from the site of production in the cerebellum and is dependent on PF stimulation frequency in a biphasic manner. NO released from PF terminals generated a synapse-specific long-term potentiation of PF-PC synapse when PF was stimulated at certain frequencies. These imaging studies clarified new aspects of the regulatory mechanisms of synaptic functions.

Keywords: Ca²⁺ signaling, inositol 1,4,5-trisphosphate, nitric oxide, Purkinje cell, synaptic plasticity

Introduction

Intracellular Ca²⁺ is one of the most ubiquitous cell signals, and it regulates various important cell functions including contraction, secretion, transcription, and immunity. In excitable cells, Ca²⁺ is a unique molecule that relays signals mediated by membrane potential changes to the cell interior. Furthermore, in response to various signals, Ca²⁺ is released from the intracellular Ca²⁺ store through Ca²⁺ release channels, ryanodine receptors, and inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃Rs). Ryanodine receptors mainly function as a Ca²⁺-induced Ca²⁺ release channel and amplify depolarization-induced Ca²⁺ influx via voltage-dependent Ca²⁺ channels. IP₃Rs are activated in the presence of IP₃, which is generated by phospholipase C downstream of

either G-protein-coupled receptors or tyrosine kinase receptors. IP₃Rs require submicromolar Ca²⁺ concentration for channel opening even in the presence of IP₃ (1). Thus, IP₃Rs are gated cooperatively by IP₃ and Ca²⁺ and function as a Ca²⁺-induced Ca²⁺ release mechanism in the presence of IP₃ (2, 3).

Ca²⁺ influx via voltage-dependent Ca²⁺ channels (VDCCs) plays a central role in neurotransmitter release as a rapid signal in presynaptic terminals. Ca²⁺ influx via NMDA receptors plays an important role in the generation of synaptic plasticity in various neurons including hippocampal neurons (4). Ca²⁺ release from intracellular stores is also involved in various neuronal functions. There are three subtypes of IP₃Rs with similar but distinct properties, which seem to underlie differential Ca²⁺ signaling patterns in various cell types (5). Type 1 IP₃ receptor (IP₃R1) is the major subtype expressed in the central nervous system (CNS). In hippocampal pyramidal cells, the coincidence of synaptic inputs and action potential induces Ca²⁺ release via IP₃R, generating a Ca²⁺ wave traveling along proximal dendrites (6).

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Published online in J-STAGE: May 9, 2006

DOI: 10.1254/jphs.CPJ06006X

Cerebellar Purkinje cells (PCs) express an extremely high amount of IP₃R1 throughout their dendrites and somata. PCs receive two excitatory inputs: one from parallel fibers (PFs) and another from climbing fibers (CFs). Ca²⁺ signaling at PF-PC synapses has been extensively studied, and local Ca²⁺ dynamics at the synaptic site has been well characterized (7, 8). The coincidence of PF activation and CF input has been shown to induce a large Ca²⁺ release from intracellular stores attributable most likely to the cooperative activation of IP₃R1 by IP₃ and Ca²⁺ (9) (Fig. 1, arrows 10 and 13). Such a Ca²⁺ signal is considered to play a pivotal role in the generation of long-term depression (LTD) at PF-PC synapses (10).

Despite our common agreement on the importance of Ca²⁺ signaling mechanisms in the regulation of neuronal functions, there remains much that need to be clarified regarding upstream and downstream Ca²⁺ signals. In our laboratory, we have been studying this issue using molecular imaging techniques. Here, I would like to summarize some aspects of upstream and downstream Ca²⁺ signaling mechanisms in cerebellar neurons on the basis of our recent findings and discuss physiological implications of these new findings.

Visualization of IP₃ signaling in PCs

Glutamate as a neurotransmitter activates both ionotropic and metabotropic glutamate receptors at synapses in the CNS. The activation of group I metabotropic

glutamate receptors (mGluR) results in the activation of phospholipase C β and the generation of IP₃ (Fig. 1, arrows 2 and 5). Accumulating evidence indicates that IP₃-induced Ca²⁺ release generates localized Ca²⁺ signals at PF-PC synapses. However, IP₃ signaling within neurons were only indirectly estimated from resulting Ca²⁺ signals, and a direct analysis of local IP₃ signaling had not been carried out before we attempted to solve this problem.

