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Galantamine Enhancement of Long-Term Potentiation Is Mediated by Calcium/Calmodulin-Dependent Protein Kinase II and Protein Kinase C Activation

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ABSTRACT: Galantamine, a novel Alzheimer's drug, is known to inhibit acetylcholinesterase activity and potentiate nicotinic acetylcholine receptor (nAChR) in the brain. We previously reported that galantamine potentiates the NMDA-induced currents in primary cultured rat cortical neurons. We now studied the effects of galantamine on long-term potentiation (LTP) in the rat hippocampal CA1 regions. The field excitatory postsynaptic potentials (fEPSPs) were induced by stimulation of the Schaffer collateral/commissural pathways in the hippocampal CA1 region. Treatment with 0.01–10 μM galantamine did not affect the slope of fEPSPs in the CA1 region. Galantamine treatment increased calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase Cα (PKCα) activities with a bell-shaped dose–response curve peaked at 1 μM, thereby increasing the phosphorylation of AMPA receptor, myristoylated alanine-rich protein kinase C, and NMDA receptor as downstream substrates of CaMKII and/or PKCα. By contrast, galatamine treatment did not affect protein kinase A activity. Consistent with the bell-shaped CaMKII and PKCα activation, galantamine treatment enhanced LTP in the hippocampal CA1 regions with the same bell-shaped dose–response curve. Furthermore, LTP potentiation induced by galantamine treatment at 1 μM was closely associated with both CaMKII and PKC activation with concomitant increase in phosphorylation of their downstream substrates except for synapsin I. In addition, the enhancement of LTP by galantamine was accompanied with α7-type nAChR activation. These results suggest that galantamine potentiates NMDA receptor-dependent LTP through α7-type nAChR activation, by which the postsynaptic CaMKII and PKC are activated. © **2009 Wiley-Liss, Inc.**

KEY WORDS: CaMKII; PKC; NMDA; LTP; galantamine; Alzheimer's disease

INTRODUCTION

The neuronal nicotinic ACh receptors (nAChRs) are downregulated in the brain of patients with Alzheimer's disease (AD) (Vidal and Changeux, 1996; Woodruff-Pak and Hinchliffe, 1997). One of the strategies for improving the cognitive deficits in the patients with AD would be attributed to stimulation of nAChRs. In fact, four of five Alzheimer's drugs including tacrine, donepezil, rivastigmine, and galantamine, which were approved for clinical use in the United States, are anticholinesteases. Although galantamine does inhibit cholinesterase, its potency is lower than the other three drugs.

In addition to inhibition of acetylcholinesterase, novel approaches to directly stimulate nAChRs in the brain have been expected. Indeed, galantamine potentiated ACh-induced currents in nAChRs (Schrattenholz et al., 1996; Maelicke and Albuquerque, 2000; Maelicke et al., 2001). The optimal concentration of galantamine to maximally potentiate ACh-induced currents was 0.1–1 μM (Schrattenholz et al., 1996; Maelike and Albuquerque, 2000; Maelike et al., 2001; Santos et al., 2002). Thus, galantamine has a unique property as compared to conventional acetylcholinesterase inhibitors.

AD is a progressive neurodegenerative disorder with cognitive dysfunction. Reduction in *N*-methyl-D-aspartate receptors (NMDARs) is also found in the brain of AD patients, possibly contributing to memory deficits (Fonnum et al., 1995). We previously reported that galantamine also potentiated NMDA-induced currents in primary rat cortical neurons (Moriguchi et al., 2004). Recently, the glycine binding site of NMDARs has been targeted to be a potential therapeutic site to treat memory deficits associated with aging and AD. The partial glycine site agonist, D-cycloserine, and the glycine prodrug, milacemide,

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Abbreviations used: α-BuTX, α-bungarotoxin; AChE, AChsterase; ACSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; AMPARs, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors; CaMKII, calcium/calmodulin-dependent protein kinase II; DARPP-32, dopamine- and cAMP-regulated phosphoprotein; DHβE, dihydro-β-erythroidine; fEPSPs, field excitatory post synaptic potentials; HFS, high frequency stimulation; LTP, long-term potentiation; MARCKS, myristoylated alanine-rich protein kinase C substrate; nAChRs, nicotinic acetylcholine (Ach) receptors; NMDARs, *N*-methyl-D-aspartate receptors; PKA, protein kinase A; PKC, protein kinase C.

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prevented memory deficit in animal behavioral tests (Hanndelmann et al., 1989; Baxter et al., 1994), and have been tested as cognitive enhancers in both healthy subjects and patients with AD (Dysken et al., 1992; Schwartz et al., 1991, 1996). Thus, NMDARs play a crucial role in learning and memory.

LTP is mediated by several protein kinases and phosphatases (Fukunaga et al., 1993; Soderling and Derkach, 2000; Lisman et al., 2002). For example, we have shown that calcium/calmodulin-dependent protein kinase II (CaMKII) autophosphorylation is required for LTP induction (Fukunaga et al., 1993, 1995; Liu et al., 1999; Fukunaga and Miyamoto, 2000). Like CaMKII, protein kinase C (PKC) activity is essential for the hippocampal LTP induction (Collingridge et al., 2004).

Here we found that galantamine treatment stimulates both CaMKII and PKCα activities, thereby leading to potentiation of NMDAR-dependent LTP. The α7-type nAChR activation was required for the enhancement of NMDAR-dependent LTP by galantamine. Thus, galantamine-enhanced NMDAR functions with concomitant activation of α7-type nAChRs underlies the enhancement of LTP, thereby improving the cognitive and learning deficits in AD patients.

MATERIALS AND METHODS

Electrophysiology

Preparation of hippocampal slices was performed as described previously (Liu et al., 1999). Briefly, brains were rapidly removed from ether-anesthetized male Wistar rats (7- to 8-week-old) and the hippocampi were dissected out. Transverse hippocampal slices (400-μm thick) prepared using a vibratome (microslicer DTK-1000) were incubated for 2 h in continuously oxygenized (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) at room temperature (28°C). After a 2-h recovery period, a slice was transferred to an interface recording chamber and perfused at a flow rate of 2 ml/min with ACSF warmed to 34°C. Field excitatory postsynaptic potentials (fEPSPs) were evoked by a 0.05 Hz test stimulus through a bipolar stimulating electrode placed on the Schaffer collateral/commissural pathway and recorded from the stratum radiatum of CA1 using a glass electrode filled with 3 M NaCl. Recording was performed using a single-electrode amplifier (CEZ-3100, Nihon Kohden, Tokyo, Japan), and the maximal value of the initial fEPSPs slope was collected and averaged every 1 min (3 traces) using an A/D converter (PowerLab 200; AD Instruments, Castle Hill, Australia) and a personal computer. After a stable base-line was obtained, high frequency stimulation (HFS) of 100 Hz with a 1-s duration was applied twice with a 10-s interval and test stimuli were continued for the indicated periods. After electrophysiological recording, slices were transferred to a plastic plate cooled on ice to dissect out the CA1 region. CA1 regions were frozen in liquid nitrogen and stored at −80°C for biochemical analysis. Each drugs treatment for 20 min before HFS stimulation or sampling for immnoblot analysis.

