Dopamine D_4 Receptors Modulate GABAergic Signaling in Pyramidal Neurons of Prefrontal Cortex

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Dopaminergic neurotransmission in the prefrontal cortex (PFC) plays an important role in regulating cognitive processes and emotional status. The dopamine D_4 receptor, which is highly enriched in the PFC, is one of the principal targets of antipsychotic drugs. To understand the cellular mechanisms and functional implications of D_4 receptors, we examined the impact of D_4 receptors in PFC pyramidal neurons on GABAergic inhibition, a key element in the regulation of "working memory." Application of the D_4 agonist *N*-(methyl)-4-(2-cyanophenyl) piperazinyl-3-methylbenzamide maleate caused a reversible decrease in postsynaptic $GABA_A$ receptor currents; this effect was blocked by the D_4 antagonist 3-[(4-[4-chlorophenyl]piperazine-1-yl)methyl]-[1H]-pyrrolo[2,3-b]pyridine but not by the D_2 antagonist sulpiride, suggesting mediation by D_4 receptors. Application of PD168077 also reduced the $GABA_A$ receptor-mediated miniature IPSC amplitude in PFC pyramidal neurons recorded from slices. The D_4 modulation of $GABA_A$ receptor currents was blocked by protein kinase A (PKA) activation and occluded by PKA inhibition. Inhibiting the catalytic activity of protein phosphatase 1 (PP1) also eliminated the effect of PD168077 on $GABA_A$ currents. Furthermore, disrupting the association of the PKA/PP1 complex with its scaffold protein Yotiao significantly attenuated the D_4 modulation of $GABA_A$ currents, suggesting that Yotiao-mediated targeting of PKA/PP1 to the vicinity of $GABA_A$ receptors is required for the dopaminergic signaling. Together, our results show that activation of D_4 receptors in PFC pyramidal neurons inhibits $GABA_A$ channel functions by regulating the PKA/PP1 signaling complex, which could underlie the D_4 modulation of PFC neuronal activity and the actions of antipsychotic drugs.

Key words: dopamine receptors; $GABA_A$ receptor channels; protein kinase A; protein phosphatase 1; Yotiao; inhibitor-1

The prefrontal cortex (PFC), a brain region highly associated with cognitive and emotional processes (Goldman-Rakic, 1995; Miller, 1999), receives a major dopaminergic input from the ventral tegmental area (Lewis et al., 1986; Berger et al., 1988). Regional depletion of dopamine in the PFC of monkeys produces impairments in working memory performance (Brozoski et al., 1979), suggesting that PFC dopaminergic transmission plays a key role in cognitive functions. Disorders in dopaminergic signaling are thought to underlie the etiology of many neuropsychiatric disorders, including schizophrenia and depression (Desimone, 1995), and almost all effective antipsychotic drugs target dopamine receptors (Lidow and Goldman-Rakic, 1994; Seeman and Van Tol, 1994). Dopamine can have both inhibitory and excitatory functions in neuronal networks through the coupling of different dopamine receptors to distinct ion channels (for review, see Nicola et al., 2000). These receptors have been classified as either D_1-like (D_1, D_5) or D_2-like (D_2, D_3, D_4), based on their sequence homology, pharmacological profiles, and distinct downstream signal transduction pathways. Although considerable evidence suggests that D_1 receptors are critically involved in regulating the working memory functions of the PFC (Sawaguchi and Goldman-Rakic, 1991; Williams and Goldman-Rakic, 1995), little is known about the cellular mechanisms and functional consequences of D_2-like receptor-mediated signaling in the PFC.

