Cyclic ADP Ribose-Dependent Ca\(^{2+}\) Release by Group I Metabotropic Glutamate Receptors in Acutely Dissociated Rat Hippocampal Neurons

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Abstract

Group I metabotropic glutamate receptors (group I mGluRs; mGluR1 and mGluR5) exert diverse effects on neuronal and synaptic functions, many of which are regulated by intracellular Ca\(^{2+}\). In this study, we characterized the cellular mechanisms underlying Ca\(^{2+}\) mobilization induced by (R5)-3,5-dihydroxyphenylglycine (DHPG; a specific group I mGluR agonist) in the somata of acutely dissociated rat hippocampal neurons using microfluorometry. We found that DHPG activates mGluR5 to mobilize intracellular Ca\(^{2+}\) from ryanodine-sensitive stores via cyclic adenosine diphosphate ribose (cADPR), while the PLC/IP3 signaling pathway was not involved in Ca\(^{2+}\) mobilization. The application of glutamate, which depolarized the membrane potential by 28.5±4.9 mV (n = 4), led to transient Ca\(^{2+}\) mobilization by mGluR5 and Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels. We found no evidence that mGluR5-mediated Ca\(^{2+}\) release and Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels interact to generate supralinear Ca\(^{2+}\) transients. Our study provides novel insights into the mechanisms of intracellular Ca\(^{2+}\) mobilization by mGluR5 in the somata of hippocampal neurons.

Introduction

The group I metabotropic glutamate receptors (mGluRs), which include mGluR1 and mGluR5, play important roles in regulating intrinsic excitability and synaptic plasticity [1,2,3]. Importantly, intracellular Ca\(^{2+}\) contributes to various aspects of mGluR-mediated effects. Enhancement of neuronal excitability [4,5,6] and long-term depression mediated by mGluR (mGluR-LTD) [7] were shown to be blocked by intracellular dialysis of BAPTA, and the involvement of Ca\(^{2+}\)-dependent proteins such as PICK1 and NCS-1 in mGluR-LTD has recently been demonstrated [8,9,10]. In addition, mGluR triggers retrograde endocannabinoid signaling, an effect that is greatly enhanced by increases in Ca\(^{2+}\) [11,12]. However, the signaling pathways and the source of Ca\(^{2+}\) that contributes to these diverse effects have not yet been clearly elucidated.

It is well known that group I mGluRs mobilize Ca\(^{2+}\) from intracellular stores in hippocampal neurons [13,14]. As group I mGluRs are coupled to Gq proteins [13,15], Ca\(^{2+}\) mobilization may involve the phospholipase C (PLC)/inositol-3-triphosphate (IP3) signaling pathways [1]. Indeed, the synergistic or supralinear Ca\(^{2+}\) release by group I mGluR stimulation paired with backpropagating action potential (AP) was shown to be from IP3 receptor (IP3R)-sensitive intracellular stores in apical dendrites of CA1 hippocampus [17,18]. However, studies in midbrain dopaminergic neurons demonstrated that intracellular Ca\(^{2+}\) mobilization by group I mGluRs required cyclic ADPR ribose (cADPR)/ryanodine receptors (RyRs) as well as IP3/IP3Rs [19]. The role of cADPR in mGluR-mediated Ca\(^{2+}\) signaling is supported by the study showing that the glutamate-induced stimulation of ADP-ribosyl cyclase occurs preferentially in NG108-15 neuroblastoma/glioma hybrid cells over-expressing mGluR1, 3, 5, and 6 [20]. It is not yet clear if this finding could also be extended to hippocampal neurons, but considering the frequent involvement of PLC-independent signaling pathways in several effects of group I mGluRs [21,22,23,24], the possibility that Ca\(^{2+}\) mobilization by group I mGluR may be mediated by signal pathways other than PLC/IP3Rs should be tested in hippocampal neurons.

Ca\(^{2+}\) signaling in neurons is highly compartmentalized, with Ca\(^{2+}\) having distinctive roles in each section [25,26,27]. Mechanisms involved in axonal and dendritic Ca\(^{2+}\) signaling have been extensively studied due to their importance in the regulation of neurotransmitter release and synaptic plasticity [28,29,30]. Somatic Ca\(^{2+}\) signals also play important roles in regulating cellular excitability, synaptic plasticity and gene expression [7,31,32], but the mechanisms involved in somatic Ca\(^{2+}\) signals are not well studied. As different neuronal compartment may have distinct Ca\(^{2+}\) signaling machinery, results obtained from dendrites or axons may not extend to the somatic Ca\(^{2+}\) signals. Therefore, separate studies of somatic Ca\(^{2+}\) signals are warranted.
In the current study, we directly investigated the signaling pathways underlying somatic Ca\textsuperscript{2+} mobilization by group I mGluRs. Using microfluorometric Ca\textsuperscript{2+} measurements in the somata of acutely dissociated rat hippocampal neurons loaded with Fura 2-AM, we discovered that stimulation of group I mGluRs induces the cADPR-dependent Ca\textsuperscript{2+} mobilization from ryanodine-sensitive stores. Our results represent a novel mechanism for Ca\textsuperscript{2+} mobilization by group I mGluRs in hippocampal neurons.