Immunoblotting Analysis

Hippocampal CA1 samples were homogenized in 70 μl of homogenizing buffer containing 50 mM Tris-HCl (pH 7.4), 0.5% Triton X-100, 4 mM EGTA, 10 mM EDTA, 1 mM Na_3VO_4, 40 mM sodium pyrophosphate, 50 mM NaF, 100 nM calyculin A, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 50 μg/ml trypsin inhibitor, and 1 mM dithiothreitol (DTT). Insoluble material was removed by a 10-min centrifugation at 15,000 rpm. After determining protein concentration in supernatants using Bradford's solution, samples were boiled 3 min in Laemmli's sample buffer (Laemmli, 1970). Samples containing equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to an Immobilon PVDF membrane for 2 h at 70 V. After blocking with TTBS solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 2.5% bovine serum albumin (BSA) for 1 h at room temperature, membranes were incubated overnight at 4°C with antiphospho CaMKII, (1:5,000, Fukunaga et al., 1988), anti-CaMKII, (1:5,000, Fukunaga et al., 1988), antiphospho-synapsin I (site3) (1:2,000, Chemicon, Temecula, CA), antisynapsin 1 (1:2,000, Fukunaga et al., 1992), antiphospho-GluR1 (Ser-831) (1:1,000, Upstate, Lake placid, MA), anti-GluR1 (1:1,000, Chemicon), antiphospho-PKCα (Ser-657) (1:2,000, Upstate), anti-PKCα (1:2,000, Upstate), antiphospho-MARCKS (Ser-152/156) (1:2,000, Chemicon), anti-MARCKS (1:2,000, Ohmitsu et al., 1999), antiphospho-NR1 (Ser-896) (1:2,000, Upstate), anti-NR1 (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA), antiphospho-NR1 (Ser-897) (1:2,000, Upstate), antiphospho-GluR1 (Ser-845) (1:1,000, Upstate), or antiphospho-DARPP-32 (Thr-34) (1:2,000, Cell signaling, Beverly, MA). Bound antibodies were visualized using the enhanced chemiluminescence detection system (Amersham Life Science, Buckinghamshire, UK) and analyzed semiquantitatively using the National Institutes of Health Image program (NIH image: version 1.63). In this study, we did not find any change in total protein levels (CaMKII, synapsin I, GluR I, PKCα, MARCKS, and NR I) by densitometric analysis of the western blots. So, we did not show the data for total protein expression level. We used β-tubulin as an internal control.

Other Chemicals

The NMDA receptor inhibitor, DL-2-amino-5-phosphonovaleric acid (APV), the CaMKII inhibitor, KN-93, the PKC inhibitor, chelerythrine, the α7-type nACh receptor inhibitor, α-bungarotoxin (α-BuTX), the α4β2-type nACh receptor inhibitor, dihydro-β-erythroidine (DhβE), physostigmine, and galantamine were purchased from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis

Data were represented as means ± standard error of mean (SEM) Comparison between two experimental groups was made using the unpaired Student's *t*-test. Multiple comparisons

were made by one-way analysis of variance (ANOVA) followed by Sheffe's test. A $P < 0.05$ was considered significant.

RESULTS

Effects of Galantamine on the fEPSPs and Phosphorylation of CaMKII, PKCα, and DARPP-32 in the Hippocampal CA1 Regions

We previously reported that galantamine potentiated NMDA-induced currents through the activation of PKC in rat cortical neurons (Moriguchi et al., 2004). The maximal potentiation was obtained by 1 μM galantamine, with a bell-shaped dose–response curve. We here examined whether galantamine treatment affected protein kinase activities in the hippocampal CA1 region. We examined autophosphorylation of CaMKII and PKCα, and phosphorylation of dopamine- and cAMP-regulated phosphoprotein (DARPP-32) as downstream targets of protein kinase A (PKA) (Edwards et al., 2002) by immunoblot analyses using phosphospecific antibodies. Consistent with our previous observations, galantamine potentiated CaMKII and PKCα autophosphorylation with a bell-shaped dose response peaked at 1 μM (CaMKII: 131.4% ± 6.4% of control, $n = 4$; PKCα: 134.1% ± 7.2% of control, $n = 4$) without changes in total protein levels of CaMKII and PKCα (Figs. 1A,B). By contrast, DARPP-32 (Thr-34) phosphorylation was not affected by galantamine treatment at 0.01–10 μM (Figs. 1A,B). We also examined the effect of galantamine on the slope of fEPSPs induced by stimulation of the Schaffer collateral/commissural pathways in the hippocampal CA1 region. Galantamine treatment for 20 min did not affect the slope of fEPSPs at concentrations of 0.01–10 μM (Fig. 1C), suggesting that AMPA current is not affected by the galantamine treatment.

Effects of Galantamine on Phosphorylation of Synapsin I (site3), GluR1 (Ser-831), MARCKS (Ser-152/156), and NR1 (Ser-896)

We next asked whether galantamine enhanced phosphorylation of downstream targets of CaMKII and PKCα activities in the hippocampal CA1 region. Phosphorylation of GluR1 (Ser-831) as downstream of CaMKII (Derkach et al., 1999) was increased by galantamine treatment at 0.1–10 μM without change in total GluR1 protein level (126.5% ± 4.8% of control, $n = 4$) (Figs. 2A,C). Phosphorylation of synapsin I (site 3) was also measured as presynaptic CaMKII substrate (Yamagata, 2003). Galantamine treatment did not affect synapsin I (site 3) phosphorylation (Figs. 2A,C). Likewise, phosphorylation of myristoylated alanine-rich protein kinase C substrate (MARCKS) (Ser-152/156) and NR1 (Ser-896) as downstream targets of PKCα (Tingley et al., 1997; Ohmitsu et al., 1999) were increased significantly by 1 μM galantamine without change in total MARCKS and NR1 protein level (pMARCKS

