Muscarinic Potentiation of $GABA_A$ Receptor Currents Is Gated by Insulin Signaling in the Prefrontal Cortex

Xiao-Hong Ma, Ping Zhong, Zhenglin Gu, Jian Feng, and Zhen Yan
Department of Physiology and Biophysics, State University of New York at Buffalo, Buffalo, New York 14214

Cholinergic neurotransmission and insulin signaling in cognitive areas, such as the prefrontal cortex (PFC), play a key role in regulating learning and memory. However, the cellular mechanisms by which this regulation occurs are unclear. Because GABAergic inhibition in the PFC controls the timing of neuronal activity during cognitive operations, we examined the potential regulation of GABA transmission by cholinergic and insulin signaling in PFC pyramidal neurons. Activation of muscarinic acetylcholine receptors (mAChRs) with carbachol produced an enhancement of $GABA_A$ receptor currents in acutely dissociated cells after a short treatment with insulin. Inhibiting phosphoinositide-3 kinase (PI3K), a downstream target of insulin signaling, eliminated this effect as well as the carbachol-induced enhancement of GABAergic miniature IPSC amplitudes in PFC slices. The muscarinic potentiation of $GABA_A$ currents was blocked by PKC inhibitors, broad-spectrum protein tyrosine kinase inhibitors, and specific inhibitors of the nonreceptor tyrosine kinase Src. Additionally, muscarinic receptors in PFC slices activated PKC and the focal adhesion kinase Pyk2 (a potential molecular link between PKC and Src) in a PI3K-dependent manner. Together, our results show that mAChR activation in PFC pyramidal neurons enhances $GABA_A$ receptor functions through a PKC-dependent, Src-mediated signaling cascade that is gated by an insulin/PI3K pathway. Given the significance of GABAergic transmission in regulating PFC functions, our results provide a novel mechanism for understanding the role of cholinergic systems and insulin signaling in learning and memory.

Key words: **modulation; m1 receptors; PKC; tyrosine phosphorylation; insulin signaling; single-cell mRNA profiling; prefrontal cortex; patch clamp**

Introduction

The cholinergic system plays a crucial modulatory role in the CNS. Degeneration of basal forebrain cholinergic neurons, together with deficits in the central cholinergic system, has been linked to cognitive and memory impairment associated with Alzheimer's disease (AD) (Coyle et al., 1983; Muir, 1997). So far, the most effective therapeutic strategy in AD treatment has been to enhance cholinergic transmission (Sitaram et al., 1978; Benzi and Moretti, 1998). Acetylcholine (ACh) exerts its functions in the brain by activating two families of receptors: nicotinic ACh receptors (nAChRs), which are ligand-gated ion channels, and muscarinic ACh receptors (mAChRs), which couple to G-protein pathways. It has been recognized for some time that mAChRs play key roles in the control of high-level cognitive processes, such as selective attention, learning, and memory (Nathanson, 1987). The muscarinic receptor–G-protein interactions, as well as the downstream second-messenger system, are impaired in AD (Pavia et al., 1998). Drugs that antagonize mAChRs impair rodent performance in various learning and memory tasks (Sutherland et al., 1982; Whishaw et al., 1987; Roldan et al., 1997), whereas drugs that activate muscarinic receptors are helpful in ameliorating the cognitive deficits of AD (Whitehouse, 1993).

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Correspondence should be addressed to Dr. Zhen Yan, Department of Physiology and Biophysics, State University of New York at Buffalo, 124 Sherman Hall, Buffalo, NY 14214. E-mail: zhenyan@buffalo.edu.

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Emerging evidence suggests that insulin, in addition to mAChRs, also has important functions in brain regions involved in cognition, and insufficient insulin in these areas can result in memory loss and even AD (Craft et al., 1996; Wickelgren, 1998). Insulin receptors are highly expressed in CNS neurons and localized to synapses (Wozniak et al., 1993; Abbott et al., 1999). Recent studies show that CNS insulin may function as an important neuromodulator to influence cognition by regulating ion channels, neurotransmitter receptors, and synaptic transmission (Wan et al., 1997b; Kanzaki et al., 1999; Man et al., 2000). Impairment of cerebral insulin signaling causes behavioral abnormalities similar to those caused by disruption of the cholinergic function (Mayer et al., 1990), suggesting the potential interaction between the two systems.

One of the major target structures of cholinergic projections from the basal forebrain is the prefrontal cortex (PFC), a brain region critically involved in the control of cognition and emotion (Goldman-Rakic, 1995). The PFC is composed primarily of glutamatergic pyramidal principal neurons and GABAergic interneurons. The GABAergic synapses on pyramidal neurons (Somogyi et al., 1983) exert powerful inhibitory control via $GABA_A$ receptors over the excitatory output of the PFC. One important function of the GABAergic inhibition in the PFC is to control the timing of neuronal activity during cognitive operations and thereby shape the temporal flow of information (Constantinidis et al., 2002). To understand the potential role of mAChRs in regulating GABAergic inhibition in the PFC, we examined the molecular and cellular mechanisms underlying muscarinic modulation of $GABA_A$ receptor-mediated currents in PFC pyramidal neurons. We found that mAChR activation enhances postsynap-

tic $GABA_A$ currents via a PKC-dependent, tyrosine kinase-mediated pathway. Additionally, this modulation is gated by insulin signaling. It provides a novel mechanism for mAChRs to modulate neuronal excitability and synaptic transmission in PFC circuits, which may contribute to the regulation of cognitive functions by ACh and insulin.

Materials and Methods

Acute dissociation procedure. PFC neurons from young adult (3–5 weeks postnatal) rats were acutely dissociated using procedures similar to those described previously (Yan and Surmeier, 1996; Feng et al., 2001; Wang et al., 2002). In brief, rats were anesthetized and decapitated; brains were quickly removed, iced, and then blocked for slicing. The blocked tissue was cut in 400 μm slices with a vibratome while bathed in a low-Ca^{2+} (100 μM), HEPES-buffered salt solution (in mM: 140 Na isethionate, 2 KCl, 4 $MgCl_2$, 0.1 $CaCl_2$, 23 glucose, 15 HEPES, 1 kynurenic acid, pH 7.4, 300–305 mOsm/l). Slices were then incubated for 1–6 hr at room temperature (20–22°C) in a $NaHCO_3$-buffered saline bubbled with 95% O_2, 5% CO_2 (in mM: 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 glucose, 1 pyruvic acid, 0.05 glutathione, 0.1 N^G-nitro-L-arginine, 1 kynurenic acid, pH 7.4, 300–305 mOsm/l). All reagents were obtained from Sigma (St. Louis, MO).

Slices were then removed into the low-Ca^{2+} buffer, and regions of the PFC were dissected and placed in an oxygenated Cell-Stir chamber (Wheaton, Inc., Millville, NJ) containing protease (type XIV, 1.2–1.4 mg/ml; Sigma) in HEPES-buffered HBSS (Sigma) at 35°C. After 35 min of enzyme digestion, tissue was rinsed three times in the low-Ca^{2+} HEPES-buffered saline and incubated for an additional 30–60 min. Then the enzyme-treated slices were mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was then plated into a 35 mm Lux Petri dish and placed on the stage of a Nikon (Tokyo, Japan) inverted microscope.

Whole-cell recordings. For whole-cell recordings of currents, we used standard voltage-clamp techniques (Yan and Surmeier, 1997). Dissociated cells were incubated with 0.5 μg/ml insulin for 10–20 min before recording in all experiments unless otherwise stated. The internal solution consisted of (in mM): 180 *N*-methyl-D-glucamine, 40 HEPES, 4 $MgCl_2$, 0.5 BAPTA, 12 phosphocreatine, 2 Na_2ATP, 0.2 Na_3GTP, 0.1 leupeptin, pH 7.2–7.3, 265–270 mOsm/l. The external solution consisted of (in mM): 135 NaCl, 20 CsCl, 1 $MgCl_2$, 10 HEPES, 0.001 TTX, 5 $BaCl_2$, 10 glucose, pH 7.3, 300–305 mOsm/l.

