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Possible involvement of group I mGluRs in neuroprotective effect of theanine

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Abstract

We investigated the molecular mechanism underlying the neuroprotective effect of theanine, a green tea component, using primary cultured rat cortical neurons, focusing on group I metabotropic glutamate receptors (mGluRs). Theanine and a group I mGluR agonist, DHPG, inhibited the delayed death of neurons caused by brief exposure to glutamate, and this effect of theanine was abolished by group I mGluR antagonists. Although the administration of glutamate alone decreased the neuronal expression of phospholipase C (PLC)-β1 and -γ1, which are linked to group I mGluRs, their expression was equal to the control levels on co-treatment with theanine. Treatment with theanine or DHPG alone for 5–7 days resulted in increased expression of PLC-β1 and -γ1, and the action of theanine was completely abolished by group I mGluR antagonists. These findings indicate that group I mGluRs might be involved in neuroprotective effect of theanine by increasing the expression levels of PLC-β1 and -γ1.

Keywords: Theanine; Rat cortical neuron; Neuroprotection; Glutamate-induced excitotoxicity; Metabotropic glutamate receptor; Phospholipase C isozyme

Overstimulation of the *N*-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methylisoxazol-4-propionic acid (AMPA)/kainate (KA) receptor is a key event in excitotoxicity, and identification of the mechanisms in this cascade is important, because inhibition of NMDA or AMPA/KA receptors has been associated with toxic effects [13] and because activation of these receptors is an early event resulting in a narrow time frame for therapeutic intervention [9,24].

One epidemiological study has shown that the incidence of stroke was significantly lower in people who consume more than five cups of green tea per day [28] and the same result was obtained in the study performed by Keli et al. [17]. There is a lot of evidence for a neuroprotective effect of its ingredients, especially catechin and theanine (γ-glutamylethylamide). It has been reported that (−)-epigallocatechin 3-gallate, the main component of green tea catechin, inhibits the death of cultured mouse cortical neurons induced by oxidative stress [23], and neurons in the hippocampus, striatum, and cortex induced in mouse and rat ischemia–reperfusion models [12,20]. This neuroprotection by catechin is suggested to be due to its strong O_2^--scavenging effect, which is similar to that of superoxide dismutase [32]. Recently, theanine was indicated to inhibit ischemic delayed neuronal death in gerbils [14,15]. Since theanine did not strongly affect the binding of AMPA, KA, and MDL105,519, an antagonist for the NMDA receptor glycine site, to glutamate receptors, the authors suggested that ionotropic glutamate receptors (iGluRs) are not involved in the neuroprotective action of theanine [16], but the molecular mechanism involved has not been clarified yet.

Metabotropic GluRs (mGluRs) are G protein-coupled receptors that bind glutamate to modulate neuronal excitability and synaptic transmission in the central nervous system. The eight known members of the

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mGluR subfamily have been divided into three groups on the basis of their sequence identity, pharmacology, and preferred signal transduction mechanism. There is evidence for both neurotoxic and neuroprotective actions of both group I mGluR agonists and antagonists ([25] and references therein). Group I mGluRs (mGluR1 and mGluR5) are primarily localized postsynaptically, where they modulate ion channel activity and neuronal excitability, and are coupled to Gαq and associated effectors, such as phospholipase C (PLC)-β1 [8,11]. Furthermore, it appears that Ca^{2+}-entry into cells via activation of G proteins depends on PLC-γ activity [26], and signaling by group I mGluR recruits PLC-γ1 to phosphoinositide 3 kinase enhancer (PIKE) to serve as a guanine nucleotide exchanger factor contributing to the antiapoptotic action of group I mGluR activation [27]. It is well known that overexpression of PLC-β1 and -γ1 protects cells from oxidative stress-induced cell death [3,21,33]. Finally, it was hypothesized that activation of group I mGluR protects neurons from oxidative stress.

Therefore, we investigated whether or not theanine prevents neuronal death due to glutamate-induced excitotoxicity, i.e., oxidative stress, via activation of group I mGluRs.