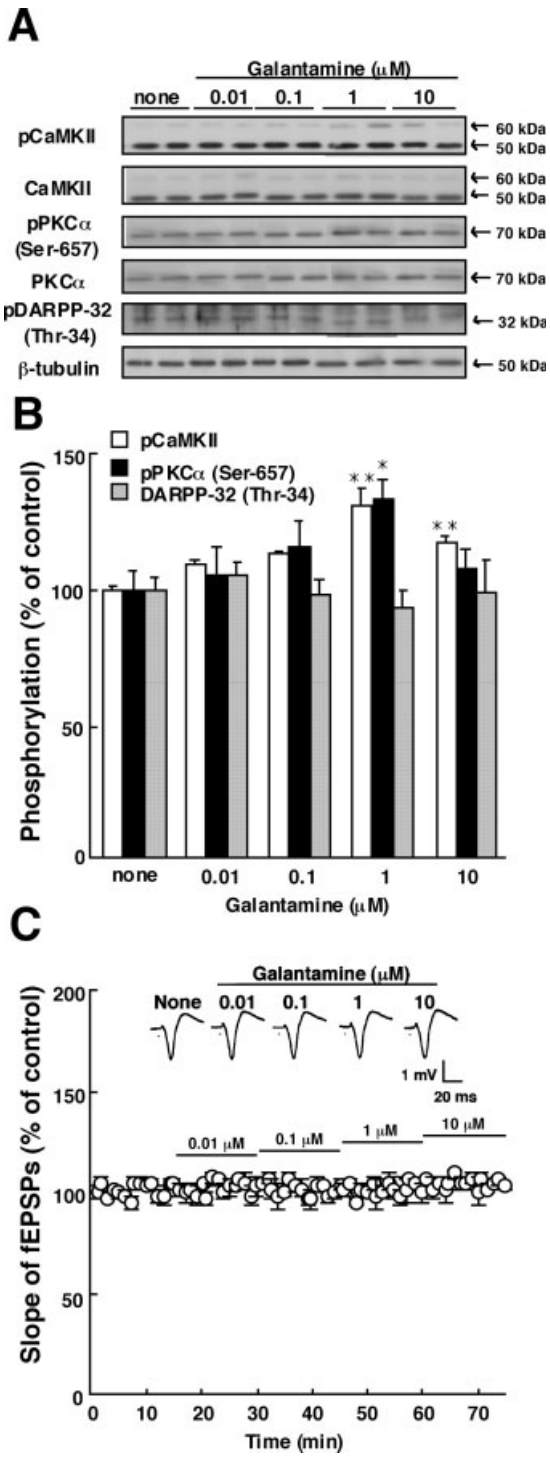

FIGURE 1. Effects of galantamine treatment on CaMKII and PKCα autophosphorylation and phosphorylation of DARPP-32. (A) Representative images of immunoblots using antibodies against pCaMKII and pPKCα and pDARPP-32 (Thr-34). Levels of proteins were unchanged following galantamine treatment. (B) Quantitative analyses of pCaMKII, pPKCα (Ser-657), and pDARPP-32 (Thr-34) by densitometry are summarized. Data are expressed as percentage of value of controls without stimulation ($n = 4$). (C) fEPSPs were recorded from the hippocampal CA1 region after sequential treatment with 0.01 to 10 μM galantamine. $^{**}P < 0.01$; $^{*}P < 0.05$ vs. the control.

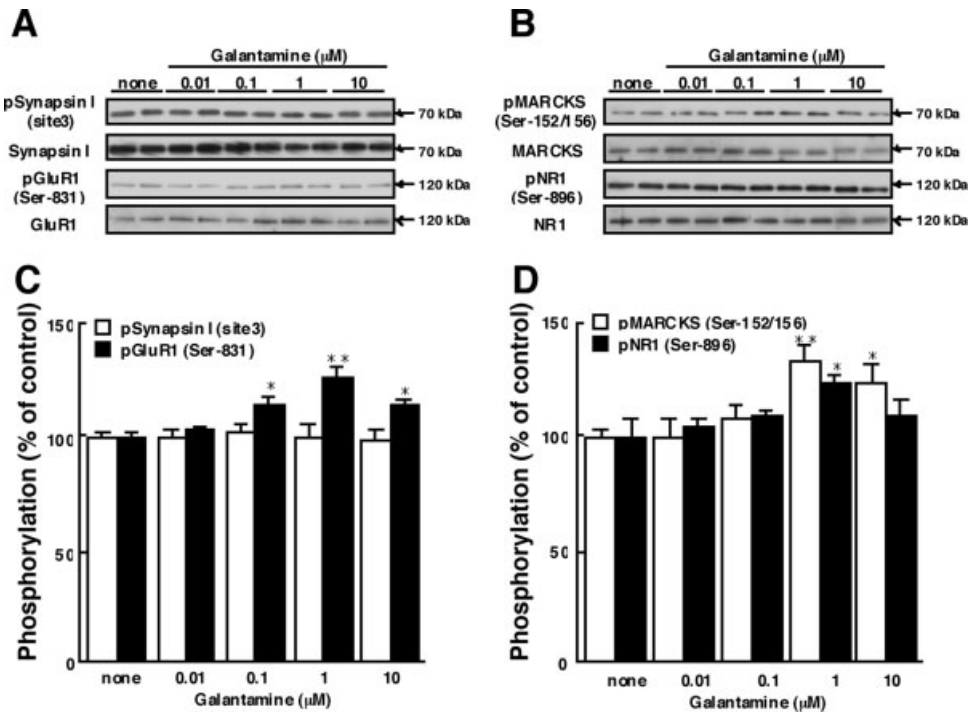

FIGURE 2. Effects of galantamine treatment on phosphorylation of GluR1 (Ser-831), synapsin I (site3), MARCKS (Ser-152/156), and NR1 (Ser-896). (A) Representative images of immunoblots using antibodies against pGluR1 (Ser-831), GluR1, pSynapsin I (Site3), and synapsin I. Protein levels were unchanged following galantamine treatment. (B) Representative images of immunoblots using antibodies against pMARCKS (Ser-152/156), MARCKS, pNR1 (Ser-896), and NR1. Protein levels were unchanged following galantamine treatment. (C) Quantitative analyses of pGluR1 (Ser-831) and pSynapsin I (site3) by densitometry are summarized. (D) Quantitative analyses of pMARCKS (Ser-152/156) and pNR1 (Ser-896) analyzed by densitometry are summarized. Data are expressed as percentage of value of controls without stimulation ($n = 4$). $^{**}P < 0.01$; $^{*}P < 0.05$ vs. the control.

(Ser-152/156): 133.5% ± 7.2% of control, $n = 4$; pNR1 (Ser-896): 124.8% ± 3.3% of control, $n = 4$) (Figs. 2B,D). The bell-shaped increase in the CaMKII and PKCα autophosphorylation and phosphorylation of substrates was correlated with the bell-shaped potentiation of NMDAR currents previously observed (Moriguchi et al., 2004).

Potentiation of NMDA Receptor-Dependent LTP by Galantamine Treatment

We next tested whether the increased both CaMKII and PKCα activities induced by galantamine affect the LTP induction/maintenance in the hippocampal CA1 region. In control slices, LTP induced by stimulation of the Schaffer colloteral/commissural lasted for at least 60 min (171.4% ± 13.4% of baseline at 60 min, $n = 7$) (Figs. 3A,B). The LTP was completely inhibited by treatment with the NMDAR antagonist APV at 50 μM (data not shown), suggesting that LTP was an NMDAR-dependent event. Galantamine was applied 20 min before HFS and included in the perfusion throughout the experiments. Galantamine at 1 μM significantly enhanced LTP, while treatment at 0.01, 0.1, or 10 μM did not (Figs. 3B,C). Significant potentiation of LTP was obtained at 1 μM galantamine (129.6% ± 11.9% of control LTP, $n = 6$) (Figs. 3B,C). The bell-shaped increases in both CaMKII and PKCα autophosphorylation likely contribute to galantamine-induced LTP enhancement.

Association of CaMKII Activation With the Enhanced LTP After Galantamine Treatment

We further confirmed that the enhanced LTP by galantamine is associated with the increases in CaMKII autophosphorylation and GluR1 phosphorylation. Consistent with our hypothesis, LTP-induced increase in CaMKII autophosphorylation was potentiated by galantamine treatment throughout LTP induction/maintenance up to 60 min after HFS without change in total CaMKII protein level (172.2% ± 2.9% in galantamine treated slices vs. 152.0% ± 3.2% in control at 60 min, $n = 4$) (Figs. 4A,B). Similarly, increased GluR1 (Ser-831) phosphorylation was potentiated by galantamine treatment without change in total GluR1 protein level (180.0% ± 3.4% in galantamine treated slices vs. 148.9% ± 1.4% in control at 60 min, $n = 4$) (Figs. 4A,D). Although Synapsin I (site3) phosphorylation was increased by LTP induction, galantamine had no effects on the phosphorylation (142.4% ± 3.6% in galantamine treated slices vs. 143.5% ± 2.2% in control at 60 min, $n = 4$) (Figs. 4A,C).