Recordings were obtained with an Axon Instruments (Union City, CA) 200B patch-clamp amplifier that was controlled and monitored with a IBM personal computer running pClamp 8 with a DigiData 1320 series interface. Electrode resistances were typically 2–4 MΩ in the bath. After seal rupture, series resistance (4–10 MΩ) was compensated (70–90%) and periodically monitored. Care was exercised to monitor the constancy of the series resistance, and recordings were terminated whenever a significant increase (>20%) occurred. The cell membrane potential was held at 0 mV. GABA (100 μM) was applied for 2 sec every 30 sec to minimize the desensitization-induced decrease in current amplitude. Drugs were applied with a gravity-fed sewer pipe system. The array of application capillaries (internal diameter of ~150 μm) was positioned a few hundred micrometers from the cell under study. Solution changes were effected by the SF-77B fast-step solution stimulus delivery device (Warner Instrument Corp., Hamden, CT).

Muscarinic receptor ligands carbachol (CCh), oxotremorine methiodide (oxo-M), atropine and pirenzepine, insulin (Sigma), as well as second-messenger reagents wortmannin, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY 294002), Go6976, chelerythrin, Go6850 (i.e., GF109203x), PKC_{19-36}, PKI[5–24], genestein, daizein, lavendustin A, and lavendustin B (Calbiochem, San Diego, CA), were made up as concentrated stocks in water or DMSO and stored at −20°C. Stocks were thawed and diluted immediately before use. The amino acid sequence for the cSrc inhibitory peptide is PASADGHRGPSAAFVPPAA.

Data analyses were performed with AxoGraph (Axon Instruments), Kaleidagraph (Albeck Software, Reading, PA), Origin 6 (OriginLab Co., Northampton, MA), and Statview (Abacus Concepts, Berkeley, CA). For analysis of statistical significance, Mann–Whitney *U* tests were performed to compare the current amplitudes in the presence or absence of CCh. ANOVA tests were performed to compare the differential degrees of current modulation by CCh between groups subjected to different treatments.

Electrophysiological recordings in slices. To evaluate the regulation of miniature IPSCs (mIPSCs) by muscarinic receptors in PFC slices, the whole-cell patch-clamp technique was used for voltage-clamp recordings. Patch electrodes (5–9 MΩ) were filled with the following internal solution (in mM): 130 Cs-methanesulfonate, 10 CsCl, 4 NaCl, 10 HEPES, 1 $MgCl_2$, 5 EGTA, 12 phosphocreatine, 5 MgATP, 0.2 Na_3GTP, 0.1 leupeptin, pH 7.2–7.3, 265–270 mOsm/l. The slice (300 μm) was placed in a perfusion chamber attached to the fixed stage of an upright microscope (Olympus Optical, Tokyo, Japan) and submerged in continuously flowing, oxygenated artificial CSF. Cells were visualized with a 40× water-immersion lens and illuminated with near infrared (IR) light, and the image was detected with an IR-sensitive CCD camera. It takes 1 min to change solutions completely in the perfusion chamber.

A Multiclamp 700A amplifier was used for these recordings (Axon Instruments). Tight seals (2–10 GΩ) from visualized pyramidal neurons were obtained by applying negative pressure. The membrane was disrupted with additional suction, and the whole-cell configuration was obtained. The access resistances ranged from 13 to 18 MΩ. Cells were held at 10 mV for the continuous recording of mIPSCs. Mini Analysis Program (Synaptosoft, Leonia, NJ) was used to analyze synaptic activity. All quantitative measurements (1 min of events) were taken 4–6 min after drug application. Statistical comparisons of the distribution of synaptic current amplitudes were made using the Kolmogorov–Smirnov (K–S) test.

Single-neuron mRNA profiling. To detect mRNAs for m1–m5 muscarinic receptors in PFC pyramidal neurons, we used the single-cell reverse transcription (RT)-PCR technique as described previously (Yan and Surmeier, 1996, 1997). A patch electrode was used to lift a dissociated neuron into a stream of control solution, and then the neuron was aspirated into the electrode by applying negative pressure. After aspiration, the electrode was broken and its contents ejected into a 0.5 ml Eppendorf tube containing 5 μl of diethyl pyrocarbonate-treated water, 0.5 μl of RNAsin (28 U/μl), 0.5 μl of dithiothreitol (DTT; 0.1 M), and 1 μl of oligo-dT primer (0.5 μg/μl). The mixture was heated to 70°C for 10 min and then incubated on ice for >1 min. Single-strand cDNA was synthesized from the cellular mRNA by adding SuperScript II reverse transcriptase (1 μl, 200 U/μl) and buffer (4 μl, 5× First Strand Buffer), RNAsin (0.5 μl, 28 U/μl), DTT (1.5 μl, 0.1 M), and mixed dNTPs (1 μl, 10 mM). The reaction mixture (20 μl) was incubated at 42°C for 50 min. The reaction was terminated by heating the mixture to 70°C for 15 min and then icing. The RNA strand in the RNA–DNA hybrid was then removed by adding 1 μl of RNase H (2 U/μl) and incubating for 20 min at 37°C.

The cDNA from the RT of RNA in single PFC neurons was amplified via PCR, which was performed with a thermal cycler (MJ Research, Inc., Watertown, MA) in thin-walled plastic tubes. Reaction mixtures contained 2.5 mM $MgCl_2$, 0.5 mM each of the dNTPs, 0.4 μM primers, 2.5 U of *Taq* DNA polymerase (Promega, Mannheim, Germany), 5 μl of 10× buffer (Promega), and 3.5 μl of the cDNA template made from the single-cell RT reaction. The thermal cycling program for the amplification was 94°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min for 45 cycles. The PCR primers for m1–m5 were as described previously (Yan and Surmeier, 1996; Yan et al., 2001). PCR products were separated by electrophoresis in ethidium bromide-stained 1.5% agarose gels. Negative controls for contamination from extraneous and genomic DNA were run for every batch of neurons.

Protein kinase assay. After incubation, brain slices were lysed in cold lysis buffer (1% Triton X-100, 5 mM EDTA, 10 mM Tris, 50 mM NaCl, 30 mM $Na_4P_2O_7 \cdot 10$ mM H_2O, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM PMSF; complete protease inhibitors were from Roche Products, Hertfordshire, UK) on ice for 30 min. Brain lysates were centrifuged and ultracentrifuged, and PKC was immunoprecipitated with mouse monoclonal anti-PKCαβγ (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr, followed by the addition of 50 μl of protein A–Sepharose beads and incubation for 1 hr at 4°C. The beads were pelleted by centrifugation, washed three times

with lysis buffer and three times with kinase buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$), and then resuspended in 30 μl of kinase buffer. *In vitro* kinase activity was measured in the PKC immunoprecipitates using myelin basic protein (MBP) as substrate. The assay was initiated by the addition of 5 μl of [γ-^{32}P]ATP (10 mCi/ml) and 1 μl of MBP (5 mg/ml), continued for 20 min at room temperature, and stopped by boiling samples in SDS-PAGE sample buffer. Samples were loaded onto a 20% polyacrylamide gel and subjected to electrophoresis. The gels were vacuum-dried and exposed to BioMax film (Eastman Kodak, Rochester, NY). Kinase activity was quantified by phosphoimager.

Immunoprecipitation and Western blotting. After incubation, slices were immediately lysed in lysis buffer on ice for 30 min. Cell lysates were centrifuged (15,000 × *g* for 20 min) and ultracentrifuged (40,000 × *g* for 30 min) to remove insoluble material. Proline-rich tyrosine kinase-2 (Pyk2) antibody (1:250; Upstate Biotechnology, Lake Placid, NY) was added to the homogenates and incubated for 1 hr at 4°C. Protein A–Sepharose beads were then added and mixed for 1 hr at 4°C. The beads were pelleted by centrifugation and washed three times with lysis buffer. After the final wash, the beads were resuspended in 50 μl of an SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.7, 10% glycerol, 2% SDS, 10% 2-mercaptoethanol, and 0.01% bromophenol blue). Proteins were separated by SDS-PAGE and subjected to Western blotting with the anti-pY antibody (1:1000; Upstate Biotechnology) for the detection of tyrosine phosphorylation of Pyk2. Next the blots were stripped for 1 hr at 50°C followed by blocking in 5% nonfat dry milk and incubated with antibodies recognizing total Pyk2. For the detection of active Src kinase, a phosphospecific antibody against the active form of this kinase, anti-Src^{Y418} (1:2000; Biosource International, Camarillo, CA) was used in the Western blot assay. Quantitation was obtained from densitometric measurements of immunoreactive bands on films. Data correspond to the mean ± SEM and were analyzed by ANOVA tests.