Results

mGluR5 is responsible for DHPG-induced Ca\textsuperscript{2+} release from intracellular stores

To investigate the mechanisms underlying Ca\textsuperscript{2+} increase by group I mGluR stimulation, acutely dissociated hippocampal CA1 neurons were loaded with 2 μM Fura 2-AM for microfluorometry experiments. The application of 50 μM (R)-3,5-dihydroxyphenylglycine (DHPG), a specific group I mGluR agonist, to these cells rapidly increased intracellular Ca\textsuperscript{2+} concentrations in the somata. The amplitude of DHPG-induced Ca\textsuperscript{2+} increase (Ca\textsubscript{DHPG}) was highly variable among cells, ranging from ~20 nM to ~500 nM (mean = 97.5 ± 7.8 nM, n = 168), but Ca\textsubscript{DHPG} values obtained from the same cell upon repetitive application of DHPG at 2 min intervals yielded consistent data (Ca\textsubscript{DHPG,2}/Ca\textsubscript{DHPG,1} = 98.6 ± 5.1%, n = 6).

To investigate the mechanism of DHPG-induced Ca\textsuperscript{2+} increase, we regarded CaDHPG,1 as the control and applied various experimental solutions (C) or 2 μM thapsigargin (D) prior to the second application of DHPG. The relative amplitude of Ca\textsubscript{DHPG,2} compared with Ca\textsubscript{DHPG,1} (Ca\textsubscript{DHPG,2}/Ca\textsubscript{DHPG,1}) was obtained to study the contribution of each variable to the DHPG-induced Ca\textsuperscript{2+} increase.

The amplitude of the second Ca\textsuperscript{2+} transient was significantly suppressed by the selective mGluR5 antagonist, MPEP (25 μM), but not by the mGluR1 antagonist LY367385 (100 μM), indicating that mGluR5 is responsible for the DHPG-induced Ca\textsuperscript{2+} increases in hippocampal CA1 neurons (Figure 1A & 1B). DHPG-induced Ca\textsuperscript{2+} transients were not affected by the removal of external Ca\textsuperscript{2+} or the inhibition of receptor-operated Ca\textsuperscript{2+} entry by SKF96365 (10 μM), but they were markedly suppressed when cells were pretreated with the sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) inhibitor thapsigargin (2 μM) for 5 min, indicating that DHPG mobilizes Ca\textsuperscript{2+} from its intracellular stores (Figure 1C–E).

mGluR5-induced Ca\textsuperscript{2+} release from intracellular stores is independent of PLC/IP\textsubscript{3}

We next tested whether the PLC/IP\textsubscript{3} signaling pathways link mGluR5 and Ca\textsuperscript{2+} mobilization. Interestingly, DHPG-induced Ca\textsuperscript{2+} release was not affected by the PLC inhibitor U73122 (1 μM; Figure 2A & 2C). Conversely, muscarinic receptor-mediated Ca\textsuperscript{2+} transients induced by the muscarinic receptor agonist carbachol (CCh; 10 μM) were completely inhibited by U73122 (Figure 2B & 2C), confirming that U73122 effectively inhibited the PLC pathway under our experimental conditions. Subsequently, we loaded intact cells with heparin (20 mg/ml in the electroporation pipette), a competitive antagonist of the IP\textsubscript{3}Rs [33], using a single-cell electroporator. Loading of heparin was confirmed by co-administration of the fluorescent compound Alexa Fluor-488 (Figure 2D). This manipulation completely inhibited the induction of Ca\textsuperscript{2+} transients by CCh, but not those by DHPG (Figure 2E).

For quantitative analyses, we measured the first DHPG-induced Ca\textsuperscript{2+} transient in a Fura 2-AM-loaded neuron, patched the same neuron with a Fura 2 (10 μM)-containing pipette with or without heparin (1 mg/ml), and re-applied DHPG to elicit a second Ca\textsuperscript{2+} transient. We confirmed that the amplitudes of the second Ca\textsuperscript{2+} transients, which were measured at a holding potential of ~60 mV, did not differ from those of the first Ca\textsuperscript{2+} transients (103.2 ± 14.7%, n = 4) (Figure 2F, left bar). The inclusion of heparin did not affect DHPG-induced Ca\textsuperscript{2+} transients (97.1 ± 24.6%, n = 4) (Figure 2F, right bar).

PLC\textsubscript{β1} and PLC\textsubscript{β4} are known to mediate group I mGluR signaling in the brain [34,35]; therefore, we tested whether DHPG induces Ca\textsuperscript{2+} transients in hippocampal CA1 neurons isolated from mice lacking the PLC\textsubscript{β1} or PLC\textsubscript{β4} subunits. As illustrated in Figure 2G, DHPG was still able to induce Ca\textsuperscript{2+} transients in cells from PLC\textsubscript{β1} or PLC\textsubscript{β4} knockout mice. These results suggest that mGluR5 induces Ca\textsuperscript{2+} release independently of PLC/IP\textsubscript{3} signaling pathways.

mGluR5 activates cADPR pathways to induce RyR-dependent Ca\textsuperscript{2+} release from intracellular stores

Alternatively, cADPR, which is metabolized from nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) by ADP-ribose cyclases [36,37,38], may be involved in mobilizing Ca\textsuperscript{2+} from intracellular stores. The involvement of ADP-riboseyl cyclase and/or cADPR in agonist-induced intracellular Ca\textsuperscript{2+} mobilization has been described in a variety of cell types [39,40,41,42,43,44,45,46]. Notably, overexpression of mGluR1 or mGluR5 in NG108-15 cells induced the activation of ADP-ribose cyclase [20]. In addition, group I mGluR-induced Ca\textsuperscript{2+} release from midbrain dopaminergic neurons was shown to be mediated by both IP\textsubscript{3} and cADPR [19]. Therefore, we tested the involvement of cADPR signal pathways in DHPG-induced Ca\textsuperscript{2+} mobilization.