Among the D_2-like receptors, D_4 receptor is expressed at the highest level in PFC pyramidal principal neurons and GABAergic interneurons (Mrzljak et al., 1996; Wedzony et al., 2000). Given its high affinity for atypical antipsychotic drugs that constitute a major improvement in the treatment of schizophrenia (Van Tol et al., 1991; Kapur and Remington, 2001), D_4 receptor has been suggested to play an important role in PFC cognitive functions and therefore to be involved in the pathophysiology of neuropsychiatric disorders (for review, see Oak et al., 2000). In agreement with this, D_4 receptor antagonists have been found to alleviate stress-induced working memory deficits in monkeys (Murphy et al., 1996) and to ameliorate the cognitive deficits exhibited by monkeys after long-term treatment with the psychotomimetic drug phencyclidine (Jentsch et al., 1997, 1999). Moreover, altered cortical excitability and reduced exploration of novel stimuli have been shown in D_4 receptor-deficient mice (Dulawa et al., 1999; Rubinstein et al., 2001), and significantly elevated D_4 receptors have been demonstrated in patients with schizophrenia (Seeman et al., 1993). To better understand the functional role of D_4 receptors under normal and pathological conditions, it is important to determine cellular substrates of D_4 receptors that are involved in the modulation of PFC neuronal activity and cognitive processes.

Recent evidence indicates that GABAergic inhibition in the PFC plays a key role in working memory by sculpting the temporal profile of activation of the neurons during cognitive operations and thereby shaping the temporal flow of information (Constan-

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tinidis et al., 2002). Computational models of the dopaminergic modulation of working memory processing predict that dopamine-mediated alterations in $GABA_A$ currents in PFC pyramidal neurons are critical for maintaining the specificity and stability of delay-period activity (Durstewitz et al., 2000). Therefore, understanding how the D_4 receptor modulates GABAergic inhibition would provide important insights into its role in cognitive functions associated with the PFC.

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; Cai et al., 2002). All experiments were performed with the approval of the State University of New York at Buffalo Animal Care Committee. In brief, rats were anesthetized by inhaling 2-bromo-2-chloro-1,1,1-trifluoroethane (1 ml/100 gm; Sigma, St. Louis, MO) and decapitated; the 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 containing (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 an $NaHCO_3$-buffered saline containing (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, and bubbled with 95% O_2 and 5% CO_2. All reagents were obtained from Sigma.

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 pronase (1–3 mg/ml) in HEPES-buffered HBSS (Sigma) at 35°C. After 30 min of enzyme digestion, tissue was rinsed three times in the low Ca^{2+}, HEPES-buffered saline and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was then plated into a 35 mm Lux Petri dish, which was then placed on the stage of a Nikon (Tokyo, Japan) inverted microscope.

Whole-cell recordings. Whole-cell recordings of currents used standard voltage-clamp techniques (Hamill et al., 1981; Yan and Surmeier, 1997). Electrodes were pulled from Corning (Corning, NY) 7052 glass and fire-polished before use. The internal solution (Yan and Surmeier, 1997) 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, 5 $BaCl_2$, 10 glucose, 0.001 TTX, pH 7.3–7.4, 300–305 mosm/l.

Recordings were obtained with an Axon Instruments (Union City, CA) 200B patch-clamp amplifier that was controlled and monitored with an IBM personal computer running pClamp (version 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; recordings were terminated whenever a significant increase (>20%) occurred. The cell membrane potential was held at 0 mV. The application of GABA (50 μM) evoked a partially desensitizing outward current with the decay rate fitted by a single or double exponential. Peak values were measured for generating the plot as a function of time and drug application. GABA was applied for 2 sec every 30 sec to minimize a desensitization-induced decrease in current amplitude. Drugs were applied with a gravity-fed "sewer pipe" system. The array of application capillaries (~150 μm inner diameter) 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 Instruments, Hamden, CT).