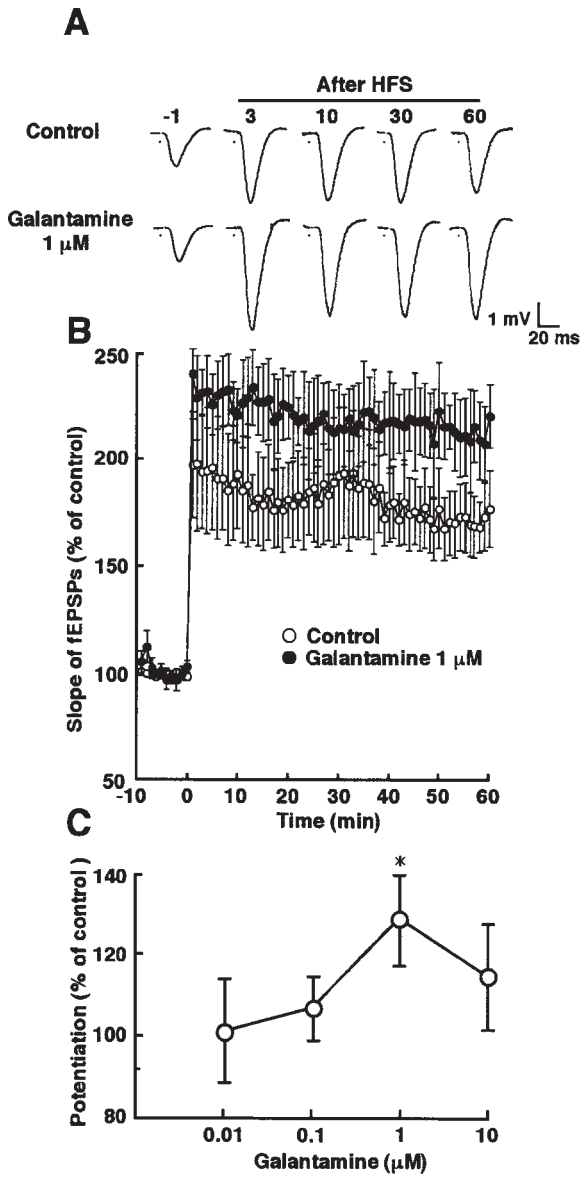

FIGURE 3. Enhancement of LTP by galantamine treatment in the hippocampal CA1 region. (A) Representative fEPSPs recorded from the CA1 region in the presence and absence of 1 μM galantamine. (B) Changes in slopes of fEPSPs following HFS in the presence and absence of 1 μM galantamine. (C) level of LTP potentiation 60 min after HFS in the presence or absence of galantamine. Potentiation by galantamine shows a bell-shaped dose response relationship ($n = 4$). $^{*}P < 0.05$ vs. the control.

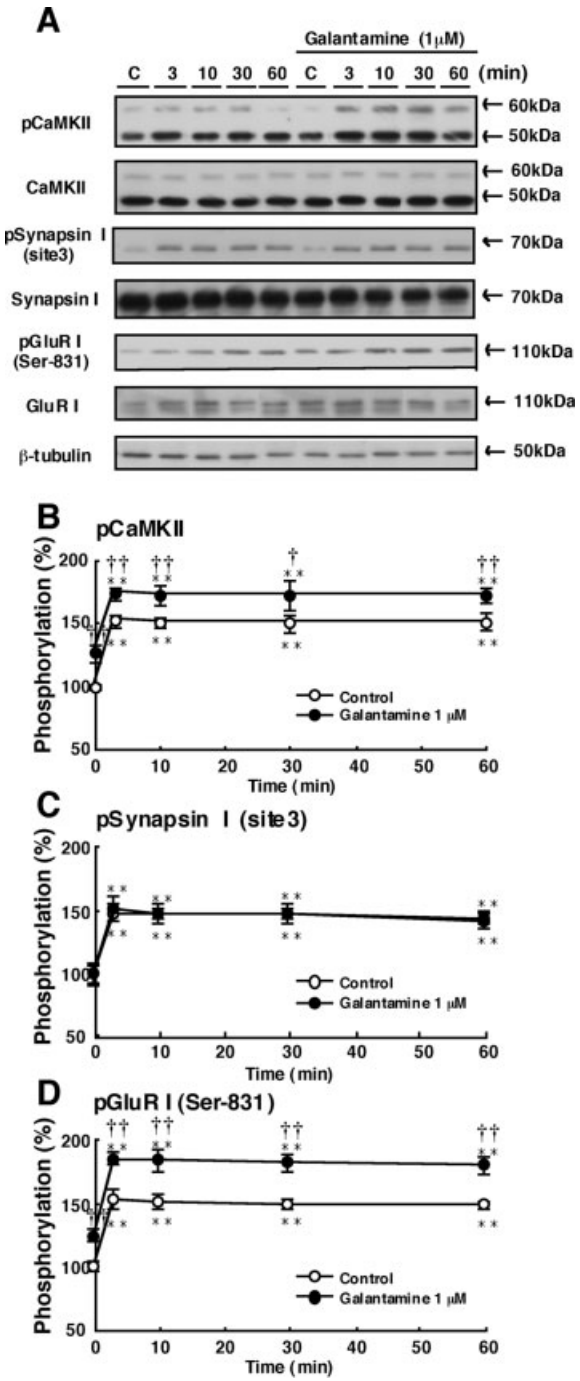

FIGURE 4. Effects of galantamine treatment (1 μM) on increases in CaMKII autophosphorylation and phosphorylation of synapsin I and GluR1(Ser-831) following LTP induction. (A) Representative images of immunoblots using antibodies against pCaMKII, CaMKII, pSynapsin I (site3), synapsin I, phosphorylated GluR1 (Ser-831), and GluR1. Protein levels were unchanged following galantamine treatment and LTP induction. (B–D) Quantitative analyses of autophosphorylated CaMKII, pSynapsin I (site3), and pGluR1 (Ser-831) determined by densitometry are summarized. Data are expressed as percentage of value of controls without HFS in the absence of galantamine ($n = 4$). $^{**}P < 0.01$ versus the control before HFS; $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ versus control without galantamine treatment.

Association of PKCα Activation by Enhancement of LTP After Galantamine Treatment

In addition to CaMKII activation by galantamine treatment, PKC pathway may also underlie galantamine-induced LTP enhancement as shown in Figure 3. LTP-induced increase in PKCα autophosphorylation was potentiated by galantamine treatment throughout LTP induction/maintenance up to 60 min after HFS without change in total PKCα protein level (189.0% ± 7.0% in galantamine treated slices vs. 159.8% ± 4.3% in

control at 60 min, $n = 4$) (Figs. 5A,B). Similarly, the increase in MARCKS (Ser-152/156) and NR1 (Ser-896) phosphorylation was potentiated by galantamine treatment without change in total MARCKS and NR1 protein level (MARCKS (Ser-152/156): 212.8% ± 7.8% in galantamine treated slices vs. 175.3% ± 5.4% in control at 60 min, $n = 4$; NR1 (Ser-896): 235.5% ± 3.8% in galantamine treated slices vs. 183.1% ± 2.4% in control at 60 min, $n = 4$) (Figs. 5A,C,D).