In 1999, we generated an indicator of IP₃, GFP-PHD, which is a fusion between the green fluorescent protein and the PH domain of phospholipase C δ 1 (11). When GFP-PHD is expressed in cells, most of it is bound to phosphatidylinositol 4,5-bisphosphate (PIP₂) on the plasma membrane at the resting state. Upon the activation of cells to induce IP₃ production, GFP-PHD translocates from the plasma membrane to the cytoplasm to bind to IP₃. Therefore, the extent of cytoplasmic translocation of GFP-PHD indicates the time course and magnitude of IP₃ signals. The use of GFP-PHD enabled the time-lapse imaging of intracellular IP₃ signaling in cultured PCs (12). A quite unexpected finding was obtained: IP₃ production was observed not only upon mGluR activation but also upon AMPA receptor (AMPA) activation, which was not expected to stimulate phospholipase C (PLC) β . It became clear that AMPAR-mediated depolarization-induced intracellular Ca²⁺ concentration increase is sufficient to induce IP₃ production in PCs. There have been several studies that suggested a depolarization-induced IP₃ production in

Fig. 1. Schematic diagram of IP₃-Ca²⁺ signaling downstream of synaptic inputs to PCs. Arrows indicate stimulatory signals.

peripheral tissues. However, it was not confirmed whether IP₃ production was a direct consequence of depolarization or an indirect effect mediated by the depolarization-induced release of agonists within tissues. The direct measurement of IP₃ in single PCs clearly showed that depolarization-induced increase in intracellular Ca²⁺ concentration results in IP₃ production. PLC δ , which translocates to the plasma membrane upon Ca²⁺ increase, may be responsible for the Ca²⁺-dependent IP₃ production (Fig. 1, arrows 15 and 8). Indeed, PLC δ subtypes are expressed in PCs. Further studies are required to clarify the underlying mechanism. To examine whether the Ca²⁺-dependent IP₃ production occurs under conditions close to the physiological environment, the translocation of GFP-PHD was observed during a train of CF-induced complex spikes in cerebellar slices. Indeed, repetitive stimulation of CF at 1 Hz caused a gradual increase in IP₃ concentration in PCs (12). These observations suggest that a continuous activity of CFs may generate a gradual buildup of both

IP₃ and diacylglycerol (DG) (Fig. 1, arrows 7 and 8).

IP₃ signaling at PF-PC synapse

Because mGluR is expressed at the rim of each PF-PC synapse, PF activity is expected to generate IP₃ signaling in postsynaptic PCs. IP₃ generation at PF-PC synapses was studied using sagittal cerebellar slice preparations after the infection of PCs with the Sindbis virus encoding GFP-PHD (13) (Fig. 2A). By two-photon excitation fluorescence microscopy, the translocation of GFP-PHD was measured in fine dendrites of PCs upon the electrical stimulation of PFs (Fig. 2: B and C). The extent of translocation of GFP-PHD depended on the number of PF pulses, and PF-induced IP₃ production was inhibited by a mGluR antagonist, 4-CPG (Fig. 2D). Surprisingly, IP₃ production was also partially inhibited in the presence of an AMPAR antagonist, NBQX (Fig. 2D). These observations suggest that AMPAR-mediated depolarization-dependent Ca²⁺ influx potentiates

Fig. 2. Measurement of PF-stimulation-induced IP₃ signaling in PCs using GFP-PHD. **A:** Sindbis virus-mediated expression of GFP-PHD in PC visualized by two-photon laser scanning microscopy at low (left panel) and high (right panel) magnifications. **B:** Line-scan image of fine dendrite showing translocation of GFP-PHD upon PF stimulation (10 pulses at 50 Hz). **C:** Time courses of fluorescence intensity changes of membrane region and cytoplasmic region for data shown in **B**. **D:** Compiled results of peak translocation of GFP-PHD upon PF stimulation. The number of pulses was varied between 1 and 40 at a constant stimulation frequency of 50 Hz in the absence and presence of glutamate receptor antagonists. Reproduced from ref. 13 with permission from the Society for Neuroscience ©2004.

PLC activity (Fig. 1, arrow 14). Taking these and previous observations together, they indicate that mGluR and AMPAR at PF-PC synapses collaborate to generate local Ca²⁺ signals via two mechanisms, namely, the cooperative activation of PLC (Fig. 1, arrows 2 and 14) and that of IP₃R (Fig. 1, arrows 10 and 12). Indeed, Ca²⁺ measurements showed that mGluR and AMPAR cooperatively generate postsynaptic Ca²⁺ signals (13).

Sensory inputs to the cerebellar cortex are mediated by mossy fibers that terminate on the somata of granule cells, which in turn extend PFs. In vivo electrophysiological measurements showed that sensory inputs cause a burst of action potentials in granule cells (3–4 pulses at approximately 80 Hz) (14). The measurement of PF-induced IP₃ signals in PCs at different pulse numbers (Fig. 2D) and frequencies suggests that mossy fiber-mediated inputs to the cerebellar cortex are such that homosynaptic mGluR and AMPAR cooperatively generate IP₃ signals within PCs (13).