Dopamine receptor ligands *N*-(methyl)-4-(2-cyanophenyl)piperazinyl-3-methybenzamide maleate (PD168077) maleate, 3-[(4-[4-chlorophenyl]piperazine-1-yl)methyl]-[1H]-pyrrolo[2,3-b]pyridine trihydrochloride (L-745870) (Tocris, Ballwin, MO), *R,S*-(±)-sulpiride and clozapine (Sigma), as well as the second messenger reagents chlorophenylthio-cAMP (cpt-cAMP), PKC_{19-36}, and PKI[5–24] (Calbiochem, San Diego, CA), microcystin, okadaic acid (OA), and okadaic acid methyl ester (OAE) (Sigma/RBI, Poole, UK) were made up as concentrated stocks in water or DMSO and stored at −20°C. The final DMSO concentration in all applied solutions was <0.1%. No change on $GABA_A$ currents has been observed with this concentration of DMSO. Stocks were thawed and diluted immediately before use. The amino acid sequence for the phosphorylated I-1 peptide p^{Thr35}I-1[7–39] is PRKIQFTVPLLEPHLD-PEAAEQIRRRRP(pT)PATL. The amino acid sequence for the PKA anchoring inhibitory peptide Yotiao[1440–1457] is LEEEVAKVIVSM-SIAFAQ. The amino acid sequence for the protein phosphatase 1 (PP1) anchoring inhibitory peptide Gm[63–75] is GRRVSFADNFGFN.

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

Electrophysiological recordings in slices. To evaluate the regulation of miniature IPSCs (mIPSCs) by D_4 receptors in PFC slices, the whole-cell patch technique was used for voltage-clamp recordings using patch electrodes (5–9 MΩ) 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 (Olympus Optical). 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 and were compensated 50–70%. Cells were held at 0 mV for the continuous recording of mIPSCs. The Mini Analysis Program (Synaptosoft, Leonia, NJ) was used to analyze synaptic activity. For each different treatment, mIPSCs of 1 min were used for analysis. Statistical comparisons of the synaptic currents were made using the Kolmogorov–Smirnov (K-S) test.

RESULTS

Activation of D_4 receptors reduces $GABA_A$ receptor currents in prefrontal cortical pyramidal neurons

To test the potential impact of D_4 dopamine receptors on postsynaptic $GABA_A$ receptor channels in PFC, we first examined the effect of PD168077, a potent and highly selective D_4 receptor agonist (Glase et al., 1997), on $GABA_A$ receptor-mediated currents in dissociated pyramidal neurons located in the intermediate and deep layers (III–VI) of the rat PFC. Acutely isolated PFC pyramidal neurons were readily distinguished from GABAergic interneurons by their distinct morphological features: a pyramidal-shaped soma and a prominent apical dendrite. A representative example is shown in Figure 1*A*. GABA (50 μM) was applied to these neurons, which were voltage-clamped using whole-cell techniques. The application of GABA evoked a partially desensitizing outward current that could be completely blocked by the $GABA_A$ receptor antagonist bicuculline (30 μM, data not shown), confirming mediation by the $GABA_A$ receptor.

As shown in Figure 1*B*, application of PD168077 (20 μM) caused a reduction in the amplitudes of $GABA_A$ currents in PFC pyramidal neurons. The modulation was not accompanied by changes in current decay kinetics. The PD168077-induced reduction of $GABA_A$ currents was reversible, and it had rapid-onset kinetics, taking 1–2 min to stabilize (Fig. 1*C*). In a sample of neurons we examined, PD168077 (20 μM) reduced the amplitude of GABA-evoked (50 μM) currents by 15.4 ± 0.6% ($n = 86$; $p < 0.01$; Mann–Whitney *U* test). After recovery from the first application, a second application of PD168077 resulted in a similar response (93.2 ± 2.4% of first response; $n = 18$). This PD168077-induced inhibition of $GABA_A$ currents did not result from an