When cells were pretreated with nicotinamide (5 mM) for 5 min to inhibit ADP-ribose cyclase [47], DHPG was no longer able to induce the production of Ca\textsuperscript{2+} transients (Figure 3A). Similarly, in...
Our results demonstrated a predominant role for the cADPR/RyR signaling pathway in mGluR5-induced Ca\(^{2+}\) release in the somata of hippocampal neurons. In the brain, glutamate is the natural neurotransmitter that stimulates mGluRs and ionotropic glutamate receptors (iGluRs), which include AMPA and NMDA receptors. NMDA receptors and the Ca\(^{2+}\)-permeable AMPA receptors are possible sources of Ca\(^{2+}\) entry into the cell. In addition, glutamate-induced membrane depolarization should activate voltage-gated Ca\(^{2+}\) channels (VGCCs) to cause Ca\(^{2+}\) influx. Indeed, we confirmed that glutamate depolarization causes Ca\(^{2+}\) influx from 29.0±4.6 mV (n = 4). As shown in Figure 4A, the application of DHPG and glutamate (30 μM) on the same cell demonstrated that the amplitude of glutamate-induced Ca\(^{2+}\) transients (Ca\(_{\text{Glu}}\)) was significantly greater than Ca\(_{\text{DHPG}}\). The average Ca\(_{\text{DHPG}}\) was 92.8±10.3 nM from 74 cells, whereas Ca\(_{\text{Glu}}\) was 284.3±23.6 nM from the same population (Figure 4B, p<0.01). The Ca\(_{\text{DHPG}}\)/Ca\(_{\text{Glu}}\) ratio was calculated to be 39.0±3.6% (n = 74).

Group I mGluR Ca\(^{2+}\) Signaling in Hippocampus

Subsequently, experiments were performed to determine the source of Ca\(^{2+}\) entry when cells were stimulated with glutamate. Repetitive application of glutamate at 2 min intervals yielded reproducible Ca\(_{\text{Glu}}\) values (Ca\(_{\text{Glu2}}\)/Ca\(_{\text{Glu1}}\) = 103.6±8.7%, n = 5). When the bath solution was replaced with a Ca\(^{2+}\)-free solution prior to the second application of glutamate, the Ca\(_{\text{Glu2}}\) was significantly decreased so that the Ca\(_{\text{Glu2}}\)/Ca\(_{\text{Glu1}}\) ratio was 50.4±9.6% (n = 6, Figure 5A). Unexpectedly, the addition of the NMDA receptor blocker AP-5 before the second application of glutamate had no effect, and the Ca\(_{\text{Glu2}}\)/Ca\(_{\text{Glu1}}\) ratio was 101.7±5.0% (n = 7, Figure 5B). In contrast, the addition of the AMPA receptor blocker CNQX prior to the second application of glutamate was as effective as the removal of Ca\(^{2+}\), producing a Ca\(_{\text{Glu2}}\)/Ca\(_{\text{Glu1}}\) ratio of 45.1±7.1% (n = 8, Figure 5C), suggesting that AMPA receptors may be involved in glutamate-induced Ca\(^{2+}\) influx. However, the addition of 1-naphthyl acetyl spermine (NASPM, 10 μM), a specific blocker of the Ca\(^{2+}\)-permeable AMPA receptor, had no significant effect on glutamate-induced Ca\(^{2+}\) transients (Figure 5D). These results suggest that neither NMDA nor AMPA receptors are involved in the observed Ca\(^{2+}\) influx pathway, but that AMPA receptor activation may trigger Ca\(^{2+}\) influx through VGCCs by depolarizing the membrane potential. In support of this, we found that glutamate-induced membrane depolarization was 5.0±1.2 mV (n = 3) in the presence of CNQX, which is significantly less than that observed in the control (29.0±4.6 mV, n = 4, p<0.05).

To identify the subtype of VGCC responsible for the calcium influx, we tested the effects of specific pharmacological inhibitors. We found that nimodipine (10 μM), an L-type Ca\(^{2+}\) channel blocker, significantly reduced glutamate-induced Ca\(^{2+}\) transients (Figure 6A). In contrast, the amplitude of Ca\(_{\text{Glu}}\) was not significantly affected by α-conotoxin GVIA (1 μM), α-agatoxin IVA (200 nM), and NiCl\(_2\) (100 μM), indicating that N-type, P/Q type, T-type and R-type Ca\(^{2+}\) channels are not involved (Figure 6B). These data indicate that in glutamate application
experiments L-type Ca²⁺ channels mediate Ca²⁺ entry triggered by AMPA receptor-mediated depolarization.

cADPR/RyR-dependent Ca²⁺ release does not interact with the Ca²⁺ influx through L-type Ca²⁺ channels

To understand the complexity of mGluR-mediated Ca²⁺ signaling, it is necessary to examine the interaction between mGluR-induced Ca²⁺ release and glutamate-induced Ca²⁺ influx. It has been shown that Ca²⁺ entry through VGCCs interacts synergistically with IP₃ to enhance mGluR-mediated Ca²⁺ release in apical dendrites of hippocampal CA1 neurons [17,18]. Supralinear Ca²⁺ release by DHPG along with either membrane potential depolarization or NMDA receptor activation was also demonstrated in primary cultured hippocampal neurons [13]. Conversely, Topolnik et al. (2009) demonstrated that dendritic L-type Ca²⁺ channels are enhanced by mGluR5-induced Ca²⁺ mobilization from ryanodine-sensitive stores in the GABAergic interneurons of the hippocampus [53]. Notably, cADPR was shown to enhance L-type Ca²⁺ channels induced by both orthograde and retrograde pathways in NG108-15 cells [54]. Therefore, we analyzed Ca²⁺ transients by DHPG and glutamate, under the assumption that CaGlut represents the sum of Ca²⁺ influx (CaInflux), Ca²⁺ mobilization by mGluR3 (CaDHPG), and the supralinear Ca²⁺ transients (CaSUP) by synergistic effects of mGluR5 and Ca²⁺ influx (CaGlut = CaInflux + CaDHPG + CaSUP).