Results

Activation of muscarinic receptors enhances GABA-evoked currents in dissociated PFC pyramidal neurons pretreated with insulin

To test the potential impact of muscarinic receptors on GABAergic signaling, we examined the effect of the cholinergic receptor agonist CCh on $GABA_A$ receptor-mediated currents in PFC pyramidal neurons. Application of GABA (50 μM) evoked a partially desensitizing outward current that was completely blocked by the $GABA_A$ receptor antagonist bicuculline (30 μM; $n = 5$; data not shown), confirming mediation by the $GABA_A$ receptor. After a brief (10–15 min) pretreatment of the dissociated cells with insulin (0.5–1 μg/ml), subsequent application of CCh (20 μM) caused a significant enhancement in the amplitudes of $GABA_A$ currents in PFC pyramidal neurons (15.1 ± 2.7%, mean ± SEM; $n = 127$; $p < 0.01$; Mann–Whitney *U* test). The modulation was not accompanied by changes in current decay kinetics.

The time course and current traces from a representative cell are shown in Figure 1, *A* and *B*. The CCh-induced enhancement of $GABA_A$ currents had slow onset kinetics, taking 4–5 min to stabilize. After recovery from the first application, a second application of CCh resulted in a similar response (Fig. 1*A*) (88.5 ± 10.4% of first response; $n = 34$).

One interesting feature of the CCh potentiation of $GABA_A$ currents is that it requires a short pretreatment with insulin of the pyramidal neurons isolated from PFC slices (which have been deprived of endogenous insulin with the enzymatic dissociation procedure). CCh failed to enhance $GABA_A$ currents significantly in nontreated dissociated cells (Fig. 1*C*) (3.9 ± 0.9%, mean ± SEM; $n = 19$; $p > 0.05$; Mann–Whitney *U* test). Because CCh had significantly different effects on $GABA_A$ currents in insulin-treated versus nontreated cells, insulin pretreatment was performed in subsequent experiments with isolated neurons unless otherwise stated.

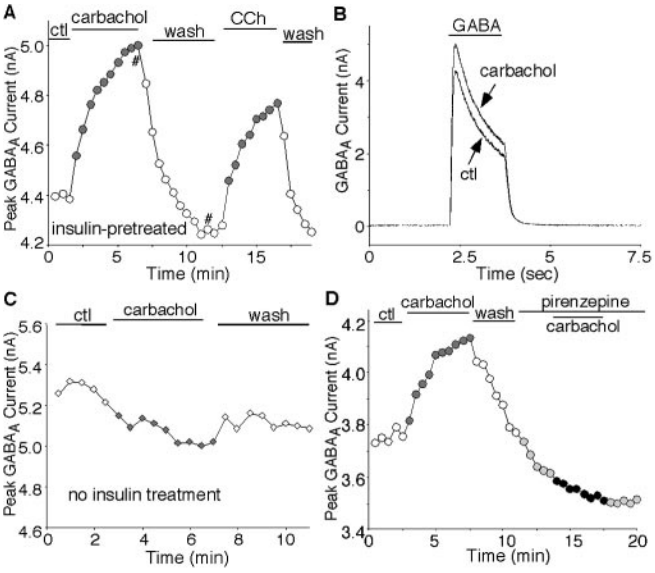

Figure 1. Activation of muscarinic receptors reversibly enhanced $GABA_A$ receptor currents in isolated PFC pyramidal neurons pretreated with insulin. *A*, *C*, Plot of peak amplitude of GABA-evoked current as a function of time and ligand application in an insulin-pretreated neuron (*A*) or a dissociated neuron with no insulin pretreatment (*C*) showing the different effects of CCh (20 μM) on $GABA_A$ receptor currents. *B*, Representative current traces taken from the records used to construct *A* (at time points denoted by #). *D*, Plot of peak $GABA_A$ current as a function of time and ligand application. In the presence of pirenzepine (10 μM), an antagonist with the highest affinity for m1/m4 receptors, CCh had little effect on $GABA_A$ currents. *ctl*, Control.

We then tested which receptors may mediate the effect of CCh on $GABA_A$ currents. Molecular cloning studies have revealed the existence of five mAChR subtypes (m1–m5) (Wess, 1996). These mAChRs can be grouped into two classes, an M1 class (m1, m3, and m5) and an M2 class (m2 and m4) based on their distinctive coupling to signal transduction pathways (Bonner et al., 1987; Peralta et al., 1988; Hulme et al., 1990). Antagonist experiments were performed to examine which mAChR subtypes potentially are involved. Application of the nonselective muscarinic receptor antagonist atropine (10 μM) abolished the enhancing effect of CCh by 90 ± 3.5% ($n = 11$), suggesting the involvement of mAChRs in the CCh action. Application of pirenzepine, an antagonist with the highest affinity for m1/m4 receptors (Hammer et al., 1980; Buckley et al., 1989), also blocked the CCh enhancement of $GABA_A$ currents (88.6 ± 3.0% block; $n = 6$). An example is shown in Figure 1*D*. CCh reversibly enhanced $GABA_A$ currents in the cell but failed to do so in the presence of pirenzepine (10 μM). Similar results were obtained with the lower concentration (1 μM) of pirenzepine (87.1 ± 2.2% block; $n = 5$). These results suggest that the CCh-induced enhancement of $GABA_A$ currents is mediated by muscarinic receptors, and probably by m1 or m4 receptors.

The m1 receptor is the most abundant subtype among the multiple muscarinic receptors expressed in PFC pyramidal neurons

Given the limited selectivity of muscarinic ligands for the individual receptor subtypes (Hulme et al., 1990), we then detected which muscarinic receptors are present in PFC pyramidal neurons. Muscarinic receptors are enriched in cortical areas, but how these receptors are coordinately expressed in different populations of cortical neurons is unclear. The expression pattern of m1–m5 mRNAs was examined using the single-cell RT-PCR technique (Lambolez et al., 1992; Monyer and Lambolez, 1995;

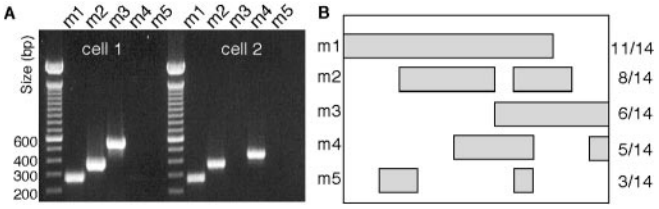

Figure 2. Multiple muscarinic receptor mRNAs were coexpressed in single PFC pyramidal neurons. *A*, Expression profiles of muscarinic receptor mRNAs in two isolated PFC pyramidal neurons showing the coexpression of m1, m2, and m3 mRNAs in one cell and the coexpression of m1, m2, and m4 mRNAs in the other cell. *B*, Bar plot showing the coordinated expression of m1–m5 receptor mRNAs in a sample of 14 PFC pyramidal neurons. The extent of coexpression is indicated by the overlap of the bars.