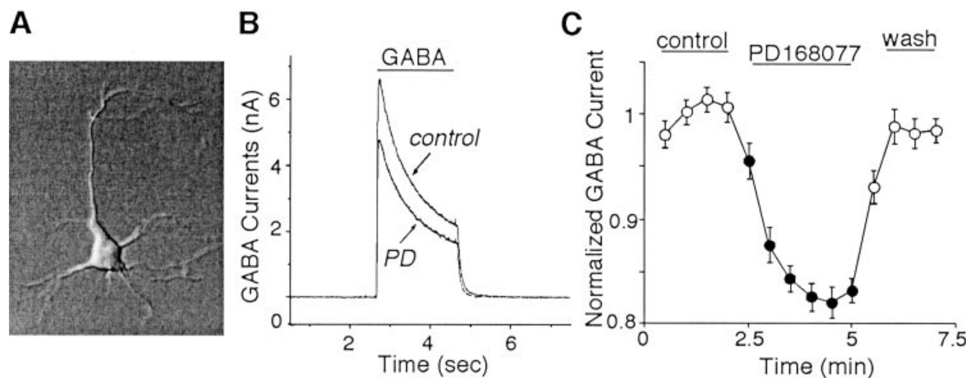

Figure 1. Application of the D_4 agonist PD168077 reversibly reduced $GABA_A$ receptor currents in PFC pyramidal neurons. *A*, Photomicrograph of an acutely isolated PFC pyramidal neuron. *B*, Current *traces* recorded from the neuron shown in *A*. The D_4 agonist PD168077 (*PD*; 20 μM) reduced GABA-evoked (50 μM) currents in the cell. *C*, Plot of peak $GABA_A$ current as a function of time and agonist application in a sample of dissociated PFC pyramidal neurons ($n = 45$).

agonist-independent rundown of the current, because no significant decrease in the current was observed in the absence of PD168077. Similar modulation was observed when different concentrations (25 μM, 100 μM, and 1 mM) of GABA were applied or membrane potentials were held at different levels (−40 mV, −20 mV, and 0 mV) (data not shown). In contrast to the inhibitory effect on GABA-evoked currents, PD168077 (20–50 μM) had no effect on glutamate-evoked (1 mM) currents in dissociated PFC pyramidal neurons tested ($n = 10$; data not shown).

To verify that D_4 receptors were mediating the modulation seen with PD168077, we examined the ability of L-745870, a highly selective D_4 antagonist (Kulagowski et al., 1996; Patel et al., 1997), to prevent the action of PD168077. Dissociated neurons were treated with L-745870 for 15 min before the examination of the PD168077 effect. As shown in Figure 2*A* and *B*, in the presence of the D_4 antagonist L-745870 (20 μM), PD168077 failed to modulate $GABA_A$ currents. In contrast, the PD168077-induced reduction of $GABA_A$ currents was still intact in neurons treated with the D_2 receptor antagonist sulpiride (20 μM). Because the atypical antipsychotic clozapine has higher affinity to D_4 receptors compared with D_2 receptors (Van Tol et al., 1991), we also examined the effect of clozapine on PD168077 modulation of $GABA_A$ currents. As shown in Figure 2*C*, treating neurons with clozapine (20 μM) blocked the PD168077-induced reduction of $GABA_A$ currents. The percentage modulation of $GABA_A$ currents by PD168077 in the absence or presence of various antagonists is summarized in Figure 2*D*. PD168077 had little effect on $GABA_A$ currents in neurons treated with L-745870 (4.2 ± 1.8%; $n = 24$; $p > 0.05$; Mann–Whitney *U* test) or clozapine (3.6 ± 0.4%; $n = 10$; $p > 0.05$; Mann–Whitney *U* test), which was significantly different from the effect of PD168077 on control cells (15.3 ± 1.1%; $n = 36$; $p < 0.005$; ANOVA) or sulpiride-treated neurons (14.8 ± 2.6%; $n = 11$; $p < 0.005$; ANOVA). The pharmacological profile of these responses thus identifies D_4 as the receptor underlying the PD168077-induced inhibition of $GABA_A$ currents.