Implications of Ca²⁺-PLC interaction

The hydrolysis of PIP₂ by PLC generates not only IP₃ but also DG, which is the upstream signal of protein kinase C (PKC) (Fig. 1, arrow 16). Furthermore, it has been shown that depolarization and receptor activation cooperatively stimulate PLC β to generate endocannabinoids (15). Thus, the cooperative activation of PLC by mGluR and AMPAR is also important for DG-PKC and endocannabinoid signaling, and hence has a broad scope of physiological significance. We also found that when PCs were dialyzed with whole-cell patch clamp solution containing 20 mM BAPTA, mGluR-mediated IP₃ generation was strongly inhibited (13). This suggests that an increase in cytoplasmic Ca²⁺ concentration is essential for PLC activation. This finding may have an impact on the interpretation of results of experiments in which intracellular Ca²⁺ concentration is strongly buffered by Ca²⁺ chelators such as BAPTA. That is, a high concentration of BAPTA introduced into neurons would block not only Ca²⁺ signals but also DG-PKC signaling. This is an important point when interpreting experimental results that are obtained in the presence of high concentrations of Ca²⁺ buffers in whole-cell patch clamp solution. It is also important to note that under experimental conditions that induce a large increase in cytoplasmic Ca²⁺ concentration in neurons, PLC may be activated, causing the simultaneous activation of the DG-PKC system.

Nitric oxide signaling in cerebellum

Nitric oxide (NO) is an intercellular messenger, the

role of which was first recognized as the endothelium-derived relaxing factor (EDRF) (16). Thus, the activation of endothelial cells by agonists induces the generation of NO, which then diffuses to adjacent vascular smooth muscle cells to cause vascular relaxation. In 1988, Garthwaite et al. showed that cell preparations isolated from the cerebellum (mainly composed of granule cells) liberate a vascular relaxing factor upon NMDA application (17). Pharmacological analyses suggested that the neuron-derived relaxing factor has the same properties as those of EDRF. This observation suggests that NO is also an intercellular signaling molecule in the CNS. Subsequently, NO synthase cDNAs were cloned and three subtypes were identified, NOS1, NOS2, and NOS3 (18). NOS1 is also called neuronal NOS (n-NOS) and is expressed mainly in the nervous system. In the cerebellum, NOS1 is expressed in granule cells but not in PCs. These findings collectively suggest that PFs generate NO during activation, and NO then spreads to neighboring neurons. Indeed, Shibuki et al. succeeded in measuring PF activity-dependent NO signals in cerebellar slices using an NO-sensitive electrode (19). It has been considered that the diffusion of NO is extremely rapid and that it diffuses >100 μ m in all directions from the site of generation. If this is indeed the case, then synaptically generated NO is not a synapse-specific messenger but rather a global modulator. To clearly define the physiological roles of NO in the CNS, it is important to clarify the spatial distribution of NO signals. However, measurements using NO-sensitive electrodes have a very low spatial resolution and it is difficult to estimate the spatial distribution of NO signals. A small molecular NO indicator, DAF-2, has been used for the imaging of NO in biological samples (20). Multistep reactions of NO with ambient O₂ and the subsequent covalent binding of the oxidized product with DAF-2 are required before the fluorescence intensity of DAF-2 increases. The complex reaction of DAF2 with NO limits the response time of DAF-2 and may interfere with the estimation of spatial distribution of the NO signal in the cerebellum. These considerations urged us to develop a new NO indicator.

New genetically encoded NO indicator

The first problem to overcome in designing a new NO indicator is the development of a method of specifically recognizing NO. The physiological target molecule of NO is soluble guanylyl cyclase (sGC), which is a heme protein and generates cyclic GMP from GTP upon binding to NO. Unlike other heme proteins that bind to both NO and O₂, sGC has a unique property of binding to NO but not to O₂. Therefore, we considered that the

sGC molecule may be used for the recognition of NO. A series of deletion mutants of sGC were generated and their heme binding capacity was assessed (21). The N-terminal region of the sGC $\beta 1$ subunit was found to be essential for heme binding and NO recognition. This region of sGC is referred to as the heme-binding region (HBR). A panel of recombinant proteins with fusion between GFP and HBR were assessed for NO-dependent fluorescence intensity change. One of such fusion proteins (HBR-GFP) was used as an NO indicator (22).