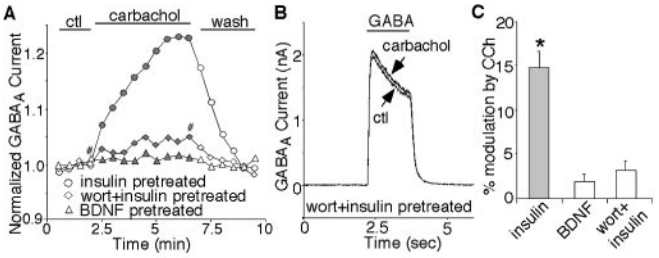

Figure 3. Muscarinic modulation of $GABA_A$ currents in isolated PFC pyramidal neurons required pretreatment with insulin, but not other growth factors, and was sensitive to the PI3K inhibitor wortmannin. *A*, Plot of peak $GABA_A$ current as a function of time and agonist application in dissociated neurons pretreated with BDNF (40 ng/ml), insulin (1 μg/ml for 15 min), or insulin plus wortmannin (*wort*) (4 μM for 15 min). CCh (20 μM) caused an enhancement of $GABA_A$ currents only in insulin-pretreated neurons. *ctl*, Control. *B*, Representative current traces in a neuron taken from the records used to construct *A* (at time points denoted by #). *C*, Histograms (mean ± SEM) showing the percentage of modulation of peak $GABA_A$ currents by CCh in neurons pretreated with insulin ($n = 27$), BDNF ($n = 10$), or insulin plus wortmannin ($n = 9$). *$p < 0.005$; ANOVA.

Yan and Surmeier, 1996, 1997). Acutely isolated pyramidal neurons located in the intermediate and deep layers (III–VI) of the rat PFC were distinguished readily from GABAergic interneurons by their distinct morphological features: a pyramid-shaped soma and a prominent apical dendrite (Feng et al., 2001). The expression of GABA-synthesizing enzyme glutamic acid decarboxylase mRNA was consistently negative in the harvested neurons (data not shown), confirming that they are not GABAergic interneurons. The mRNA expression profiles for muscarinic receptor subtypes of two representative PFC pyramidal neurons are shown in Figure 2*A*. The m1, m2, and m3 receptor mRNAs were coexpressed in one cell, and the m1, m2, and m4 receptor mRNAs were coexpressed in the other. In 14 individual PFC pyramidal neurons tested, ~80% of them expressed the m1 receptor mRNA (11 of 14), and ~60% of these cells expressed the m2 receptor mRNA (8 of 14). A subset of these cells expressed the m3 (6 of 14) or m4 (5 of 14) receptor mRNA. The m5 receptor mRNA rarely was detectable (3 of 14). The coordinated expression of muscarinic receptor mRNAs in the sample of 14 PFC pyramidal neurons is summarized in Figure 2*B*. The prominent expression of m1 mRNA is consistent with previous anatomical studies showing the abundant presence of m1 receptors in cortical areas (Buckley et al., 1988; Levey et al., 1991).

Muscarinic modulation of $GABA_A$ currents is gated by the insulin/phosphoinositide-3 kinase pathway

Because muscarinic potentiation of $GABA_A$ currents in dissociated PFC neurons requires insulin pretreatment, we also tested whether other growth factors that share some common signaling pathways as insulin can elicit the same effect. Acutely isolated neurons were pretreated with BDNF (40 ng/ml) for 15 min, and then the CCh effects on $GABA_A$ currents were examined. As shown in Figure 3*A*, CCh could not enhance $GABA_A$ currents in BDNF-treated neurons. In dissociated neurons pretreated with neurotrophin-3 (40 ng/ml), CCh also failed to enhance $GABA_A$ currents ($-4.2 \pm 1.8\%$, mean ± SEM; $n = 9$; $p > 0.05$; Mann–Whitney *U* test). These results suggest that insulin signaling plays a unique role in facilitating muscarinic potentiation of $GABA_A$ currents.

Among the multiple insulin-regulated signal transduction pathways (for review, see Myers and White, 1996), a prominent downstream target of insulin receptors is phosphoinositide-3 kinase (PI3K). To test whether PI3K is involved in muscarinic modulation of $GABA_A$ currents, we pretreated PFC-dissociated neurons with the PI3K inhibitor wortmannin. As shown in Figure 3, *A* and *B*, wortmannin pretreatment largely eliminated the CCh-induced enhancement. The percentage of modulation of peak $GABA_A$ currents by CCh in cells pretreated with insulin (1 μg/ml), BDNF (40 ng/ml), or wortmannin (4 μM) plus insulin (1 μg/ml) is summarized in Figure 3*C*. CCh significantly enhanced $GABA_A$ currents in insulin-pretreated cells (14.9 ± 1.6%, mean ± SEM; $n = 27$; $p < 0.01$; Mann–Whitney *U* test) but had little effect in BDNF-pretreated cells (2.0 ± 1.3%; $n = 10$; $p > 0.05$; Mann–Whitney *U* test) or in cells pretreated with insulin plus wortmannin (3.6 ± 1.2%; $n = 9$; $p > 0.05$; Mann–Whitney *U* test). Pretreatment with another PI3K inhibitor, LY 294002 (40 μM), gave similar results, attenuating most of the enhancing effect of CCh on $GABA_A$ currents (4.1 ± 1.5% compared with insulin-treated cells; $n = 6$; $p < 0.01$; ANOVA). The significantly different effects of CCh on $GABA_A$ currents in cells pretreated with insulin versus BDNF (Fig. 3*C*) ($p < 0.005$; ANOVA), as well as in insulin-pretreated cells in the absence versus presence of wortmannin (Fig. 3*C*) ($p < 0.005$; ANOVA), indicate that muscarinic regulation of $GABA_A$ receptors is dependent on insulin via its activation of PI3K.

Postsynaptic modulation of GABAergic synaptic transmission by mAChRs in PFC slices is gated by PI3K activation

To understand the impact of muscarinic receptors on GABAergic synaptic transmission, we examined the effect of CCh on $GABA_A$ receptor-mediated IPSCs in PFC slices. Because CCh can induce the excitation of GABAergic interneurons (Kawaguchi, 1997; Kondo and Kawaguchi, 2001), PFC slices were exposed to TTX (1 μM), and mIPSCs were recorded in PFC pyramidal neurons. mIPSCs are believed to be caused by the random release of single neurotransmitter packets (quanta), and a significant effect on their amplitude is usually considered good evidence for a modification of postsynaptic $GABA_A$ receptor properties. Application of bicuculline (30 μM) blocked the mIPSCs ($n = 5$), indicating that these synaptic currents are mediated by $GABA_A$ receptors. A representative experiment is shown in Figure 4*A–C*. Bath application of CCh induced a reversible enhancement of the mIPSC amplitude by 26.7% ($p < 0.001$; K–S test). The second application of CCh caused a comparable response, enhancing mIPSC amplitude to a similar extent (25.6%). In a sample of PFC pyramidal neurons that we examined, CCh increased the mean amplitude of mIPSCs by 23.3 ± 2.1% (mean ± SEM; $n = 20$; $p < 0.01$; Mann–Whitney *U* test). In contrast to the significant enhancement of mIPSC amplitude, CCh did not produce a consistent modulatory effect on mIPSC frequency in the PFC, with an