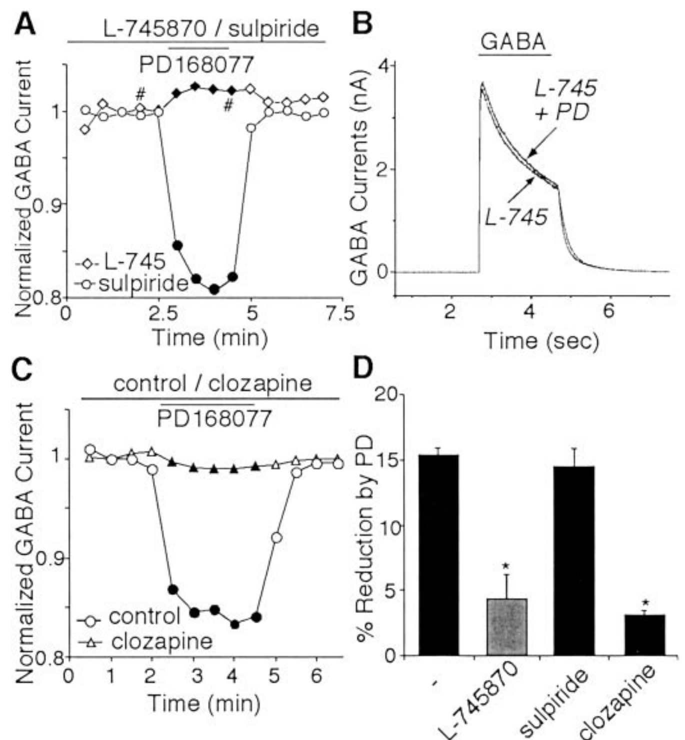

Figure 2. The effect of PD168077 on $GABA_A$ receptor currents was mediated by D_4 receptors. *A*, Plot of peak $GABA_A$ current as a function of time and agonist application in an L-745870-treated neuron (*L-745*; *diamonds*) and a sulpiride-treated neuron (*circles*). The selective D_4 antagonist L-745870 (20 μM) but not the D_2 antagonist sulpiride (20 μM) blocked PD168077-induced reduction of $GABA_A$ currents. *B*, Representative current *traces* taken from the records used to construct *A* (at time points denoted by #). *C*, Plot of peak $GABA_A$ current as a function of time and agonist application in a clozapine-treated (20 μM) neuron (*triangles*) and a nontreated control neuron (*circles*). *D*, Cumulative data (means ± SEM) showing the percentage modulation of $GABA_A$ currents by PD168077 in the absence ($n = 36$) or presence of L-745870 ($n = 24$), sulpiride ($n = 11$), or clozapine ($n = 10$). *$p < 0.005$; ANOVA.

Activation of D_4 receptors decreases $GABA_A$ receptor-mediated synaptic transmission in prefrontal cortex

We then examined the effect of PD168077 on $GABA_A$ receptor-mediated IPSCs, indicative of the impact of D_4 receptors on GABAergic synaptic transmission. PFC slices were exposed to TTX (1 μM), and mIPSCs were recorded in PFC pyramidal neurons to better isolate the postsynaptic effect of D_4 receptors. Application of bicuculline (30 μM) blocked the mIPSCs ($n = 5$), indicating that these synaptic currents are mediated by $GABA_A$ receptors. As shown in Figure 3*A–C*, bath application of PD168077 to the PFC slice caused a significant and reversible leftward shift on the distribution of mIPSC amplitudes ($p < 0.001$; K-S test), but not the distribution of mIPSC frequencies, indicating that PD168077 reduced postsynaptic responses to GABA. In a sample of PFC pyramidal neurons we examined, PD168077 decreased the mean amplitude of mIPSCs by 21.1 ± 1.1% (Fig. 3*D*) (means ± SEM; $n = 27$; $p < 0.01$; Mann–Whitney *U* test), whereas the frequency of mIPSCs recorded from PFC pyramidal neurons in slices was not significantly changed by PD168077 (Fig. 3*D*) (6.4 ± 4.1%; $n = 27$; $p > 0.05$; Mann–