Lack of Association of PKA Activation in Galantamine Treatment

Because PKA activity contributes to LTP induction (Frey et al., 1993), we also examined phosphorylation of downstream targets of PKA after LTP induction in the presence or absence of 1 μM galantamine. DARPP-32 phosphorylation was not changed following LTP induction in the presence or absence of galantamine (Figs. 6A,B). On the other hand, phosphorylation of GluR1 (Ser-845) (Roche et al., 1996) and NR1 (Ser-897) was significantly increased following LTP induction (GluR1 (Ser-845): 137.4% ± 2.4% of control at 3 min, $n = 4$; NR1 (Ser-897): 130.0% ± 2.3% of control at 3 min, $n = 4$). However, phosphorylation of GluR1 (Ser-845) and NR1 (Ser-897) were not affected by treatment with 1 μM galantamine (Figs. 6A,C,D). Protein levels of GluR1 and NR1 were not changed following LTP and galantamine treatment (Fig. 6A).

Effects of KN-93 and Chelerythrine on Galantamine-Induced Enhancement of LTP in the Hippocampal CA1 Region

Because galantamine-induced enhancement of LTP was associated with increased autophosphorylation of CaMKII and PKCα in the hippocampal CA1 region (Figs. 4 and 5), we examined the effect of KN-93 (10 μM) or chelerythrine (3 μM) on galantamine-enhanced LTP. The kinase inhibitors were applied 20 min before HFS and included in the perfusion throughout the experiments. KN93 inhibited LTP induction after HFS (92.3% ± 3.5% of baseline at 60 min, $n = 4$) (Figs. 7A,B). Similarly, treatment with chelerythrine partially inhibited galantamine-enhanced LTP induction (113.9% ± 13.0% of baseline at 60 min, $n = 4$) (Figs. 7A,B).

Effects of KN-93 and Chelerythrine on Activity of CaMKII or PKC in the Hippocampal CA1 Region

Because galantamine-enhanced LTP was inhibited by treatments with KN-93 or chelerythrine in the hippocampal CA1 regions (Fig. 7), we confirmed that the effects of kinase inhibitors were due to inhibition of CaMKII or PKC activities in the hippocampal slices. The LTP-induced enhancement of CaMKII autophosphorylation and GluR1 (Ser-831) phosphorylation markedly decreased by KN-93 treatment to 105.7% ± 5.3% and 97.3% ± 3.7% of control, respectively, 3 min after HFS without change in total CaMKII and GluR1 protein level (Figs. 8A,B). Similarly, the LTP-induced enhancement of PKCα (Ser-657) and NR1 (Ser-896) phosphorylation also

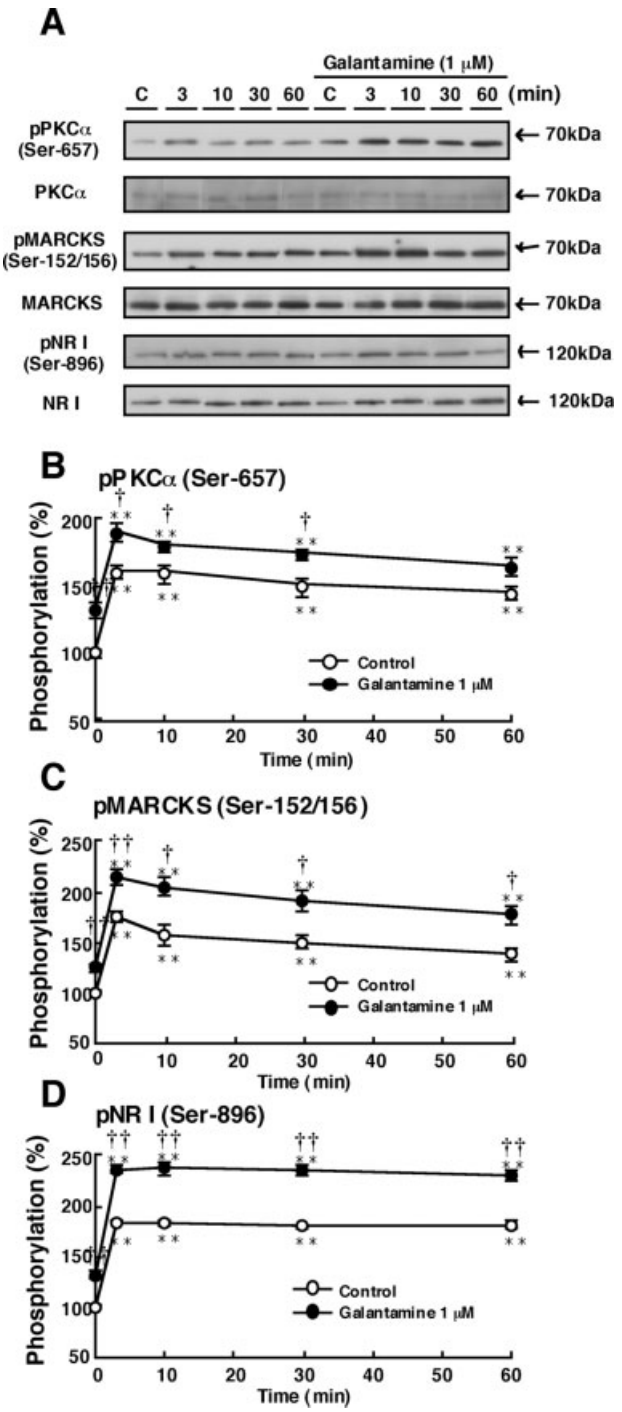

FIGURE 5. Effects of galantamine treatment (1 μM) on increases in PKCα autophosphorylation and phosphorylation of MARCKS and NR1 (Ser-896) following LTP induction. (A) Representative images of immunoblots using antibodies against pPKCα (Ser-657), PKCα, pMARCKS (Ser-152/156), MARCKS, pNR1 (Ser-896), and NR1. Protein levels were unchanged following galantamine treatment and LTP induction. (B–D) Quantitative analyses of pPKCα (Ser-657), pMARCKS (Ser-152/156), and pNR1 (Ser-896) determined by densitometry are summarized. Data are expressed as percentage of value of controls without HFS in the absence of galantamine ($n = 6$). $^{**}P < 0.01$ versus the control before HFS; $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ versus control without galantamine treatment.