Materials and methods

Materials. Theanine was obtained from Funakoshi (Tokyo, Japan). MK801, (+)-α-amino-4-carboxy-α-methylbenzeneacetic acid (MCPG), 2-methyl-6-(phenylethynyl)-pyridine (MPEP), and (+)-2-methyl-4-carboxyphenylglycine (LY367385) were purchased from Tocris Cookson (Langford, Bristol, UK). All other chemicals were of reagent grade and were obtained commercially.

Primary cortical neuronal cultures. Primary cultured cortical neurons were prepared by the method reported previously with slight modification [29]. All experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University and were performed according to the Guidelines for Animal Experimentation of Kyoto Pharmaceutical University. Briefly, E18 rat embryos (Japan SLC, Hamamatsu, Japan) were decapitated, and the brains were rapidly removed and placed in a petri dish half-filled with cold HBSS (137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose, and 2.5 mM Hepes). The cortices were dissected out under a dissecting microscope, and then placed in another dish containing ice-cold HBSS to thoroughly remove blood vessels and membrane from cortical tissues. The tissues were mechanically dissociated with a pipette. The cell suspension was filtered through a mesh with a pore size 150 μm, followed by resuspension in an appropriate volume of B-27 Supplement Minus AO (Invitrogen, Carlsbad, CA)-supplemented Eagle's minimum essential medium (EMEM; Nissui, Tokyo, Japan). The cells obtained were seeded into polyethyleneimine-coated dishes at the density of 2.3×10^6 cells/dish, the medium being changed every 2 days, and used for experiments after culturing for 7 days or more. With this procedure the cultured cells comprised 90% or more neurons, with a negligible amount of glial cells, as judged immunohistochemistry for MAP2 and GFAP (data not shown).

Treatment of neurons. Cultured neurons were washed with Mg^{2+}-free Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, and 5 mM Hepes, pH 7.4) twice, and then incubated with the indicated concentrations of L-glutamate with or without inhibitor(s) in the same solution for 10 min at 37 °C. For long-term treatment, theanine and other reagents were added to the culture medium and were freshly added when the medium was changed. After the neurons were cultured to subconfluence, they were washed with EMEM twice and cultured in 10% horse serum containing EMEM (HS-EMEM) for 24 h.

Cytotoxicity assay. After glutamate treatment, the MTT assay was performed. We confirmed that the decrease in cell viability observed in the MTT assay was not caused by mitochondrial dysfunction, by comparison with the results obtained on trypan blue exclusion.

Reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA was extracted from cultured neurons with a Gen Elute Mammalian Total RNA kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA by means of Oligo-T priming and Moloney murine leukemia virus reverse transcriptase, and then the cDNA was polymerase chain reaction (PCR)-amplified for 40 cycles of 94 °C for 15 s, 58 °C 30 s, and 72 °C for 60 s with r-*Taq* DNA polymerase (TAKARA, Shiga, Japan). The primer sets for mGluR1, mGluR5, mGluR5a, and mGluR5b used were the same as those reported by Biber et al. [6], and their sequences and sizes of the expected PCR products are summarized in Table 1.

Immunoblotting. The neurons recovered were suspended in extraction buffer (0.32 M sucrose, 50 mM benzamidine, 4 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM diisopropylfluorophosphate, 1 mM dithiothreitol, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 10 μg/mL aprotinin, 1 mM orthovanadate, and 10 mM Hepes, pH 7.4) and homogenized, and then the protein concentration was determined using a Coomassie brilliant blue reagent. After the addition of the same volume of Laemmli sample buffer and boiling for 5 min, the samples were electrophoresed on sodium dodecyl sulfate–polyacrylamide gels, blotted onto nitrocellulose membranes, and blocked with 5% dehydrated skim milk (Dafco Laboratories, Detroit, MI), and then immunoblotting was carried out using antibodies against PLC-β1 (1:500; Santa Cruz Biotechnology), PLC-γ1 (1:500; Santa Cruz Biotechnology), and β-actin (1:5000; Sigma). The membranes were then incubated with horseradish peroxidase (HRP)-linked antibodies against rabbit immunoglobulin. Subsequently, membrane-bound HRP-labeled proteins were detected with an enhanced chemiluminescence detection system (ECL kit, Amersham). Protein bands reacting