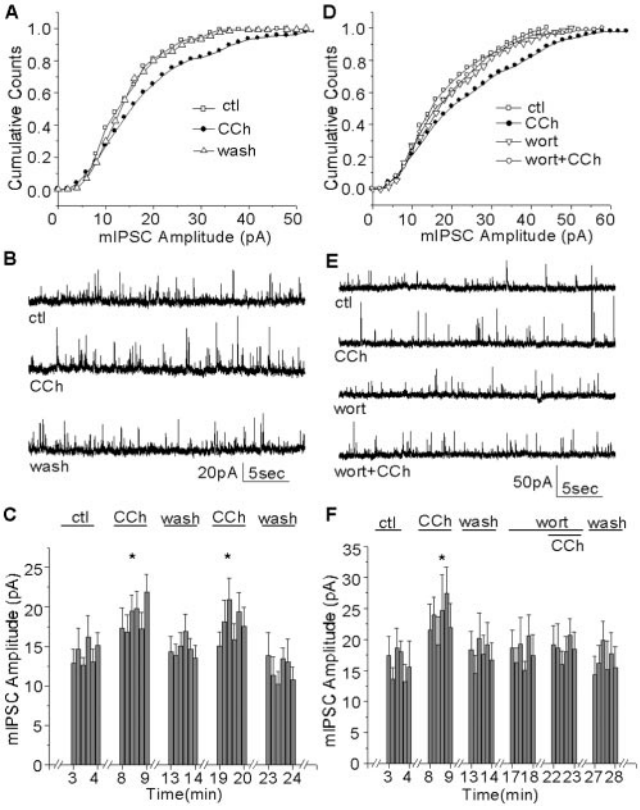

Figure 4. Muscarinic receptors enhanced the mIPSC amplitude of pyramidal neurons in PFC slices, and this effect was blocked by wortmannin (*wort*) treatment. *A*, Cumulative plots indicating that the distribution of mIPSC amplitude was reversibly increased by CCh (20 μM). *B*, mIPSC recorded under control conditions, during bath application of CCh, and after washing off the agonist. *C*, Histograms of average mIPSC amplitudes (mean ± SEM) before, during, and after two applications of CCh. In each case, 1 min of continuous recordings was calculated. *$p <$ 0.01; Mann–Whitney *U* test. *D*, Cumulative plots indicating that the CCh-induced increase in the distribution of mIPSC amplitude was eliminated in the presence of wortmannin (4 μM). *E*, Representative mIPSC traces recorded under control conditions and during application of CCh (20 μM), wortmannin (4 μM), and CCh plus wortmannin. *F*, Histograms of average mIPSC amplitudes (mean ± SEM) before, during, and after application of CCh in the absence and presence of wortmannin. *$p < 0.01$; Mann–Whitney *U* test. *ctl*, Control.

increase in mIPSC frequency observed in a subset of cells and no change found in others. In the sample of PFC pyramidal neurons that we examined, CCh slightly increased the mean frequency of mIPSCs (14.9 ± 5.3%, mean ± SEM; $n = 20$).

We then examined the involvement of PI3K in muscarinic modulation of mIPSC amplitude in pyramidal neurons in PFC slices. To suppress the basal activation of PI3K by endogenous insulin, we treated PFC slices with wortmannin. A representative example is shown in Figure 4 *D–F*. In the absence of wortmannin, CCh increased the mean mIPSC amplitude by 27.7% ($p < 0.001$; K–S test), whereas in the presence of wortmannin (4 μM), CCh failed to change the mean mIPSC amplitude significantly (−4.7%; $p > 0.05$; K–S test). Similar results were obtained in other neurons tested. In the absence of wortmannin, CCh increased the mean amplitude of mIPSCs by 22.1 ± 3.1% (mean ± SEM; $n = 5$; $p < 0.01$; Mann–Whitney *U* test), whereas CCh had little effect on the mean amplitude of mIPSCs after treatment with wortmannin (−6.3 ± 2.4%; $n = 5$; $p > 0.05$; Mann–Whitney *U* test). The significant ($p < 0.001$; ANOVA) attenuation of the CCh enhancement of mIPSC amplitude by wortmannin suggests that muscarinic receptors modulate postsynaptic GABA signaling through an insulin/PI3K-gated pathway.

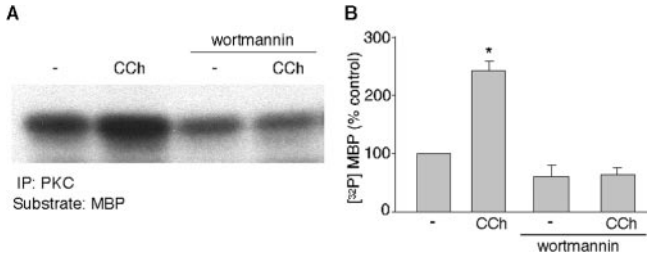

Figure 5. Muscarinic receptors in PFC slices increased PKC activity in a PI3K-dependent manner. *A*, *In vitro* kinase activity of PKC immunoprecipitates. PFC slices were pretreated with or without wortmannin (1 μM) for 15 min, followed by incubation with or without CCh (40 μM) for 7 min. Lysates of these slices were used for immunoprecipitation (*IP*) with anti-PKC. PKC kinase activity of the immune complex was measured using MBP as the substrate. *B*, Histogram summary of the phosphorylation of PKC substrate in PFC slices under different treatment conditions ($n = 6$; *$p < 0.01$; ANOVA).

Muscarinic modulation of $GABA_A$ currents is dependent on PKC activation

We subsequently examined the potential cellular mechanisms underlying the insulin/PI3K gating of muscarinic modulation of $GABA_A$ currents. It is known that activation of m1 receptors stimulates the hydrolysis of membrane phosphoinositol lipids, leading to the release of inositol-1,4,5-triphosphate and diacylglycerol (DAG). An increase in the intracellular Ca^{2+} triggers the association of PKC isozymes with the plasma membrane where DAG binds to PKC. Once PKC is membrane translocated, it is converted into an effector-independent form for sustained activation after the Ca^{2+} and DAG signals dissipate (Huang, 1989). The catalytic competence of PKC requires its phosphorylation at the activation loop by another kinase: phosphoinositide-dependent kinase 1 (PDK1) (Dutil et al., 1998; Le Good et al., 1998). Coexpression of PDK1 enhances PKC activity and activation-loop phosphorylation in a PI3K-dependent manner (Le Good et al., 1998). One potential mechanism for the m1 modulation of $GABA_A$ channels is via the activation of PKC, which is operated by PI3K/PDK1-dependent phosphorylation of sites on its activation loop. If so, then inhibiting PKC activation should eliminate the effect of muscarinic receptors on $GABA_A$ currents.

To test these hypotheses, we first examined whether the muscarinic activation of PKC was gated by the insulin/PI3K pathway. PFC slices were pretreated with wortmannin (1 μM) to suppress the basal activity of PI3K afforded by endogenous insulin. *In vitro* kinase assays then were performed on PFC slices incubated with or without CCh. A representative example is shown in Figure 5*A*. CCh potently increased the PKC activity in PFC slices, and this effect was blocked almost completely by pretreatment with wortmannin. Another muscarinic agonist, oxo-M (20 μM), gave similar results ($n = 7$; data not shown). As summarized in Figure 5*B*, CCh significantly increased PKC activity under control conditions (2.30 ± 0.15-fold; $n = 6$; $p < 0.01$; ANOVA) but had little effect on PKC activity in PFC slices treated with wortmannin (1.12 ± 0.08-fold; $n = 6$; $p > 0.05$; ANOVA). Equal loading of PKC was confirmed by blotting one-fifth of the PKC immunoprecipitates with anti-PKC (data not shown). Pretreatment with another PI3K inhibitor, LY 294002 (40 μM), attenuated the CCh-induced increase in PKC activity by 75.0 ± 5.0% (mean ± SEM; $n = 4$; data not shown). The significant ($p < 0.01$; ANOVA) difference in the effects of CCh on PKC activation in the absence versus the presence of PI3K inhibitors suggests that the insulin/PI3K pathway plays a key role in facilitating mAChR activation of PKC in the PFC.

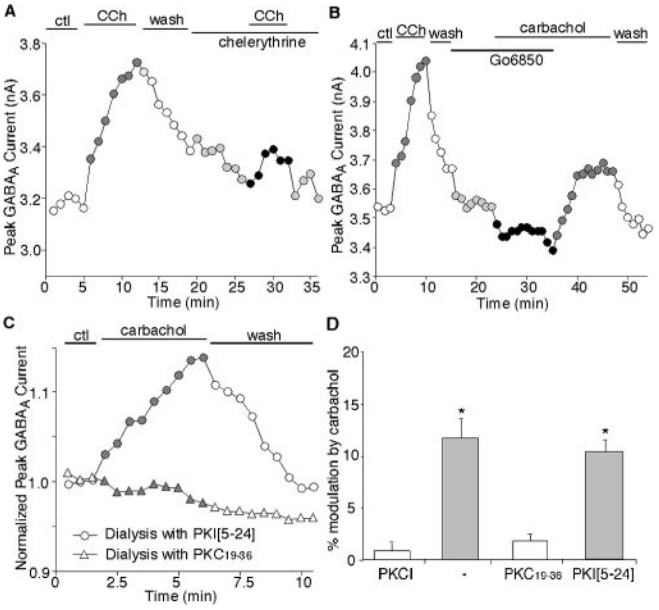

Figure 6. Muscarinic modulation of $GABA_A$ currents was dependent on activation of PKC. *A, B*, Plot of peak $GABA_A$ current as a function of time and drug application. CCh (20 μM) reversibly enhanced $GABA_A$ currents, and this effect was blocked by PKC inhibitors chelerythrin (20 μM) (*a*) and Go6850 (1 μM) (*b*). *C*, Plot of peak $GABA_A$ current as a function of time and agonist application with PKC-inhibitory peptide PKC_{19-36} (20 μM) or PKA-inhibitory peptide PKI[5–24] (20 μM) in the recording pipette. *D*, Histograms (mean ± SEM) showing the percentage of modulation of $GABA_A$ currents by CCh in the absence ($n = 18$) or presence ($n = 12$) of membrane-permeable PKC inhibitors (*PKCI*; data pooled together), as well as the CCh effect in cells dialyzed with PKC_{19-36} ($n = 9$) or PKI[5–24] ($n = 6$). *$p < 0.01$; ANOVA. *ctl*, Control.