We estimated CaSUP by subtracting the sum of CaDHPG (representing RyR-dependent release; indicated by the red boxes in Figure 7A & 7B) and CaInflux, which was estimated from the glutamate-induced Ca²⁺ transients in the presence of MPEP or ryanodine (indicated by blue boxes, Figure 7A & 7B) from CaGlut in the control condition (CaSUP = CaGlut - CaDHPG - CaInflux). In this

Figure 3. DHPG-induced intracellular Ca²⁺ mobilization occurs via the cADPR/RyR signaling pathways. DHPG-induced Ca²⁺ transients were completely inhibited when cells were pretreated with 5 mM nicotinamide (A), 100 µM 8-NH₂-cADPR (B), or 20 µM ryanodine (C) prior to the second application of DHPG. (D) Bar graphs represent the relative peak amplitude of second Ca²⁺ transients to those of first (CaDHPG₂/CaDHPG₁). Scale bars indicate 10 sec (horizontal) and 50 nM (vertical). EP = electroporation, NA = nicotinamide, cADPR = 8-NH₂-cADPR, RYA = ryanodine. ** indicates p<0.01.

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Figure 4. Glutamate-induced Ca²⁺ transients are significantly larger than DHPG-induced Ca²⁺ transients. (A) Cells were treated with both DHPG (50 µM) and glutamate (30 µM). (B) Bar graphs summarizing the average amplitudes of DHPG- and glutamate-induced Ca²⁺ transients from 74 cells. Scale bars indicate 10 sec (horizontal) and 50 nM (vertical). Glu = glutamate. ** indicates p<0.01.

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Figure 5. AMPA receptors, but not NMDA receptors, are responsible for glutamate-induced Ca²⁺ influx. (A) The amplitudes of glutamate-induced Ca²⁺ transients (CaGlut) were significantly attenuated in Ca²⁺-free solutions. CaGlut was not affected by AP-5 (B), but was significantly decreased by the pretreatment with CNQX (C). (D) CaGlut was not inhibited by NASPM. (E) Bar graphs represent the ratio between first and second CaGlut. Scale bars indicate 10 sec (horizontal) and 50 nM (vertical). ** indicates p<0.01.

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significant effect. Ca Glu,2 in the presence of U73122 was found to be 58.9%

The estimated Ca influx was found to be 58.9 ± 0.2% of CaGlu (n = 6, Figure 7A) and 39.7 ± 0.6% of CaGlu (n = 4, Figure 7B), which is comparable to the values shown in Figure 4 (39.0 ± 3.6%, n = 74). The estimated CaInflux was found to be 58.9 ± 10.2% of CaGlu (n = 6, Figure 7A) in MPEP and 52.4 ± 19.0% of CaGlu (n = 4, Figure 7B) in ryanodine. Thus, the sum of CaDHGP and CaInflux was close to CaGlu (93.2 ± 10.5% in Figure 7A and 92.1 ± 9.1% in Figure 7B), and CaSUP was found to be negligible (Figure 7C).

Because previous reports have demonstrated the role of IP3Rs in synergetic Ca2+-release by mGluR and backpropagating APs [17,18], we tested the effect of U73122 (1 μM) but found no significant effect. CaGlu2, in the presence of U73122 was 95.6 ± 8.8% of CaGlu1 (n = 8, Figure 7D), suggesting that the PLC/IP3 pathway does not contribute to either CaDHGP or CaSUP. Taken together, these results suggest that, in the somata of hippocampal neurons, cADPR/RyR-dependent Ca2+ mobilization by mGluR5 and Ca2+ influx through the L-type Ca2+ channels do not interact to generate supralinear Ca2+ transients.

Series of experiments, CaDHGP was 34.3 ± 10.2% of CaGlu (n = 6, Figure 7A) and 39.7 ± 12.9% of CaGlu (n = 4, Figure 7B), which is comparable to the values shown in Figure 4 (39.0 ± 3.6%, n = 74). The estimated CaDHGP was found to be 58.9 ± 10.2% of CaGlu (n = 6, Figure 7A) in MPEP and 52.4 ± 19.0% of CaGlu (n = 4, Figure 7B) in ryanodine. Thus, the sum of CaDHGP and CaInflux was close to CaGlu (93.2 ± 10.5% in Figure 7A and 92.1 ± 9.1% in Figure 7B), and CaSUP was found to be negligible (Figure 7C).

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Discussion

We have demonstrated the mechanisms underlying the mGluR5-induced Ca2+ mobilization in the somata of hippocampal neurons. Our results indicate that the cADPR signaling pathways are responsible for the mGluR5-induced Ca2+ mobilization from ryanodine-sensitive stores. In addition, we found that glutamate-induced Ca2+ influx via the L-type Ca2+ channels does not interact with mGluR5-induced Ca2+ mobilization to cause a supralinear Ca2+ increase. These results provide novel insights into the mechanisms for group I mGluR-induced Ca2+ mobilization in the somata of hippocampal neurons.

cADPR has long been known to be an endogenous Ca2+-releasing messenger [45,46,51], and the role of cADPR in neuronal Ca2+ signaling has previously been identified [41,43,44]. Involvement of cADPR in mGluR-mediated Ca2+ signaling was previously demonstrated in midbrain dopamine neurons [19]. This study demonstrated that, in the presence of synaptic blockers (except for mGluR), synaptic stimulation of the dopamine neurons evoked Ca2+ waves originating in dendrites 10–50 μm away from the soma, and that the mGluR-induced Ca2+ waves were inhibited only when both cADPR and PLC/IP3 signaling pathways were inhibited. It was thus concluded that mGluR-mediated Ca2+ mobilization involves two pathways mediated by cADPR and IP3 in a redundant manner. Our results differ, in that only the cADPR signaling pathway and ryanodine-sensitive stores contributed to Ca2+ mobilization by mGluR5 in the somata of hippocampal neurons (Figures 2 & 3). However, these results do not mean that the Ca2+ stores in hippocampal neuron somata are insensitive to IP3, as muscarinic receptor-mediated Ca2+ mobilization was mediated by PLC/IP3 signaling pathway (Figure 2). Possibly, Ca2+ stores in hippocampal neurons are fundamentally sensitive to both IP3 and cADPR, but signaling pathways that regulate these mediators can differ depending on cell types and subcellular localization. It will be of interest to test the contribution of the cADPR-mediated Ca2+ releases from RyRs to dendritic Ca2+ signaling in the hippocampus.