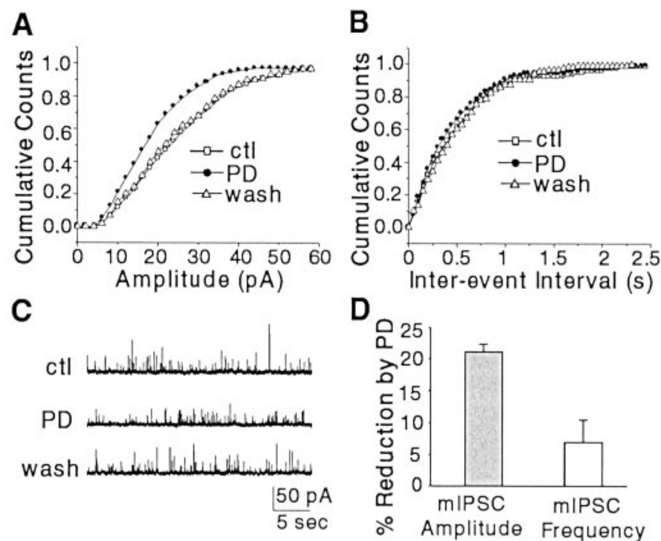

Figure 3. Activation of D_4 receptors reduced the amplitude of mIPSCs recorded from pyramidal neurons in PFC slices. *A*, Cumulative plots of the amplitude of mIPSCs in a PFC pyramidal neuron. Note that PD168077 (*PD*; 20 μM) caused a reversible leftward shift on the distribution of mIPSC amplitudes, indicative of a reduction in the sizes of mIPSCs by PD168077. *B*, Cumulative plots of the frequency of mIPSCs in the same cell demonstrating that the distribution of mIPSC frequency was not changed by PD168077 (20 μM). *C*, Representative *traces* of mIPSCs recorded from the cell before (control, *ctl*), during bath application of PD168077, and after washout of the agonist. *D*, Cumulative data (means ± SEM) showing the percentage modulation of mIPSC amplitude and mIPSC frequency by PD168077 in a sample of PFC pyramidal neurons ($n = 27$).

Whitney *U* test). These results suggest that activation of D_4 receptors could downregulate $GABA_A$ receptor functions by a postsynaptic mechanism.

D_4 modulation of $GABA_A$ currents is dependent on the inhibition of protein kinase A

We subsequently examined the signal transduction pathways mediating the modulation of $GABA_A$ currents by D_4 receptors. $GABA_A$ channels are thought to be heteropentameric structures, composed of different subunits (Macdonald and Olsen, 1994). PKA phosphorylation of $GABA_A$ receptor subunits exerts a powerful impact on recombinant and native $GABA_A$ channels (Porter et al., 1990; Moss et al., 1992a,b). Activation of D_4 receptors can inhibit adenylate cyclase and cAMP formation in transfected cell lines (Chio et al., 1994). This led us to speculate that the D_4 reduction of $GABA_A$ currents is through the inhibition of PKA. If that is the case, then the effect of D_4 receptors on $GABA_A$ receptor currents should be blocked by stimulating PKA and occluded by inhibiting PKA. To test this, we applied selective PKA activators and inhibitors.

As shown in Figure 4*A* and *B*, application of the membrane-permeable cAMP analog cpt-cAMP (200 μM) caused a small increase in basal $GABA_A$ currents. In the presence of cpt-cAMP, PD168077 failed to reduce $GABA_A$ currents. Removing cpt-cAMP restored the ability of PD168077 to inhibit $GABA_A$ currents. To further confirm the involvement of PKA in D_4 modulation of $GABA_A$ currents, we dialyzed neurons with the specific PKA inhibitory peptide PKI[5–24] (Knighton et al., 1991). The PKC inhibitory peptide PKC_{19-36} (20 μM) was used as a control. After ~5 min of dialysis to allow the peptide to enter the cell to inhibit kinase activity, the effect of the subsequent application of