Table 1
Forward and reverse oligonucleotide primer sequences used for RT-PCR screening of group I mGluRs in rat primary cultured cortical neurons [6]

Isoform	Direction	Sequence	Product (bp)
mGluR1	Forward	5′-GTACAGCTCATGATGAGGAGTC-3′	870
	Reverse	5′-GGTATGACATTATGAATCTGCAG-3′	
mGluR5	Forward	5′-CTAGAATCCTTGCAATACGGTTGG-3′	740
	Reverse	5′-ATGCGATGAAGCCAATTGATGGAC-3′	
mGluR5a	Forward	5′-TCCCGAAGGTGTACATCATCC-3′	312
mGluR5b	Reverse	5′-CTCTTGGGAAAGGGTTTGATGAC-3′	408

with the antibodies were detected on radiographic film, and the integrated optical density of each band was measured with a scanning densitometer. Each protein expression level was corrected as to the corresponding β-actin one.

Statistical analysis. The data are expressed as means ± SE. Comparisons between two or more groups were performed by means of analysis of variance (followed by Fischer's PLSD or the Bonferroni/Dunnett (Control) test), differences with a p value of 0.05 or less being considered statistically significant.

Results

First, we checked whether neuroprotective effect of theanine was observed in our in vitro system. Treatment of cultured neurons with 100 μM glutamate for 10 min caused significant neuronal death, which was inhibited by 10 μM MK801, a noncompetitive NMDA receptor antagonist (Fig. 1). This glutamate-induced neuronal death was inhibited by theanine at the concentrations of 8 or more micromolars. Similarly, 5 μM DHPG showed the neuroprotection against glutamate-neurotoxicity (Fig. 1), suggesting involvement of group I mGluRs.

In order to confirm the expression of group I mGluRs in our cultured neurons, RT-PCR analysis using total RNA obtained from cultured cortical neurons was performed. As depicted in Fig. 2, the messages of mRNAs for mGluR1 and mGluR5 were observed for the expected sizes, as reported by Biber et al. [6]. In addition, mGluR5a and mGluR5b were expressed in the neurons. On the basis of these findings, we performed the following experiments focusing on group I mGluRs.

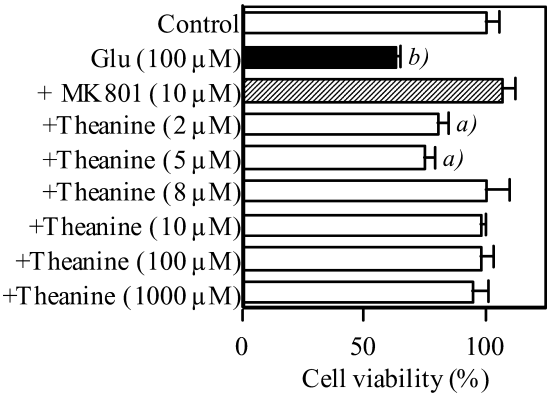

Fig. 1. Effect of theanine and MK801 on the decrease in neuron viability induced by glutamate application. After neurons had been cultured to subconfluence, they were treated with 100 μM glutamate in the presence or absence of the indicated concentrations of theanine or 10 μM MK801 in Mg^{2+}-free Locke's solution for 10 min, and then cultured in HS-EMEM for 24 h. Thereafter, the MTT assay was performed. Each column represents the mean ± SE of three to five independent experiments. (*a* and *b*) $p < 0.05$ and 0.001, significantly different from the control, respectively.