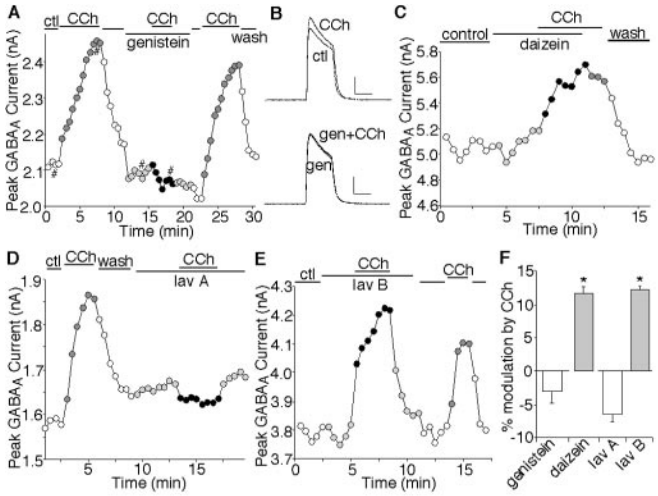

Figure 7. Muscarinic enhancement of $GABA_A$ currents was blocked by tyrosine kinase inhibitors. *A, C–E*, Plot of peak $GABA_A$ current as a function of time and drug application. *B*, Representative current traces taken from the records used to construct *A* (at time points denoted by #). Calibration: 0.5 nA, 1 sec. The CCh (20 μM)-induced enhancement of $GABA_A$ currents was eliminated in the presence of the tyrosine kinase inhibitor genestein (*gen*; 10 μM) (*A, B*) but was not affected by its inactive analog daizein (10 μM) (*c*). Another tyrosine kinase inhibitor, lavendustin A (*lav A*; 0.2 μM), also blocked CCh enhancement of $GABA_A$ currents (*D*), but its inactive analog lavendustin B (*lav B*; 0.2 μM) had no effect (*E*). *F*, Histograms (mean ± SEM) showing the percentage modulation of $GABA_A$ currents by CCh in the presence of genestein ($n = 6$), daizein ($n = 7$), lavendustin A ($n = 5$), or lavendustin B ($n = 8$). *$p < 0.001$; ANOVA. *ctl*, Control.

Next we applied a panel of structurally different membrane-permeable PKC inhibitors to test the role of PKC in the muscarinic enhancement of $GABA_A$ currents. Representative examples are shown in Figure 6. The selective PKC inhibitor chelerythrin (20 μM) mostly blocked the CCh-induced enhancement of $GABA_A$ currents (Fig. 6*A*) ($n = 4$). A more potent PKC inhibitor, Go6850 (1 μM), with high selectivity for PKCα, PKCβ, PKCγ, PKCδ, and PKCϵ isozymes, also eliminated the CCh effect, and washing off Go6850 led to recovery of the CCh enhancement of $GABA_A$ currents (Fig. 6*B*) ($n = 5$). Similarly, in the presence of another structurally different PKC inhibitor, Go6976 (1 μM), CCh failed to enhance $GABA_A$ currents ($n = 3$; data not shown).

To provide additional evidence for the involvement of PKC, we dialyzed neurons with the PKC inhibitory peptide PKC_{19-36} (20 μM). Because m4 receptors are coupled to the inhibition of adenylyl cyclase, we also dialyzed neurons with the PKA inhibitory peptide PKI[5–24] (20 μM) to test the potential involvement of m4/PKA. As shown in Figure 6*C*, CCh had little effect on $GABA_A$ currents in the presence of PKC_{19-36}, whereas the CCh-induced enhancement was intact in the presence of PKI[5–24]. The effects of CCh on $GABA_A$ currents in the presence or absence of PKC inhibitors are summarized in Figure 6*D*. CCh had little effect on $GABA_A$ currents in the presence of membrane-permeable PKC inhibitors (1.1 ± 1.0%, mean ± SEM; $n = 12$; $p > 0.05$; Mann–Whitney *U* test; data pooled together), which was significantly smaller than the effect of CCh alone (11.9 ± 1.4%; $n = 18$; $p < 0.01$; ANOVA). Likewise, CCh failed to enhance $GABA_A$ currents in the presence of PKC_{19-36} (2.1 ± 0.7%; $n = 9$; $p > 0.05$; Mann–Whitney *U* test) but had intact enhancing effects in cells loaded with PKI[5–24] (10.2 ± 0.9%; $n = 6$; $p < 0.01$; ANOVA). These data suggest that muscarinic modulation of $GABA_A$ channels depends on the m1-mediated activation of PKC.

Muscarinic enhancement of $GABA_A$ currents is mediated by a tyrosine kinase

Previous studies have shown that PKC phosphorylation of $GABA_A$ channels leads to a reduction of GABA-activated currents in transfected cell lines and native neurons (Kellenberger et al., 1992; Krishek et al., 1994; Feng et al., 2001). On the contrary, tyrosine phosphorylation of $GABA_A$ channels causes a potentiation of GABA-activated currents (Moss et al., 1995). To test whether muscarinic enhancement of $GABA_A$ currents is mediated by a tyrosine kinase, we applied the broad-spectrum tyrosine kinase inhibitor genestein. Its inactive analog daizein was used as a negative control. As shown in Figure 7, *A* and *B*, CCh failed to elicit any enhancement in the presence of genestein (10 μM), and washing off genestein led to recovery of the CCh effect. In some cells, CCh even caused a small reduction of $GABA_A$ currents in the presence of genestein. However, CCh enhanced $GABA_A$ currents in the presence of daizein (10 μM) to an extent similar to that seen with CCh alone (Fig. 7*C*).

To provide additional evidence showing the involvement of tyrosine phosphorylation, we tested another tyrosine kinase inhibitor, lavendustin A, which has a structure different from genestein. As shown in Figure 7*D*, lavendustin A (0.2 μM) also blocked the CCh-induced enhancement of $GABA_A$ currents, whereas its inactive analog lavendustin B (0.2 μM) had no effect (Fig. 7*E*). In some cells, a CCh-induced small reduction of $GABA_A$ currents was observed in the presence of lavendustin A. The effects of CCh in the absence or presence of various tyrosine kinase inhibitors and their inactive analogs are summarized in Figure 7*F*. CCh had little effect on $GABA_A$ currents in the presence of genestein (−3.5 ± 1.3%, mean ± SEM; $n = 6$; $p > 0.05$;

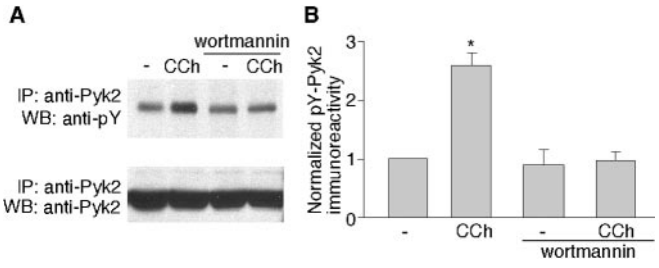

Figure 8. Muscarinic receptors increased the tyrosine phosphorylation of Pyk2 in a PI3K-dependent manner in PFC slices. *A*, Immunoblots of phosphotyrosine of Pyk2. PFC slices were pretreated with or without wortmannin (1 μM) for 15 min, followed by incubation with or without CCh (40 μM) for 7 min. Lysates of the slices were immunoprecipitated (*IP*) with anti-Pyk2 and were blotted with anti-pY (*top*). *WB*, Western blotting. Equal loading of Pyk2 was shown by reblotting with anti-Pyk2 (*bottom*). *B*, Quantitation of tyrosine phosphorylation of Pyk2 in the absence ($n = 10$) or presence ($n = 5$) of wortmannin. $*p < 0.01$; ANOVA.