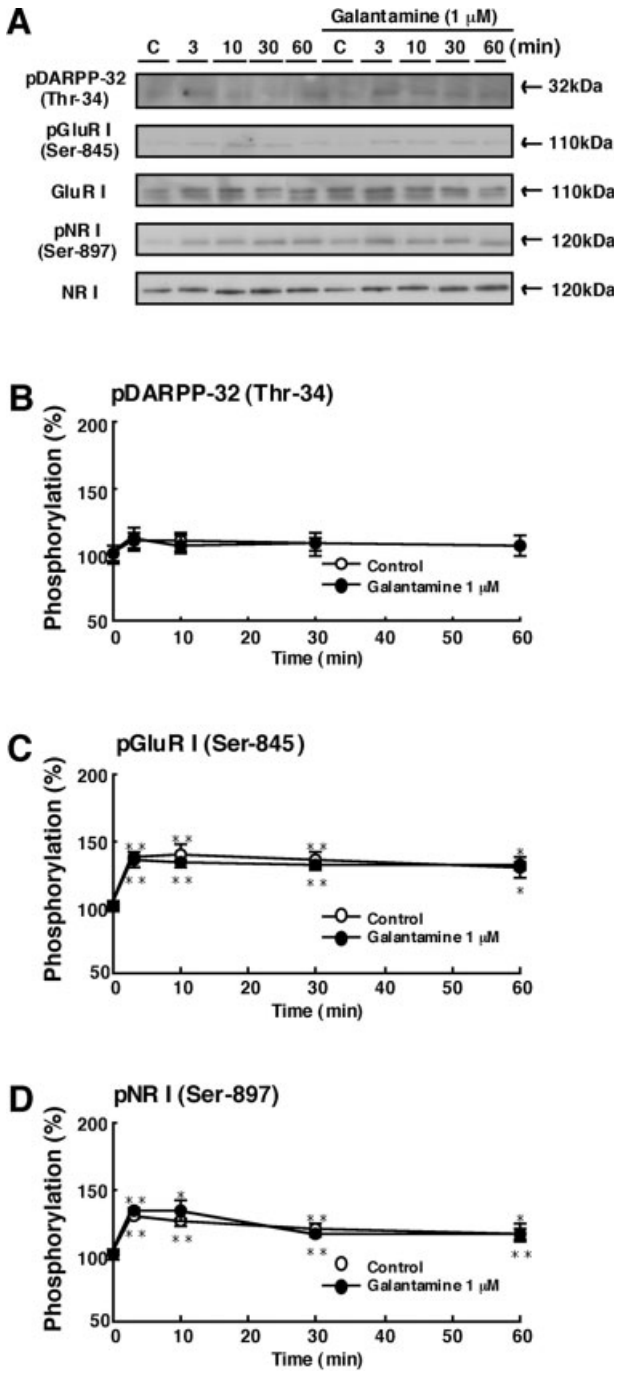

FIGURE 6. Effects of galantamine treatment (1 μM) on phosphorylation of DARPP-32 (Thr-34), GluR1 (Ser-845), and NR1 (Ser-897) following LTP induction. (A) Representative images of immunoblots using antibodies against pDARPP-32 (Thr-34), pGluR1 (Ser-845), GluR1, pNR1 (Ser-897), and NR1. Levels of GluR1 protein were unchanged following galantamine treatment and LTP induction. (B–D) Quantitative analyses of pDARPP-32 (Thr-34), pGluR1 (Ser-845), and pNR1 (Ser-897) performed by densitometry are summarized. Data are expressed as percentage of value of controls without HFS in the absence of galantamine ($n = 4$). $^{**}P < 0.01$, $^{*}P < 0.05$ versus the control before HFS.

significantly decreased by chelerythrine treatment to 101.7% ± 3.8% and 114.4% ± 3.3% of control, respectively, 3 min after HFS without change in total PKCα and NR1 protein level (Figs. 8C,D).

Effects of α-BuTX, DHβE, and Physostigmine on Galantamine-Induced Enhancement of LTP in the Hippocampal CA1 Region

Finally, we examined that whether the potentiation of NMDA receptor function by galantamine is due to nAChRs activation or AChsterase (AChE) inhibition in the hippocampal CA1 region (Fig. 9). In the hippocampal neurons, α7-type

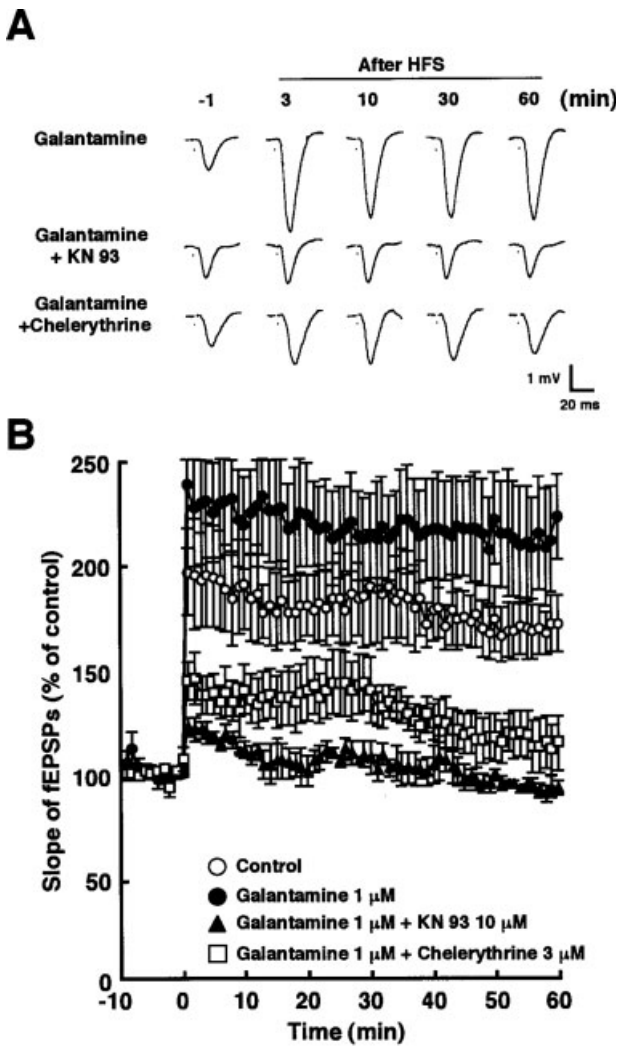

FIGURE 7. Effects of KN-93 or chelerythrine on galantamine-induced enhancement of LTP in the hippocampal CA1 region. (A) Representative fEPSPs recorded from the CA1 region with galantamine treatment in the presence or absence of KN-93 and chelerythrine before and after HFS. (B) Changes in slopes of fEPSPs following HFS are represented with or without (control) application of 1 μM galantamine in the presence and absence of KN-93 (10 μM) or chelerythrine (3 μM).