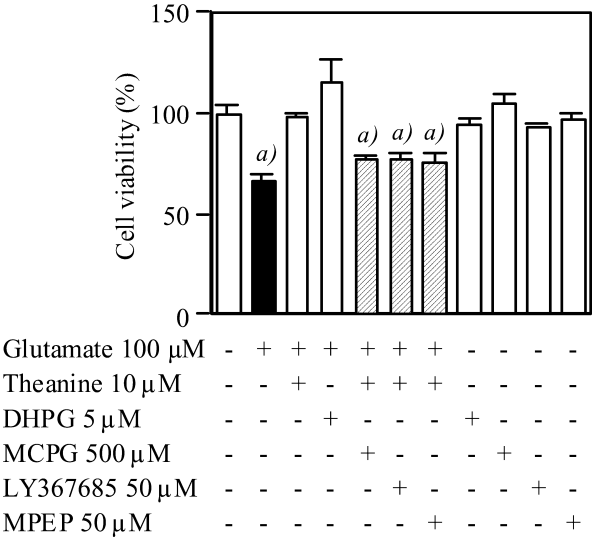

Fig. 3. Effects of theanine and DHPG on glutamate-induced excitotoxity in the presence or absence of an antagonist for group I mGluRs. After neurons had been cultured to subconfluence, they were treated with 100 μM glutamate and/or 10 μM theanine or 5 μM DHPG in the presence or absence of the indicated concentrations of an antagonist in Mg^{2+}-free Locke's solution for 10 min, and then cultured in HS-EMEM for 24 h. Thereafter, the MTT assay was performed. Each column represents the mean ± SE of three independent experiments. (*a*) $p < 0.001$ significantly different from the control.

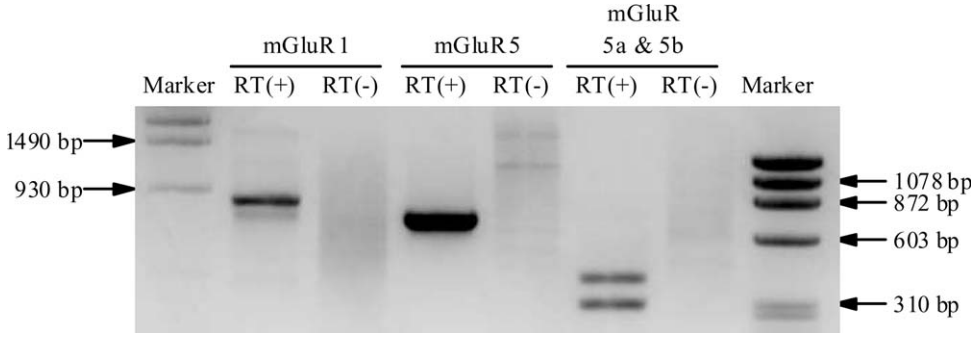

Fig. 2. Detection of mRNAs for group I mGluRs in primary cultured rat cortical neurons with the RT-PCR method. A PCR product was electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. RT(+) and RT(−) represent the presence and absence of reverse transcriptase, respectively.

In this experiment, 500 μM MCPG, 50 μM LY367385, and 50 μM MPEP were used as selective antagonists for group I mGluRs, mGluR1 and mGluR5, respectively [10]. The recovery of the viability of glutamate-treated neurons caused by theanine was prevented by simultaneous treatment with glutamate and MCPG, LY367385 or MPEP, the viability of neurons not being affected by the application of an antagonist alone (Fig. 3).

Next, we used expression levels of PLC-β1 and -γ1 as a biochemical marker of group I mGluR activity. Excitotoxic glutamate treatment resulted in significant decreases in the cellular expression levels of PLC-β1 and -γ1, but there were no changes in the either PLC isozyme level in the glutamate and theanine treatment groups (Fig. 4).

Whether or not theanine alone affects the expression levels of the two PLC isozymes was evaluated. When cortical neurons were treated with theanine alone, theanine significantly increased the expression of PLC-β1 and -γ1 in 7 days, but not 10 min (Fig. 5). As similar to theanine, an agonist for group I mGluRs, DHPG, also increased the PLC-β1 and -γ1 expression in neurons by its 5 days treatment. On the other hand, there was no alteration of either PLC isozyme level in neurons treated with theanine and MCPG, a group I mGluR antagonist (Fig. 6). In order to clarify involvement of group I mGluRs in the increases in PLC-β1 and -γ1 caused by theanine, we examined the effect of a selective antagonist for mGluR1 and mGluR5 on the expression (Fig. 6). The increased levels of PLC-β1 and -γ1 in neurons treated with theanine alone decreased to the control levels in neurons