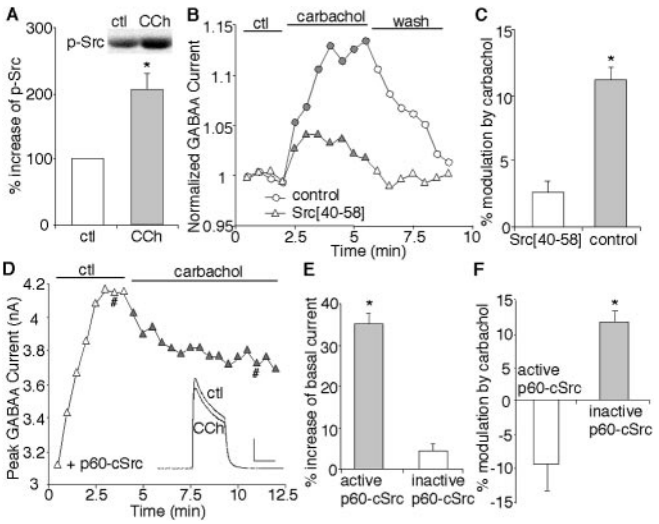

Figure 9. Muscarinic modulation of $GABA_A$ currents required the protein tyrosine kinase Src. *A*, Bar graph showing the Src phosphorylation in brain slices incubated in the absence or presence of CCh (20 μM, 10 min). *Inset*, Immunoblots of phospho-Src. After treatment, extracts of slices were immunoblotted with the phosphospecific antibody $pSrc^{(pY418)}$ against the active form of Src. $*p < 0.01$; ANOVA. *B*, Plot of peak $GABA_A$ current as a function of time and agonist application with Src[40–58] peptide (30 μM) or the scrambled control peptide sSrc[40–58] (30 μM) in the recording pipette. Inhibiting Src with the Src[40–58] peptide blocked the CCh effect on $GABA_A$ currents. The scrambled control peptide sSrc[40–58] was without effect. *C*, Histograms (mean ± SEM) showing the percentage of modulation of $GABA_A$ currents by CCh in the presence of Src[40–58] peptide ($n = 9$) or the scrambled control peptide sSrc[40–58] ($n = 10$). $*p < 0.01$; ANOVA. *D*, Plot of peak $GABA_A$ current as a function of time and agonist application with the active enzyme p60-cSrc (37.5 U/ml) in the recording pipette. Activating Src potentiated the basal $GABA_A$ currents and eliminated the enhancing effect of CCh. *Inset*, Representative current traces taken from the records used to construct *C* (at time points denoted by #). Calibration: 1 nA, 1 sec. *E*, Histograms (mean ± SEM) showing the percentage of enhancement of basal $GABA_A$ currents by the active enzyme p60-cSrc ($n = 21$) or heat-inactivated p60-cSrc ($n = 13$). $*p < 0.001$; ANOVA. *F*, Histograms (mean ± SEM) showing the percentage of modulation of $GABA_A$ currents by CCh in neurons dialyzed with the active enzyme p60-cSrc ($n = 8$) or heat-inactivated p60-cSrc ($n = 6$). $*p < 0.001$; ANOVA.

Mann–Whitney *U* test), which was significantly different from the effect of CCh in the presence of daizein (11.3 ± 0.9%; $n = 7$; $p < 0.001$; ANOVA). Moreover, CCh failed to enhance $GABA_A$ currents in the presence of lavendustin A (−6.2 ± 0.8%; $n = 5$; $p > 0.05$; Mann–Whitney *U* test), which was also significantly different from the effect of CCh in the presence of lavendustin B (12.1 ± 0.6%; $n = 8$; $p < 0.001$; ANOVA). These results suggest that muscarinic enhancement of $GABA_A$ currents occurs via phosphorylation of $GABA_A$ channels by a tyrosine kinase.

Muscarinic receptors in PFC slices activate Pyk2 in a PI3K-dependent manner

We then tried to identify the tyrosine kinase that is stimulated by mAChRs in a PKC-dependent manner. The nonreceptor tyrosine kinase Pyk2 (Girault et al., 1999), which is highly expressed in the CNS and rapidly activated by PKC (Lev et al., 1995), is a potential molecular link in this cascade. Studies in cell lines have found that m1 muscarinic receptors can activate Pyk2, inducing the association of cSrc with Pyk2 (Felsch et al., 1998). These lines of evidence led us to speculate that muscarinic enhancement of $GABA_A$ currents in PFC pyramidal neurons may occur via protein tyrosine kinase Src, which is activated by the m1/PKC/Pyk2 cascade.

To test this hypothesis, we first examined whether muscarinic receptors in the PFC could induce the activation of Pyk2. PFC slices were treated with or without CCh (40 μM), and tyrosine phosphorylation of Pyk2 in these slices was compared. Because the mAChR enhancement of PKC activity and $GABA_A$ receptor functions is gated by the insulin/PI3K pathway, we also tested whether muscarinic activation of Pyk2 used a similar mechanism. PFC slices were pretreated with wortmannin to suppress the basal insulin/PI3K signaling, and then tyrosine phosphorylation of Pyk2 in the absence or presence of CCh was measured. A representative example is shown in Figure 8*A*. CCh caused a strong increase in the tyrosine phosphorylation of Pyk2 but failed to induce Pyk2 activation in the presence of wortmannin (1 μM). As summarized in Figure 8*B*, CCh significantly increased Pyk2 phosphorylation under control conditions (2.50 ± 0.14-fold; $n = 10$; $p < 0.01$; ANOVA) but had little effect in PFC slices treated with wortmannin (1.15 ± 0.09-fold; $n = 5$; $p > 0.05$; ANOVA). These results suggest that muscarinic receptors can elevate the kinase activity of Pyk2 in PFC slices, and this effect can be attenuated significantly by inhibiting the insulin/PI3K pathway (CCh effects in the absence vs presence of wortmannin: $p < 0.01$; ANOVA).

Muscarinic receptors modulate $GABA_A$ channels via protein tyrosine kinase Src

Because Pyk2 can lead to the activation of protein tyrosine kinase Src (Dikic et al., 1996; Thomas and Brugge, 1997; Xu et al., 1997), we then tested the role of Src in the muscarinic potentiation of $GABA_A$ currents. We first examined whether activation of muscarinic receptors can increase Src activity. Because full catalytic activity of Src requires phosphorylation of Tyr418 that is located in the Src catalytic domain and is one of the autophosphorylation sites (Cooper and MacAuley, 1988), we used the phosphospecific antibody $pSrc^{(pY418)}$ to detect activated Src. As shown in Figure 9*A*, treating cortical slices with CCh significantly increased the phosphorylated Src (2.10 ± 0.18-fold; $n = 5$; $p < 0.01$; ANOVA), suggesting that mAChRs can activate Src kinase activity.

We then dialyzed neurons with an Src-inhibitory peptide, Src[40–58] (Yu et al., 1997; Lu et al., 1999; Huang et al., 2001). If the effect of CCh depends on Src, then blocking Src function should prevent the enhancement of $GABA_A$ currents by CCh. A control peptide with scrambled sequences, sSrc[40–58], was used to ensure the specificity of Src[40–58]. As shown in Figure 9*B*, CCh had little effect in Src[40–58] (30 μM)-loaded cells, whereas it elicited a strong enhancement of $GABA_A$ currents in cells infused with the control peptide sSrc[40–58] (30 μM). Comparing the percentage of CCh modulation in two groups of cells dialyzed with Src[40–58] or the control peptide sSrc[40–58] (Figure 9*C*),

it is evident that the effect of CCh in the presence of Src[40–58] (2.6 ± 0.9%, mean ± SEM; $n = 9$; $p > 0.05$; Mann–Whitney U test) is significantly different from that in the presence of the control peptide sSrc[40–58] (11.5 ± 1.1%; $n = 10$; $p < 0.01$; ANOVA).