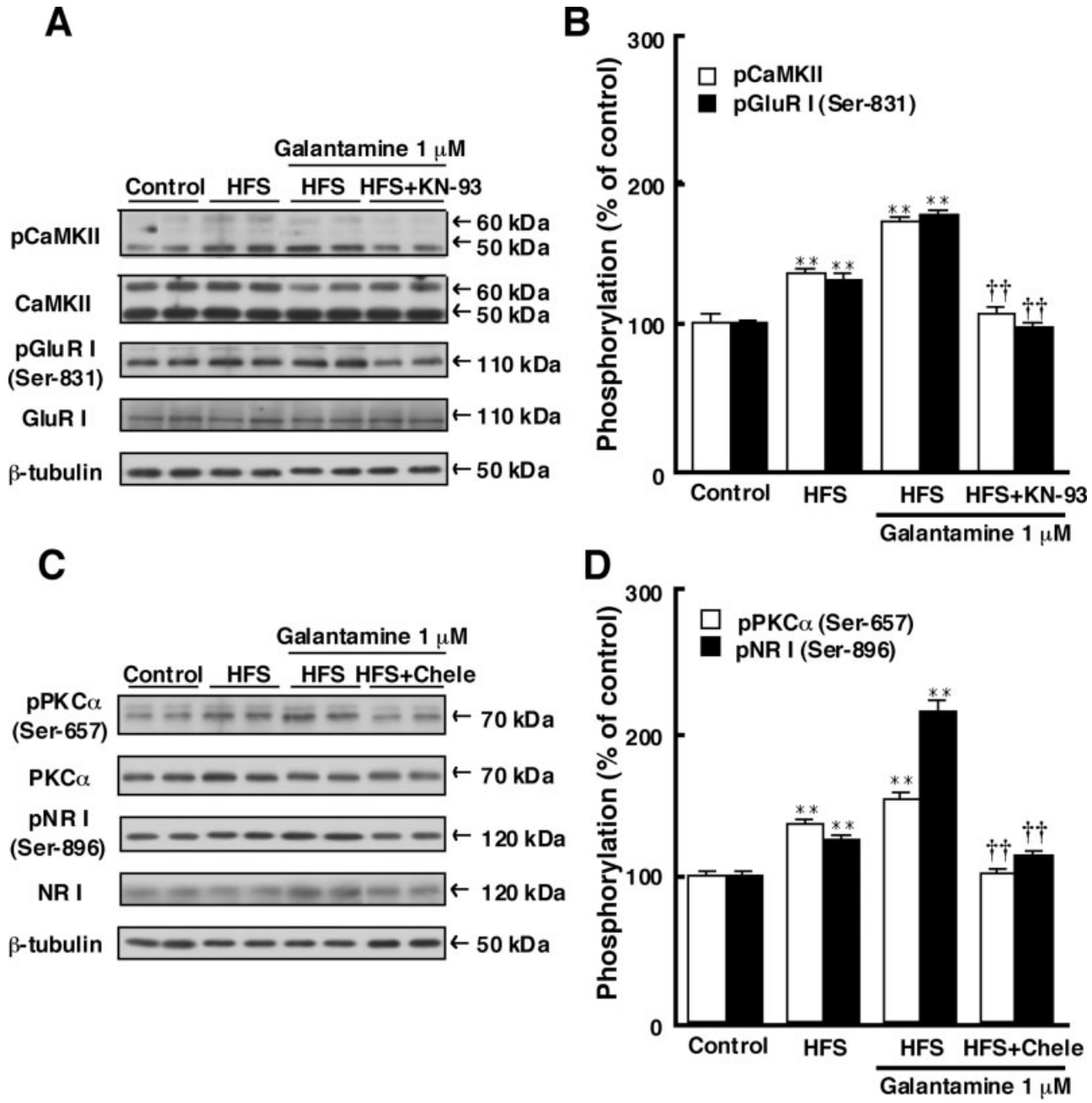

FIGURE 8. Effects of KN-93 or chelerythrine on galantamine-induced enhancement of CaMKII and PKCα (Ser-657) autophosphorylation and phosphorylation of their endogenous substrates in the hippocampus. (A) Representative images of immunoblots using antibodies against pCaMKII, CaMKII, pGluR1 (Ser-831), and GluR1. Protein levels were unchanged following galantamine treatment and LTP induction. (B) Quantitative analyses of pCaMKII and pGluR1 (Ser-831) determined by densitometry are summarized. Data are expressed as percentage of value of controls without HFS in the absence of galantamine. $^{**}P < 0.01$ versus the control before HFS; $^{\dagger\dagger}P < 0.01$ versus HFS with galantamine treatment. (C) pPKCα (Ser-657), PKCα, pNR1 (Ser-896), and NR1. Protein levels were unchanged following galantamine treatment and LTP induction. (D) Quantitative analyses of pPKCα (Ser-657) and pNR1 (Ser-896) determined by densitometry are summarized. Data are expressed as percentage of value of controls without HFS in the absence of galantamine. $^{**}P < 0.01$ versus the control before HFS; $^{\dagger\dagger}P < 0.01$ versus HFS with galantamine treatment.

nAChRs are widely expressed (Morales et al., 2008). The α7-type nAChR inhibitor, α-bungarotoxin (α-BuTX), α4β2-type nAChR inhibitor, dihydro-β-erythroidine (DHβE) and AChE inhibitor, physostigmine were applied 20 min before HFS and included in the perfusion medium throughout the experiments. α-BuTX at 25 nM significantly inhibited galantamine enhancement of LTP induction (168.9% ± 20.3% of baseline at 60 min, $n = 5$) compared with galantamine-potentiated LTP (218.8% ± 17.9% of baseline at 60 min, $n = 7$) (Figs. 9A–C). By contrast, treatments with DHβE and physostigmine did

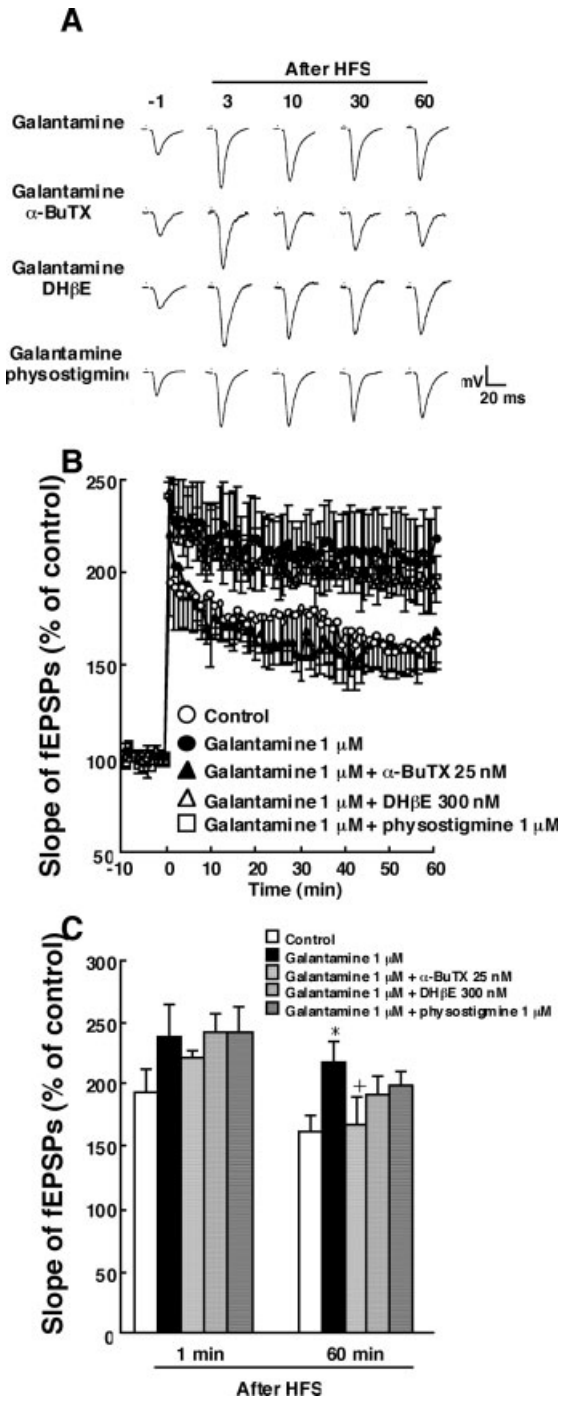

FIGURE 9. Effects of α-BuTX, DHβE, and physostigmine on galantamine-induced enhancement of LTP in the hippocampal CA1 region. (A) Representative fEPSPs were recorded from the CA1 region with galantamine treatment in the presence or absence of α-BuTX, DHβE, and physostigmine before and after HFS. (B) Changes in slopes of fEPSPs following HFS were represented with or without (control) application of 1 μM galantamine in the presence and absence of α-BuTX (25 nM), DHβE (300 nM), and physostigmine (1 μM). (C) Changes in slopes of fEPSPs following HFS were represented with or without (control) application of 1 μM galantamine in the presence and absence of α-BuTX (25 nM), DHβE (300 nM), and physostigmine (1 μM) at 1 or 60 min. *$P < 0.05$ versus the control; †$P < 0.05$ versus galantamine treatment.

not affect the galantamine-enhanced LTP induction (DHbE: 193.5% ± 14.4% of baseline at 60 min, $n = 5$; physostigmine: 199.8% ± 13.4% of baseline at 60 min, $n = 5$) (Figs. 9A–C).