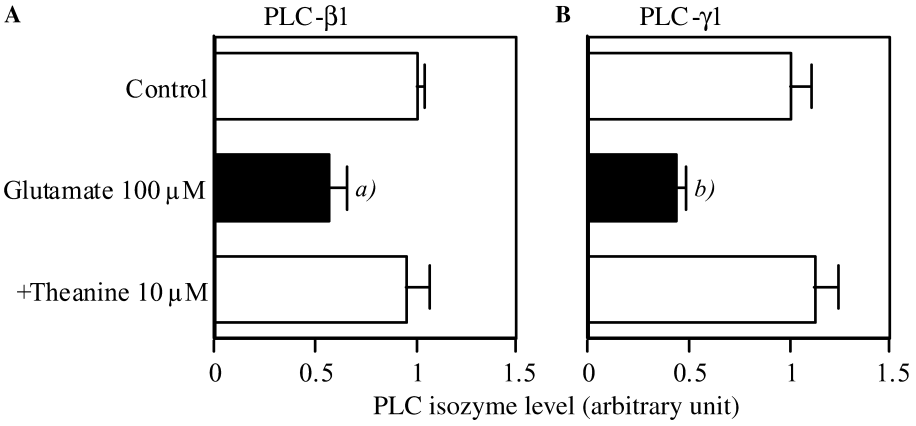

Fig. 4. Effects of glutamate and theanine on expression levels of PLC-β1 and -γ1 in neurons. After neurons had been cultured to subconfluence, they were treated with 100 μM glutamate in the presence or absence of 10 μM theanine in Mg^{2+}-free Locke's solution for 10 min, and then cultured in HS-EMEM for 24 h. Thereafter, Western blotting was performed as described under Materials and methods. Each column represents the mean ± SE of three independent experiments. (*a* and *b*) $p < 0.05$ and 0.01 significantly different from the control, respectively.

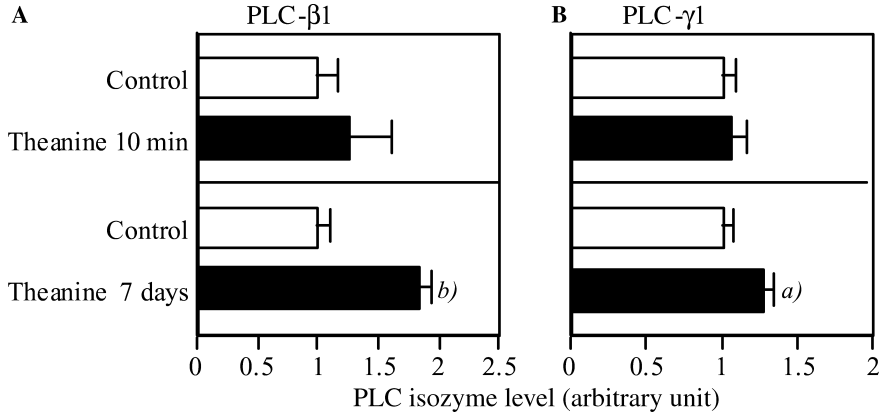

Fig. 5. Effect of theanine alone on expression levels of PLC-β1 and -γ1 in neurons. As for 10 min treatment, after neurons had been cultured for 7 days, they were treated with 10 μM theanine for 10 min. In the case of 7-day treatment, after starting culture of neurons for a day, they were cultured with 10 μM theanine for 7 days. Thereafter, the cells were cultured in HS-EMEM for 24 h, and then Western blotting was performed as described under Materials and methods. Each column represents the mean ± SE of three independent experiments. (*a* and *b*) $p < 0.05$ and 0.01 significantly different from the control, respectively.