To provide additional evidence showing the involvement of Src in muscarinic potentiation of $GABA_A$ currents, we injected the active enzyme p60-cSrc (37.5 U/ml) into PFC neurons and examined the impact of this kinase on basal $GABA_A$ currents and muscarinic modulation. $GABA_A$ currents were upregulated by p60-cSrc (Fig. 9*D*) but not by the heat-inactivated enzyme (data not shown). As summarized in Figure 9*E*, the basal $GABA_A$ currents were potentiated by 35.1 ± 3.0% when dialyzing with p60-cSrc (mean ± SEM; $n = 21$) and potentiated by only 4.7 ± 2.2% when dialyzing with heat-inactivated p60-cSrc ($n = 13$; $p < 0.001$; ANOVA). The cSrc-induced potentiation of $GABA_A$ currents also occluded the enhancing effect of subsequent CCh application, turning it into a small current reduction in most cells (Fig. 9*F*) (−9.6 ± 4.4%; $n = 8$). The CCh-induced reduction of $GABA_A$ currents in cSrc-loaded cells could be attributable to the direct depressing effect of PKC (Kellenberger et al., 1992; Krishek et al., 1994; Feng et al., 2001), because cSrc already has phosphorylated the tyrosine sites of $GABA_A$ receptors. These data suggest that activation of Src is necessary for the potentiation of $GABA_A$ currents by muscarinic receptors.

Discussion

Both mAChR and insulin signaling have been implicated in the regulation of locomotor activity, learning, and memory (Brown and Taylor, 1996; Craft et al., 1996, 1999; Wess, 1996); however, neither the cross talk between the two systems nor the cellular mechanism of their actions is clear. In this study, we show that, by converging on PKC, the two systems could be interconnected, leading to muscarinic potentiation of the postsynaptic response to GABA in PFC neurons. Because GABAergic inhibition plays a key role in controlling neuronal activity and synaptic transmission in PFC circuits, the mAChR-mediated regulation of $GABA_A$ channels should enable ACh to exert an important impact on PFC functions. Behavioral tests have suggested that combined impairment of both muscarinic cholinergic and $GABA_A$ systems could model some aspects of human AD (Cain et al., 2000). By linking these two neurotransmitters mechanistically, we could understand better the involvement of both systems in cognition.

The consistent expression of muscarinic receptors in most PFC pyramidal neurons led us to explore their potential functional roles in these cells. One candidate is the $GABA_A$ receptor channel, the activity of which is subject to regulation by protein phosphorylation, which could be triggered by the mAChR signaling. However, direct application of the mAChR agonist CCh had little effect on $GABA_A$ currents in acutely dissociated neurons. Interestingly, after a short (10–15 min) treatment with insulin, subsequent application of CCh induced an enhancement of $GABA_A$ currents. A previous study has found that insulin causes a rapid recruitment of functional $GABA_A$ receptors to the postsynaptic plasma membrane, thereby increasing the amplitude of the $GABA_A$ receptor-mediated mIPSCs (Wan et al., 1997b). In our studies, insulin pretreatment was used to prime the acutely isolated PFC pyramidal neuron to manipulate intracellular components. Insulin was washed off before recording $GABA_A$ currents. Peak amplitudes before and during CCh application were compared; therefore, the recorded differences were caused by mAChR activation. In PFC slices, blocking the basal activation of insulin/PI3K signaling eliminated muscarinic potentiation of mIPSC amplitude, further confirming that mAChR potentiation of postsynaptic $GABA_A$ receptor functions requires priming by insulin signaling.

The involvement of PI3K prompted us to speculate that the missing link between mAChR and insulin signaling could be PKC. PKC is activated via two mechanisms. One mechanism involves displacement of an inhibitory domain from the kinase catalytic domain, which is achieved by binding to DAG, a component of m1 signaling. The other mechanism involves phosphorylation at the activation loop, which is achieved by PDK1, a downstream target of PI3K (Newton, 1997; Le Good et al., 1998). In transfected cell lines, all members of the PKC family form complexes with PDK1. PDK1 phosphorylates the activation loop sites of PKC in a PI3K-dependent manner, leading to the increased catalytic capacity of PKC (Le Good et al., 1998; Belham et al., 1999). Pretreatment with insulin triggers the PI3K/PDK1 pathway, which could facilitate the activation of PKC by the subsequent application of muscarinic agonists. This hypothesis was confirmed by the finding that PI3K inhibitors blocked the muscarinic enhancement of PKC enzymatic activity and ensuing increase in $GABA_A$ currents in dissociated neurons and mIPSC amplitudes in PFC slices.

Muscarinic modulation of $GABA_A$ currents was blocked by inhibiting PKC activity, suggesting that it is a PKC-dependent event. If direct phosphorylation of $GABA_A$ receptors by PKC mediates the muscarinic effect, then current inhibition is expected (Kellenberger et al., 1992; Krishek et al., 1994). The enhancing effect of CCh led us to test the involvement of protein tyrosine kinases, because tyrosine phosphorylation has been shown to potentiate $GABA_A$ currents (Moss et al., 1995). As expected, muscarinic enhancement of $GABA_A$ currents was eliminated in the presence of tyrosine kinase inhibitors. Specific inhibition of the nonreceptor protein tyrosine kinase Src mostly blocked muscarinic enhancement of $GABA_A$ currents, suggesting that Src is directly involved in this process. This result is consistent with previous findings on the Src regulation of $GABA_A$ receptors in transfected cell lines and native neurons (Moss et al., 1995; Wan et al., 1997a). A similar PKC-dependent activation of the tyrosine kinase signaling cascade also exists in muscarinic regulation of Kv1.2 potassium channels (Huang et al., 1993; Lev et al., 1995) and NMDA receptors (Lu et al., 1999). Because the Src-mediated potentiating effect has to overcome the PKC-induced direct depressing effect on $GABA_A$ currents (Kellenberger et al., 1992; Krishek et al., 1994; Feng et al., 2001), the net enhancement of $GABA_A$ receptor currents produced by CCh is relatively modest, ranging from 10 to 30%. However, this effect is not only statistically significant but also of the typical size of channel modulation by neurotransmitters in central neurons (Surmeier et al., 1995; Lu et al., 1999; Cai et al., 2002). Similar enhancement of mIPSC amplitudes by CCh found in PFC slices further confirmed that the mAChR regulation of postsynaptic $GABA_A$ receptor channels could have a significant impact on GABAergic synaptic transmission in PFC circuits.

The molecular link between PKC and Src could be Pyk2, a member of the focal adhesion kinase family, which can be tyrosine phosphorylated and activated by PKC in central neurons (Lev et al., 1995). Like PKC activation by mAChRs, muscarinic activation of Pyk2 in PFC slices was also sensitive to PI3K inhibitors, suggesting that the focal point of the cross talk between muscarinic and insulin signaling is PKC/Pyk2. Activated Pyk2 autophosphorylates on Tyr402 (Girault et al., 1999), creating an Src homology 2 (SH2) ligand through which Pyk2 binds to the SH2 domain of Src (Dikic et al., 1996) and activates Src kinase by

relieving autoinhibition (Thomas and Brugge, 1997; Xu et al., 1997). Similar to the case with NMDA receptors (Huang et al., 2001), Pyk2 is likely to be upstream of Src in the signaling cascade by which tyrosine phosphorylation enhances the function of $GABA_A$ receptors.

In summary, this study shows that muscarinic receptors enhance postsynaptic $GABA_A$ receptor functions in PFC pyramidal neurons via PKC-dependent activation of the protein tyrosine kinase Src signaling cascade. Additionally, this cascade is gated by an insulin/PI3K/PDK1 pathway, which facilitates muscarinic activation of PKC and Pyk2. In light of the significant roles of the cholinergic system, insulin signaling, and GABAergic transmission in learning and memory, our results could provide a framework for understanding their interactions and could offer potential novel targets for AD and related disorders.

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