Dendritic Ca2+ signaling induced by group I mGluR has been extensively studied in CA1 hippocampus, and the results obtained in these studies suggest the involvement of the PLC/IP3 signaling pathway [17,18]. Remarkably, large amplitude Ca2+ increases induced by repetitive synaptic stimulation, which were considered to be attributable to IP3-induced Ca2+ release, were precisely confined to the large apical dendrite shaft at the branch point of oblique dendrites [55]. This result indicates that even in dendrites of the same neuron, Ca2+ signaling mechanisms are spatially segregated. In the present study, we used acutely dissociated hippocampal neurons with thick apical dendrites of ~50 μm. We measured Ca2+ signals only from somata in response to bath application of mGluR agonist or glutamate. Thus, our results represent somatic Ca2+ release mechanisms without interference from dendritic Ca2+ signaling mechanisms. We have provided solid evidence that in the somata of hippocampal CA1 pyramidal neurons, cADPR-mediated Ca2+ releases from RyRs serve as the predominant mechanism in mGluR-induced Ca2+ release. It should also be noted that there has not been a direct examination of somatic Ca2+ release machinery despite the fact that somatic Ca2+ signals have distinctive roles, such as protein synthesis and gene expression [31,32]. Further studies are required to test the possibility that dendritic and somatic Ca2+ release mechanisms may be distinct from each other in hippocampus.

One of the interesting features reported for mGluR-mediated Ca2+ release in dendrites is that Ca2+ entry through VGCCs interacts synergistically with IP3, and supralinearly increase the GluR-mediated Ca2+ release in apical dendrites of hippocampal neurons.
CA1 neurons [17,18]. Supralinear Ca2+ release by DHPG and either membrane potential depolarization or NMDA receptor activation was also demonstrated in primary cultured hippocampal neurons [13]. However, we showed that cADPR/RyR-dependent Ca2+ release by mGluR5 was not supralinearly increased by Ca2+ influx in the somata of CA1 pyramidal neurons (Figure 7). This suggests that, unlike IP3-dependent Ca2+ releases, cADPR-dependent Ca2+ release through RyRs is not potentiated by Ca2+ influx. However, we still need to consider another type of possible synergism between cADPR/RyR-dependent Ca2+ release and L-type Ca2+ channels, as demonstrated in previous studies. In NG108-15 cells transfected with mGluRs, direct applications of cADPR enhanced Ca2+ influx through L-type Ca2+ channels [54]. In addition, dendritic Ca2+ transients evoked by back-propagating action potentials, which are mediated by VGCCs, were potentiated by mGluR3-mediated Ca2+ release and PKC activation in hippocampal oriens-aleuves interneurons [53]. These findings suggested that the RyRs-sensitive Ca2+ releases enhance L-type Ca2+ channels via PKC-dependent mechanisms. An interesting observation in this study is that the potentiation occurs exclusively in specific microdomains of dendrites; possible absence of similar microdomains in the somata of CA1 pyramidal neurons, which needs to be determined in future studies, would explain the lack of a synergistic interaction found in this study (Figure 7).

In this study, the experiments were performed using acutely dissociated neurons, as dissociated neurons have several advantages in studying signaling mechanisms in the somata. In this preparation, indirect effects or presynaptic components can be excluded. Furthermore, rapid application and wash-out of drugs are guaranteed. It is very difficult to obtain healthy cells by enzymatic dissociation method from rats over 2 weeks old, and therefore we used immature rats (P7-P14). However, glutamate signaling is still developing at this age, and the Ca2+ mobilization mechanisms found in the current study may not extend into the somatic mechanism of adult neurons. To exclude this possibility, we examined the DHPG-induced Ca2+ release mechanisms in the somata of CA1 pyramidal neurons in brain slices from 4-week-old rats (data not shown), and found that the mechanisms were consistent with those found in acutely dissociated immature neurons. Therefore, the mechanisms of mGluR3-induced somatic Ca2+ mobilization found in the current study may be extended into at least young adult neurons.

We have demonstrated that AMPA receptors and L-type Ca2+ channels, but not NMDA receptors, are responsible for the Ca2+ influx by glutamate. It was shown previously that NMDA-dependent Ca2+ entry evokes Ca2+ increases primarily in spines, which are more concentrated with oblique dendrites [56,57]. Nakamura et al. [55] also showed that synaptic stimulation evoked Ca2+ influx by NMDA receptors exclusively at oblique dendrites, whereas backpropagating APs evoke Ca2+ increase at all dendritic locations. Thus, in acutely dissociated neurons that usually lack oblique dendrites the role of NMDA receptors in Ca2+ influx should be limited and membrane potential depolarization by AMPA receptors and the opening of L-type Ca2+ channels may be responsible for Ca2+ influx instead. Another notable finding is that the contribution of Ca2+ influx to CaGlu was larger than that of Ca− release (Figure 7), signifying the importance of L-type Ca2+ channels in somatic Ca2+ signaling.