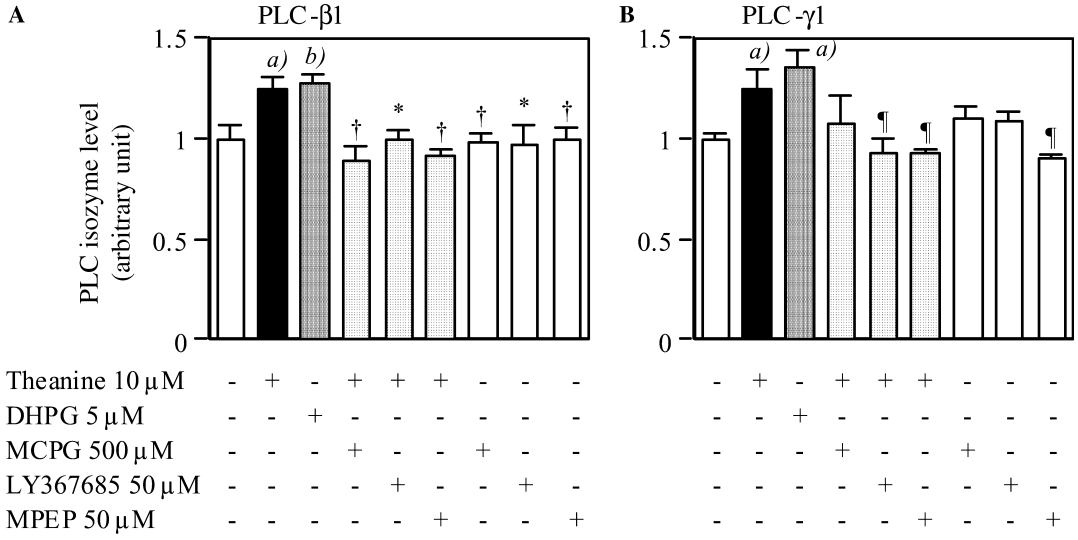

Fig. 6. Effects of theanine and DHPG on expression levels of PLC-β1 and -γ1 in neurons in the presence or absence of an antagonist for group I mGluRs. After starting culture of neurons for a day, they were cultured with 10 μM theanine or 5 μM DHPG in the presence or absence of the indicated concentrations of an antagonist for 5 days. Thereafter, the cells were cultured in HS-EMEM for 24 h, and then Western blotting was performed as described under Materials and methods. Each column represents the mean ± SE of three to five independent experiments. (*a* and *b*) $p < 0.05$ and 0.01 significantly different from the control, respectively. $^{*,\dagger,\P}p < 0.05$, 0.01, and 0.001, significantly different from the theanine group, respectively.

treated with theanine plus 50 μM LY367385 or 50 μM MPEP, by which the expression levels of the PLC isozymes were not changed.

Discussion

The neuronal death induced by glutamate was completely inhibited by 10 μM MK801, judging this death to comprise delayed death of neurons as reported previously [29], and theanine completely inhibited this death (Fig. 1), and DHPG, a representative agonist for group I mGluRs, also gave the same results (Fig. 3). In addition, the expression of mRNAs for mGluR1 and mGluR5, especially mGluR5b, an adult neuronal isoform of the latter was confirmed in these neurons (Fig. 2). Together, these findings led us to study whether or not group I mGluRs are responsible for the neuroprotective effect of theanine.

The favorable effect of theanine against glutamate excitotoxicity was almost completely abolished by co-administration of an antagonist of group I mGluRs, MCPG, and furthermore, either LY367385, a selective mGluR1 antagonist, or MPEP, a selective mGluR5 antagonist, also inhibited the effect of theanine. Also, we confirmed DHPG, an agonist for group I mGluRs, could protect the neurons from the glutamate toxicity (Fig. 3), suggesting that group I mGluRs might be responsible for the neuroprotective effect of theanine.