HBR-GFP was expressed in the mouse cerebellum using the same viral method for the expression of GFP-PHD. HBR-GFP expressed in PCs in sagittal cerebellar slices was imaged using a confocal microscope. HBR-GFP imaging clearly showed PF stimulation-induced NO signals within PCs (22). Signal intensity decreased steeply with distance from activated synapses (Fig. 3A). Indeed, the spatial distribution of NO signals measured with HBR-GFP was similar to that of the mGluR-mediated Ca^{2+} response. Furthermore, the amplitude of PF-induced NO signals was dependent on stimulation frequency. A burst stimulation (BS) of five pulses at 50 Hz (mimicking physiological firing patterns) was repeated 60 times at different frequencies. There was a frequency-dependent increase in NO signal intensity between 0.25 and 1 Hz (Fig. 3B). Quite unexpectedly, BS at higher frequencies (2–4 Hz) resulted in a decrease in NO signal intensity (Fig. 3B). The inhibitory effect of high-frequency BS is due to retrograde endocannabinoid signaling, which then causes CB1 receptor-mediated inhibition of Ca^{2+} influx through VDCCs in preterminals (23). The resulting decrease in presynaptic Ca^{2+} concentration is responsible for the decreased NOS activity. Indeed, the application of the CB1 receptor antagonist AM281 reversed the inhibitory effect at 4-Hz BS (Fig. 3B, red square). Taken together, NO signaling at PF-PC synapses functions as a frequency discriminator converting specific patterns of PF activity to an NO signal.

NO generation and synaptic plasticity

What then is the physiological role of PF-induced NO signals in PCs? Lev-Ram et al. (24) showed that PF stimulation at 1 Hz for 5 min induces a long-term potentiation (LTP) of PF-PC synapses. They used single pulses to stimulate PFs, and this type of synaptic plasticity is hereafter referred to as 1-Hz-SP LTP. They also showed that 1-Hz-SP LTP is NO-dependent because it is blocked by a NOS inhibitor and NO donor application induces LTP in cerebellar slice preparations. Furthermore, it was shown that a postsynaptic mechanism rather than a presynaptic one is involved in 1-Hz-

SP LTP. Because 1-Hz BS generates NO, we examined its effect on PF-PC synapses. Indeed, we found that 1-Hz BS for 60 s generated LTP, which was inhibited in the presence of a NOS inhibitor. Paired-pulse ratio was not changed before and after the induction of LTP, suggesting that 1-Hz-BS LTP is also generated postsynaptically. These observations suggest that both 1-Hz SP (for 5 min) and 1-Hz BS (for 1 min) generate the same NO-dependent LTP at PF-PC synapses.

Because NO has been regarded as a far-reaching messenger, the spatial distribution of 1-Hz-BS LTP was examined. Two separate beams of PFs were stimulated using two stimulating electrodes. LTP was generated only in PF-PC synapses that received BS, but not in adjacent nonoverlapping synapses (Fig. 3C). Therefore, 1-Hz-BS LTP is generated in a synapse-specific manner, which is consistent with the notion that a NO signal decays steeply with distance from activated synapses (Fig. 3A). Furthermore, BS-induced LTP is dependent on PF stimulation frequency in a biphasic manner with a peak at 1 Hz (Fig. 3D). The BS frequency dependence of LTP closely parallels that of NO production (Fig. 3B). Indeed, the inhibition of both NO production and LTP generation by 4-Hz BS is reversed by the CB1-receptor antagonist AM281 (Fig. 3: B and D, red symbols).

Potential physiological roles of NO-mediated LTP

Because both LTD and 1-Hz SP and BS-induced LTP are generated postsynaptically, it is possible that LTP extinguishes LTD (25). That is, 1-Hz LTP may eliminate LTD to remodel neural circuit connections. It is quite interesting that a certain frequency of PF activity is required to induce LTP. This suggests that a specific form of input is required to remodel cerebellar circuits.

Another possibility is that 1-Hz LTP causes “lateral potentiation” during LTD formation. When 1-Hz PF stimulation is paired with CF stimulation, LTD is generated (26). Each PF innervates multiple PCs as shown in Fig. 4. Along a PF activated at 1 Hz, LTD of a PF-PC synapse is generated in a PC that receives concomitant CF inputs. On the other hand, in adjacent PCs, which do not receive simultaneous CF inputs, PF-PC synapses undergo LTP. These synaptic plasticities in opposite directions enhance the spatial gradient of synaptic strength in neighboring PCs (Fig. 4). Deep cerebellar nuclei receive GABAergic inhibitory inputs from PCs. Therefore, the spatial gradient in the PF-PC synapses results in “lateral inhibition” at the level of deep cerebellar nuclei (Fig. 4); and this might be important for the tuning of cerebellar circuits.

Future directions

The findings obtained using new indicators of signaling molecules have clarified new aspects of cellular signaling at central synapses. These findings would have been difficult to obtain using conventional methods only. Therefore, imaging methods will be promising for the elucidation of neuronal signaling mechanisms. These results highlighted the importance of Ca^{2+} signals in the CNS. It is, therefore, important to further clarify the roles of Ca^{2+} signals. We showed that IP_3 5-phosphatase overexpression specifically blocks the IP_3 R-mediated Ca^{2+} signaling (11, 12). Such a method is expected to allow us to obtain novel insights into the role of Ca^{2+} signaling in various neuronal functions.

Acknowledgments

I thank Drs. Sho Kakizawa and Yohei Okubo for reading the manuscript. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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