In summary, we investigated the Ca2+ mobilization mechanisms by group I mGluRs using acutely dissociated hippocampal neurons. As discussed, the signaling pathways revealed in the current study may represent what occurs in the somata of hippocampal CA1 neurons, and this may be distinct from dendritic Ca2+ release machinery, which has been extensively characterized by other groups. The nucleus, as well as other important intracellular organelles, resides in the somata. Therefore, Ca2+-dependent molecules regulating cellular excitability and synaptic plasticity may be regulated by the cADPR/RyR-dependent Ca2+ release by group I mGluRs in hippocampal CA1 neurons.

Materials and Methods

Ethics Statement

Protocols were approved by the Animal Care Committee at Seoul National University (SNU-080107-7). Animal handling was conducted in accordance with national and international guidelines. The number of animals used was minimized, and all necessary precautions were taken to mitigate pain or suffering.

Preparation of acutely isolated hippocampal neurons

Hippocampal CA1 pyramidal neurons were isolated as described previously [21]. Briefly, 7 to 14-day-old Sprague-Dawley rats (14 to 34 g) were decapitated under pentobarbital anesthesia. The brain was quickly removed and submerged in ice-cold artificial cerebrospinal fluid (ACSF, see below) saturated with 95% O2 and 5% CO2. Transverse hippocampal slices (400 μm thick) were prepared using a vibratome (VT1200, Leica). After a 30 min recovery period at 32°C, the slices were treated with protease type XIV (1 mg/5 ml, Sigma) for 30–60 min, and with protease type X (1 mg/5 ml, Sigma) for 10–15 min at 32°C. The slices were allowed to recover during a 1-hour incubation period at room temperature. The CA1 region was identified and punched out under a binocular microscope (SZ40, Olympus), placed in a recording chamber containing normal Tyrode (NT) solution (see below) and mechanically dissociated using a Pasteur pipette to release individual neurons. The dissociated neurons were allowed to adhere to the bottom of the recording chamber for 10–20 min. Cells were identified as pyramidal neurons by their typical large pyramidal-shaped cell body with a thick apical dendritic stump of ~50 μm under an inverted microscope (IX70, Olympus). The isolation of hippocampal CA1 neurons from PLCβ1 or PLCβ4 knockout mice (generated as described in [35]) was performed as above.

Solutions and drugs

ACSF contained (in mM): NaCl 125, NaHCO3 25, KCl 3, Na2HPO4 1.25, CaCl2 2, MgCl2 1, glucose 10, sucrose 5, vitamin C 0.4, and was bubbled with a mixture of 95% O2 and 5% CO2 to a final pH of 7.4. NT solution contained (in mM): NaCl 150, KCl 5, CaCl2 2, MgCl2 1, glucose 10, Hepes 10, and was adjusted to pH 7.4 with Tris-OH. To make Ca2+-free NT solutions, CaCl2 was replaced with equimolar MgCl2 and 0.1 mM EGTA. Pipette solutions used for electrophysiology studies contained (in mM) K-glucuronate 110, KCl 30, Hepes 20, Mg-ATP 4, Na-vitamin C 4, Na-GTP 0.3, EGTA 0.1 titrated to pH 7.3 with KOH.

(RS)-3,3-DHPG, LY367385, MPEP, SKF96365, CNQX, AP-5, ryanodine, TTX were purchased from Tocris. U73122 was from Biomol. Fura 2, Fura 2-AM and 8-NH2-cADPR were obtained from Molecular Probes, and α-conotoxin-GVIA and α-agatoxin-IVA were from Anygen. All other drugs were purchased from Sigma. Stock solutions of drugs were made by dissolving in deionized water or DMSO according to manufacturer’s specifications and were stored at −20°C. On the day of the experiment one aliquot was thawed and used. The final concentration of DMSO in solutions was maintained below 0.1%.

Calcium measurements

Acutely dissociated hippocampal CA1 neurons were loaded by incubation with 2 μM Fura 2-AM plus 0.01% Pluronic F-127 in...
NT solution for 10 min at room temperature. For fluorescence excitation, we used a polychromatic light source (xenon-lamp based, Polychrome-IV, TILL-Photonics), which was coupled to the epillumination port of an inverted microscope (IX70, Olympus) via a quartz light guide and a UV condenser. Microfluorometry was performed with a 40× water immersion objective (NA 1.15, UAP0 40× W/340, Olympus) and a photodiode (TILL-Photonics).

Calibration of Ca²⁺ measurements

Calibration parameters were determined using in vivo calibration as described in [38]. The effective dissociation constant of Fura 2 (K-diss) was calculated from K-diss = [Ca²⁺] / (Rmax - Rmin) where [Ca²⁺] was entered as 231 nM (assuming a dissociation constant (K-diss) of BAPTA of 222 mM at pH 7.2). The estimated Rmax, Rmin, and K-steady (μM) measured using an inverted microscope were typically 0.27, 3.95 and 0.93, respectively. A standard two-wavelength protocol was used for fluorescence measurement of cells. Fluorescence intensity was measured at 1 Hz with double wavelength excitation at 340 nm (F340) and 380 nm (F380). The ratio R = F340/F380 was converted to [Ca²⁺] values using the equation [Ca²⁺]eff = [Ca²⁺] / (R-Rmin)/([Rmax-R]).

Electrophysiology

Current clamp recordings of membrane potential were performed using an EPC-10 amplifier (HEKA Elektronik) at room temperature. Membrane potentials were recorded from acutely dissociated hippocampal CA1 neurons in a conventional whole cell configuration at a sampling rate of 10 kHz filtered at 1 kHz. Data were acquired using an IBM-compatible computer running Pulse software v8.67 (HEKA Elektronik). The patch pipettes were pulled from borosilicate capillaries (Hilgenberg GmbH) using a Narishige puller (PC-10, Narishige). The patch pipettes had a resistance of 3–5 megohms when filled with above-mentioned K-based pipette solutions.