In order to clarify the involvement of group I mGluRs more clearly, we examined effect of theanine on the protein expression levels of PLC-β1 and -γ1, which are linked to group I mGluRs, as already described [8,11,26,27]. In neurons treated with glutamate, the expression levels of the two PLC isozymes were decreased (Fig. 4). This is the first demonstration that glutamate excitotoxicity causes the decreases in the PLC-β1 and -γ1 protein levels in neurons, although the details of the mechanisms underlying these decreases remained unknown. When theanine was administered to neurons with glutamate, the levels of PLC-β1 and -γ1 returned to the control levels (Fig. 4). Furthermore, theanine increased the expression levels of PLC-β1 and -γ1 in 7 day, but not 10 min, -treated neurons (Fig. 5). Similar to theanine, treatment with DHPG, an agonist for group I mGluRs, for 5 days increased the PLC-β1 and -γ1 protein levels in neurons (Fig. 6). As the case shown in Fig. 3, the increases in PLC-β1 and -γ1 expression in the theanine-treated neurons were inhibited by either LY367385 or MPEP (Fig. 6). Therefore, it is demonstrated that theanine exerts its neuroprotective effect, at least in part, via group I mGluRs, leading to the increased expression of the two PLC isozymes. It is well known that group I mGluRs cause an increase in PLC activity, as judged on measuring the hydrolysis activity in the presence or absence of representative inhibitors [11,22], there has been no report demonstrating increases in the protein levels of PLC isozymes are linked to group I mGluRs, and thus this is thought to be a new finding. Although the results shown in Figs. 3 and 6 might imply the involvement of both mGluR1 and mGluR5 in the action of theanine, detailed studies using mGluR1- and mGluR5-expressing system are necessary for the definite conclusion.

Hannan et al. [11] found that group I mGluR-stimulated phosphoinositide hydrolysis appeared to be drastically reduced in PLC-β1-knockout mice, but to our limited knowledge, whether or not other PLC isozyme(s) including PLC-γ1 are activated by group I mGluRs has not been clearly demonstrated yet. It has been reported that moderate increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$) via activation of group I mGluRs cause inactivation of NMDA receptors, resulting in neuroprotection [4,19,31], and that such a $[Ca^{2+}]_{in}$ increase via G proteins, that is, inositol-1,4,5-trisphosphate receptor-mediated Ca^{2+} release from its intracellular store (such as the endoplasmic reticulum) [5], depends on PLC-γ activity [26], and group I mGluRs require PLC-γ1 to exert their antiapoptotic action [27]. These findings together explained our findings that activation of group I mGluRs, which are G protein-coupled receptors, increased the PLC-β1 level, elevated $[Ca^{2+}]_{in}$, and then the expression of PLC-γ1 was increased. Therefore, we think that activation of group I mGluRs increases both PLC-β1 and -γ1 expression levels, although the molecular mechanism involved needs to be clarified.

Overexpression of PLC-β1 and -γ1 is thought to protect cells from oxidative stress-induced cell death [3,21,33]. It has been reported that mRNA for c-*fos*, of which the gene product is a component of the AP-1 transcription factor [1] and is induced by oxidative stress, causing apoptosis, was not induced in PLC-β1-overexpressing cells, and that thus this c-*fos* induction was a potential mechanism for PLC-β1-mediated cell protection [21]. As for PLC-γ1, Bai et al. [3] suggested that PLC-γ1 mediated survival signaling in the oxidative stress response through protein kinase C-dependent phosphorylation of Bcl-2 and inhibition of caspase-3. Although the neuroprotective effect of theanine might result, at least in part, from the above mechanisms, the molecular details of how PLC-β1 and -γ1 protect cells from oxidative stress remain to be worked out. In particular, as for PLC-γ1, careful evaluation is needed, because it is overexpressed in some human tumors and induces tumors after injection into nude mice [2,7,30].

It has been indicated that theanine administered intragastrically is absorbed by a Na^{+}-coupled cotransporter in the brush-border membrane [18], and the absorbed theanine is transported into the brain through the blood–brain barrier via the leucine-preferring transport system [34]. In addition, the administration of theanine has been reported to cause a significant increase in the dopamine concentration [34] and a decrease in the serotonin concentration [35] in the rat brain. These findings strongly expect that the neuroprotection by theanine can be observed when it is administered orally in vivo. In our laboratory, attempts to elucidate the in vivo action of theanine are currently underway.

The current study revealed that theanine protects cultured cortical neurons from the excitotoxicity of glutamate by increasing the expression levels of PLC-β1 and -γ1 via group I mGluR(s).

Acknowledgments

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