DISCUSSION

Galantamine, an anticholinesterase drug for AD, indeed potentiates ACh-induced currents in nAChRs (Schrattenholz et al., 1996; Maelicke and Alburquerque, 2000; Maelicke et al., 2001; Santos et al., 2002). The potency of galantamine to inhibit cholinesterase (IC_{50} = 600–800 nM) is lower than that of other cholinesterase inhibitors such as donepezil (26 nM) and rivastigmine (4.3 nM). Earlier studies showed that galantamine at 1–10 μM opened single AChR channels in the rat hippocampus and in the chicken α4β2 receptors expressed in mouse fibroblast (M10) cells but failed to evoke detectable whole-cell currents of AChR channel (Pereira et al., 1993, 1994). However, ACh-induced whole-cell currents were potentiated by galantamine (0.1–1 μM) in PC12 cells (α3-type AChRs), in hippocampal α 7-type AChRs (Schrattenholz et al., 1996; Maelicke and Albuquerque, 2000), in α4β2 AChRs expressed in HEK cells (Samochocki et al., 2003), and in α7 AChRs and in α7/5HT$_3$ chimera (Maelicke et al., 2001). Because galantamine-induced AChR potentiation was blocked by the monoclonal antibody FK1 that recognizes the α subunit of nAChRs, galantamine likely binds to a site distinct from the ACh binding site (Schrattenbolz et al., 1996), thereby acting as an allosterically potentiating ligand. A bell-shaped dose–response relationship was obtained for the potentiation peaked at 1 μM (Schrattenbolz et al., 1996), but the underlying mechanism has not been elucidated.

In addition to AChR dysfunction, reduced NMDAR function in the brain of AD was reported, possibly contributing to cognition and memory deficits (Fonnum et al., 1995). Recently, we reported that galantamine potentiated NMDAR-induced currents to 130% of controls through activation of PKC pathway in cultured rat cortical neurons. The galantamine concentration required to maximally potentiate was 1 μM (Moriguchi et al., 2004). In addition, cognitive enhancer nefiracetam potentiated LTP through activation of CaMKII and PKC in rat hippocampal CA1 region (Moriguchi et al., 2008). In this study, galantamine treatment at 0.01–10 μM did not affect the slope of fEPSPs in rat hippocampal CA1 region under the normal condition, suggesting no effect on AMPAR currents. This observation disagrees with a previous observation in which increased GluR1 (Ser-831) phosphorylation by CaMKII was associated with LTP induction (Barria et al., 1997). However, phosphorylation of GluR1 (Ser-845) phosphorylation by PKA is known to be essential for the potentiation of AMPAR by LTP induction (Roche et al., 1996). Because the GluR1 (Ser-845) phosphorylation was not stimulated by galantamine treatment, the stimulatory effect of galantamine on

GluR1 (Ser-831) phosphorylation was not sufficient for potentiation of AMPA current as measured by fEPSPs. By contrast, PKC activation and increased phosphorylation of MARCKS (Ser-152/156) and NR1 (Ser-896) were closely related to the bell-shaped potentiation of LTP by galantamine treatment (Fig. 3). Thus, galantamine enhanced NMDA receptor through PKC activation predominantly mediates LTP potentiation by galantamine in rat hippocampal CA1 region. We previously demonstrated that nefiracetam, a cognitive enhancer, also potentiated the NMDA-induced currents in PKC-dependent manner in rat cortical neurons (Moriguchi et al., 2003, 2007). We hypothesize that activation of NMDARs by cognitive enhancers through PKC activation provides a novel target for AD therapeutics.

The physiological relevance of NMDAR potentiation by PKC activation in hippocampus is also confirmed by NMDAR-dependent LTP in the hippocampal CA1 region. Interestingly, galantamine significantly potentiated NMDAR-dependent LTP to about 120% of control in rat hippocampal CA1 regions. Potentiation of LTP induced by galantamine showed a bell-shaped dose–response curve that was peaked at 1 μM in the same manner as CaMKII and PKC activation. This result is consistent with our previous observations, in which galantamine potentiated NMDA-induced currents in cultured rat cortical neurons exhibiting the bell-shaped dose–response relationship (Moriguchi et al., 2004). Furthermore, galantamine enhancement of NMDAR-dependent LTP was accompanied with α7-type nAChR activation (Fig. 9). Thus, the synergestic activation both of NMDARs and α7-type nAChRs by galantamine accounts for the enhancement of LTP in the hippocampal CA1 region. We also found that PKC-dependent phosphorylation of NR1 contributed to the enhancement of NMDAR function induced by galantamine in the hippocampal CA1 region. The bell-shaped increase in PKCα autophosphorylation and phosphorylation of MARCKS (Ser-152/156) and NR1 (Ser-896) was closely associated with the bell-shaped enhancement of LTP by galantamine in the hippocampal CA1 region. The activation of PKCα by galantamine triggers enhancement of NMDAR functions, because PKCα phosphorylates the Ser-896 of NMDAR NR1 subunit (NR1-Ser896) (Tingley et al., 1997). However, the mechanisms underlying the bell-shaped dose–response curve in activation of NMDAR currents and in LTP enhancement by galantamine are not clear at present.

Galantamine seems to contribute to improvement of the cognition, learning, and memory of patients with AD via multiple pathways. This study showed that galantamine potentiation of NMDAR-dependent LTP in the hippocampal CA1 region involved CaMKII and PKC activation. We already reported that galantamine potentiated NMDA-induced currents in cultured rat cortical neurons through the activation of PKC (Moriguchi et al., 2004). In addition to inhibition of AChsterase (Thomson et al., 1991), galantamine could allosterically potentiate the activity of nAChRs (Pereira et al., 1993, 1994; Schrattenholz et al., 1996; Maelicke and Alburquerque, 2000; Samochocki et al., 2003). In the brain of patients with AD, not only nAChR activity (Vidal and Changeux, 1996; Woodruff-Pak and Hinchliffe, 1997) but also the NMDAR is impaired (Greenamyre et al., 1987; Fonnum et al., 1995). In this study, we found that galantamine enhancement of NMDAR-dependent LTP is concomitant with α7-type nAChR activation (Fig. 9). Thus, galantamine stimulation of NMDARs with concomitant α7-type nAChR activation is deemed to work in concert for the improvement of the AD patient's condition. Notably, expression of CaMKIIα containing neurons were selectively lost in the hippocampal CA1 subfield of patients with AD (Wang et al., 2005). The activation of CaMKII by galantamine indeed improves cognition, learning, and memory function of patients with AD.

In conclusion, this study showed that galantamine treatment significantly potentiated NMDAR-dependent LTP through the activation of CaMKII and PKC in rat hippocampal CA1 region. Enhancement of LTP by galantamine through α7-type nAChR and NMDAR activation improves likely mediates improvement of cognitive, learning, and memory deficit in patients with AD.

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