Single cell electroporation

The loading of heparin and β-NH₂-cADPR was performed by single cell electroporation. Micropipettes were pulled as described above and were filled at their tips with NT solutions containing heparin (20 mg/ml) or β-NH₂-cADPR (100 μM) plus Alexa Fluor 488 (200 μM, Molecular Probes). Micropipettes were controlled by a micromanipulator (Burleigh) to reach cells, and square electric pulses generated with an electroporator (Axoporator 800A; Molecular Devices/MDS Analytical Technologies) were applied to transfer the mixture into the cells.

Data analysis

Data were analyzed using IgorPro (version 4.1, WaveMetrics) and Origin (version 6.0, Microcal) software. Statistical data are expressed as the mean ± S.E., where n represents the number of cells studied. The significance of differences between the means was evaluated using a Student’s t-test with confidence levels of p<0.01 (**), p<0.05 (*)

Author Contributions

Conceived and designed the experiments: J-WS W-KH. Performed the experiments: J-WS W-JY. Analyzed the data: J-WS DL S-HL. Contributed reagents/materials/analysis tools: H-SS S-HL. Wrote the paper: J-WS W-KH.

References

1. Conn PJ, Pin JP (1997) Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37: 205-237.
2. Lascher C, Huber KM (2010) Group 1 mGluR-dependent synaptic long-term depression: mechanisms and implications for circuitry and disease. Neuron 65: 445-459.
3. Nakamura S (1994) Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity. Neuron 13: 1031-1037.
4. Sidiropoulou K, Liu FM, Fowler MA, Xiao R, Phillips C, et al. (2009) Dopamine modulates an mGluR5-mediated depolarization underlying prefrontal persistent activity. Nat Neuroscience 12: 190-199.
5. Sourdet V, Russier M, Daoudal G, Anki N, Debanne D (2003) Long-term enhancement of neuronal excitability and temporal fidelity mediated by metabotropic glutamate receptor subtype 5. J Neuroscience 23: 10238-10248.
6. D’Ascenzo M, Podda MV, Fellin T, Azzena GB, Haydon P, et al. (2009) Activation of mGluR5 induces spike afterdepolarization and enhanced excitability in medium spiny neurons of the nucleus accumbens by modulating persistent Na⁺ currents. J Physiology 587: 3233-3250.
7. Brager DH, Johnston D (2007) Plasticity of intrinsic excitability during long-term depression is mediated through mGluR-dependent changes in L2 in hippocampal CA1 pyramidal neurons. J Neuroscience 27: 13926-13937.
8. Jo J, Heon S, Kim MJ, Son GH, Park Y, et al. (2008) Metabotropic glutamate receptor-mediated LTD involves two interacting Ca²⁺ sensors, NCS-1 and PICK1. Neuron 60: 1095-1111.
9. Bellone C, Lascher C (2006) Cocaine triggered AMPA receptor redistribution is reversed in vivo by mGluR-dependent long-term depression. Nat Neuroscience 9: 636-641.
10. Xia J, Chung HJ, Whiler C, Hagan RI, Linden DJ (2000) Cerebellar long-term depression requires PKC-regulated interactions between GluR2/3 and PSD-95 domain-containing protein. Neuron 20: 499-510.
11. Macjina T, Oka S, HashimotoTani Y, Ohno-Shosaku T, Aba A, et al. (2005) Synaptically driven endocannabinoid release requires Ca²⁺-assisted metabotropic glutamate receptor subtype 1 to phospholipase Cβ signaling cascade in the cerebellum. J Neuroscience 25: 6286-6305.
12. HashimotoTani Y, Ohno-Shosaku T, Tsubakova H, Ogata H, Emoto K, et al. (2005) Phospholipase Cβeta serves as a coincidence detector through its Ca²⁺ dependence for triggering retrograde endocannabinoid signal. Neuron 45: 257-268.
13. Rae MG, Marin DJ, Collingridge GL, Irving AJ (2000) Role of Ca²⁺ stores in metabotropic L-glutamate receptor-mediated supralinear Ca²⁺ signaling in rat hippocampal neurons. J Neuroscience 20: 8628-8636.
14. Rae MG, Irving AJ (2004) Both mGluR1 and mGluR5 mediate Ca²⁺ release and inward currents in hippocampal CA1 pyramidal neurons. Neuropharmacology 46: 1037-1069.
15. Kleppisch T, Voigt V, Allmann R, Offermanns S (2001) Gn1α-deficient mice lack metabotropic glutamate receptor-dependent long-term depression but show normal long-term potentiation in the hippocampal CA1 region. J Neuroscience 21: 4943–4950.
16. Krause M, Offermanns S, Stocker M, Pedarzani P (2002) Functional specificity of Gqα and G11 in the cholinergic and glutamatergic modulation of potassium currents and excitability in hippocampal neurons. J Neuroscience 22: 666-673.
17. Nakamura T, Barbara JG, Nakamura K, Ross WN (1999) Synergistic release of Ca²⁺ from IP₃-sensitive stores evoked by synaptic activation of mGluRs paired with backpropagating action potentials. Neuron 24: 727-737.
18. Nakamura T, Nakamura K, Lasser-Ross N, Barbara JG, Sandler VM, et al. (2000) Inositol 1,4,5-trisphosphate (IP₃)-mediated Ca²⁺ release evoked by metabotropic agonists and backpropagating action potentials in hippocampal CA1 pyramidal neurons. J Physiology 523: 8365-8376.
19. Morikawa H, Khodakhah K, Williams JT (2003) Two intracellular pathways mediate metabotropic glutamate receptor-induced Ca²⁺ mobilization in dopamine neurons. J Neuroscience 23: 149-157.
20. Higashida H, Zhang JS, Mochida S, Chen XL, Shin Y, et al. (2003) Syringe-specific coupling with ADPribosyl cyclase of metabotropic glutamate receptors in retina, cervical superior ganglion and NG108-15 cells. J Neurochem 85: 1148-1158.
21. Sohn JW, Lee D, Cho H, Lim W, Shin HS, et al. (2007) Receptor-specific inhibition of GABAergic activated K⁺ currents by muncarnitine and metabotropic glutamate receptors in immature rat hippocampus. J Physiology 580: 411-422.
22. Young SR, Chuang SC, Wong KK (2004) Modulation of afterpotentials and firing pattern in guinea pig CA1 neurons by group I metabotropic glutamate receptors. J Physiology 554: 371-383.
23. Ireland DR, Abraham WC (2002) Group I mGluRs increase excitability of hippocampal CA1 pyramidal neurons by a PLC-independent mechanism. J Neurophysiol 88: 107-116.
24. Ireland DR, Guevremont D, Williams JM, Abraham WC (2004) Metabotropic glutamate receptor-mediated depression of the slow afterhyperpolarization is gated by tyrosine phosphatases in hippocampal CA1 pyramidal neurons. J Neurophysiol 92: 2811–2819.
25. Augustin GJ, Santamaria F, Tanaka K (2003) Local calcium signaling in neurons. Neuron 40: 331-346.
26. Hardingham GE, Arnold EF, Bading H (2001) A calcium microdomain near NMDA receptors: on switch for ERK-dependent synapse-to-nucleus communication. Nat Neurosci 4: 565–566.

27. Fäkle B, Adelman JP (2006) Control of K<sub>Ca</sub> channels by calcium nano-/microdomains. Neuron 50: 873–881.

28. Bloodgood BL, Sabatini BL (2008) Regulation of synaptic signalling by postsynaptic, non-glutamate receptor ion channels. J Physiol 586: 1473–1480.

29. Xu J, He L, Wu LG (2007) Role of Ca<sup>2+</sup> channels in short-term synaptic plasticity. Curr Opin Neurobiol 17: 352–359.

30. Lee D, Lee KH, Ho WK, Lee SH (2007) Target cell-specific involvement of presynaptic mitochondria in post-tetanic potentiation at hippocampal mossy fiber synapses. J Neurosci 27: 13603–13613.

31. Finkbeiner S, Greenberg ME (1998) Ca<sup>2+</sup> channel-regulated neuronal gene expression. J Neurobiol 37: 171–189.

32. Barnes SJ, Opitz T, Merkens M, Kelly T, von der Brelie C, et al. (2010) Stable presynaptic mitochondria in post-tetanic potentiation at hippocampal mossy fiber synapses. Neuron 59: 187–199.

33. Ghosh TK, Eis PS, Mullaney JM, Ebert CL, Gill DL (1988) Competitive, reversible, and potent antagonism of inositol 1,4,5-trisphosphate-activated calcium release by heparin. J Biol Chem 263: 11075–11079.

34. Chuang SC, Bianchi R, Kim D, Shin HS, Wong RK (2001) Group I mGluR Ca<sup>2+</sup> channel-regulated neuronal calcium release. PLoS ONE 2: e797.

35. Kim D, Jun KS, Lee SB, Kang NG, Min DS, et al. (1997) Phospholipase C isozymes selectively couple to specific neurotransmitter receptors. Nature 389: 290–293.

36. Churamani D, Boulware MJ, Geach TJ, Martin AC, Moy GW, et al. (2007) Molecular characterization of a novel intracellular ADP-ribosyl cyclase. PLoS ONE 2: e797.

37. Churamani D, Boulware MJ, Ramakrishnan L, Geach TJ, Martin AC, et al. (2008) Molecular characterization of a novel cell surface ADP-ribosyl cyclase from the sea urchin. Cell Signal 20: 2347–2355.

38. Malavasi F, Deaglio S, Funaro A, Ferrero E, Horenstein AL, et al. (2008) Molecular characterization of a novel cell surface ADP-ribosyl cyclase from the sea urchin. Cell Signal 20: 2347–2355.

39. Reyes-Harde M, Potter BV, Galione A, Stanton PK (1999) Induction of hippocampal LTD requires nitric-oxide-stimulated PKG activity and Ca<sup>2+</sup> release from cyclic ADP-ribose-sensitive stores. J Neurophysiol 82: 1569–1576.

40. Lopatina O, Liu MX, Aminu S, Hashii M, Higashida H (2010) Oxytocin-induced elevation of ADP-ribosyl cyclase activity, cyclic ADP-ribose or Ca<sup>2+</sup> concentrations is involved in autoregulation of oxytocin secretion in the hypothalamus and posterior pituitary in male mice. Neuropharmacology 58: 50–55.

41. Yue J, Wei W, Lam CM, Zhao YJ, Dong M, et al. (2009) CD38/cADPR/Ca<sup>2+</sup> signaling in hippocampal mossy fiber long-term potentiation requires calcium influx at the granule cell soma, protein synthesis, and mitochondria-dependent axonal transport. J Neurosci 30: 12996–13004.

42. Zheng J, Wenzhi B, Miao L, Hoi Y, Zhang X, et al. (2010) Ca<sup>2+</sup> release induced by cADP-ribose is mediated by FKBP12.6 proteins in mouse bladder smooth muscle. Cell Calcium 47: 449–457.