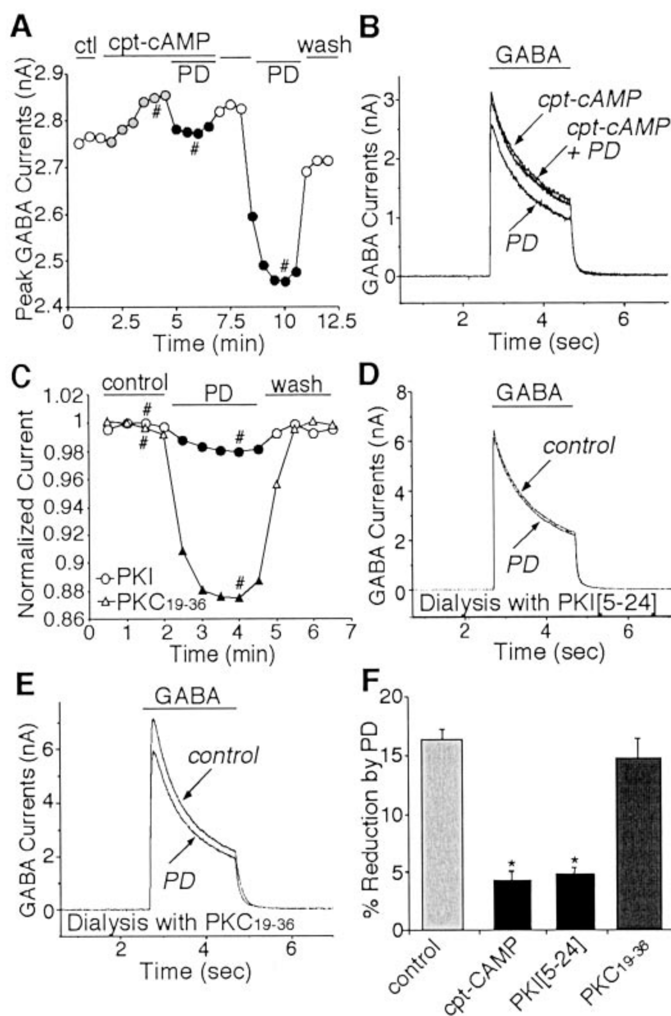

Figure 4. The effect of PD168077 on $GABA_A$ currents was blocked by PKA activation and occluded by PKA inhibition. *A*, Plot of peak $GABA_A$ currents as a function of time and drug application. In the presence of the membrane-permeable cAMP analog cpt-cAMP (200 μM), PD168077 (*PD*; 20 μM) failed to reduce $GABA_A$ currents. After washing off cpt-cAMP, the effect of PD168077 emerged. *B*, Representative current *traces* taken from the records used to construct *A* (at time points denoted by #). *C*, Plot of peak $GABA_A$ currents as a function of time and drug application in neurons dialyzed with PKI[5–24] or PKC_{19-36}. The specific PKA inhibitory peptide PKI[5–24] (20 μM), but not the PKC inhibitory peptide PKC_{19-36} (20 μM), eliminated PD168077-induced reduction of $GABA_A$ currents. *D, E*, Representative current *traces* taken from the records used to construct *C* (at time points denoted by #). *F*, Cumulative data (means ± SEM) showing the percentage modulation of $GABA_A$ currents by PD168077 in the absence ($n = 13$) or presence of cpt-cAMP ($n = 12$), PKI[5–24] ($n = 8$). or PKC_{19-36} ($n = 14$). $^*p < 0.005$; ANOVA. *ctl*, Control.

PD168077 on $GABA_A$ currents was examined. As shown in Figure 4*C–E*, the PD168077-induced reduction of $GABA_A$ currents was largely abolished in neurons dialyzed with PKI[5–24] but was almost intact in neurons loaded with PKC_{19-36}. Figure 4*F* compares the effects of PD168077 in the absence or presence of various kinase activators and inhibitors. PD168077 reduced peak $GABA_A$ currents by 4.3 ± 0.8% in the presence of cpt-cAMP ($n = 12$; $p > 0.05$; Mann–Whitney *U* test) and 4.8 ± 0.8% in the presence of PKI[5–24] ($n = 8$; $p > 0.05$; Mann–Whitney *U* test), both of which were significantly smaller than the effect of PD168077 in the absence of these agents (16.2 ± 1.0%; $n = 13$;

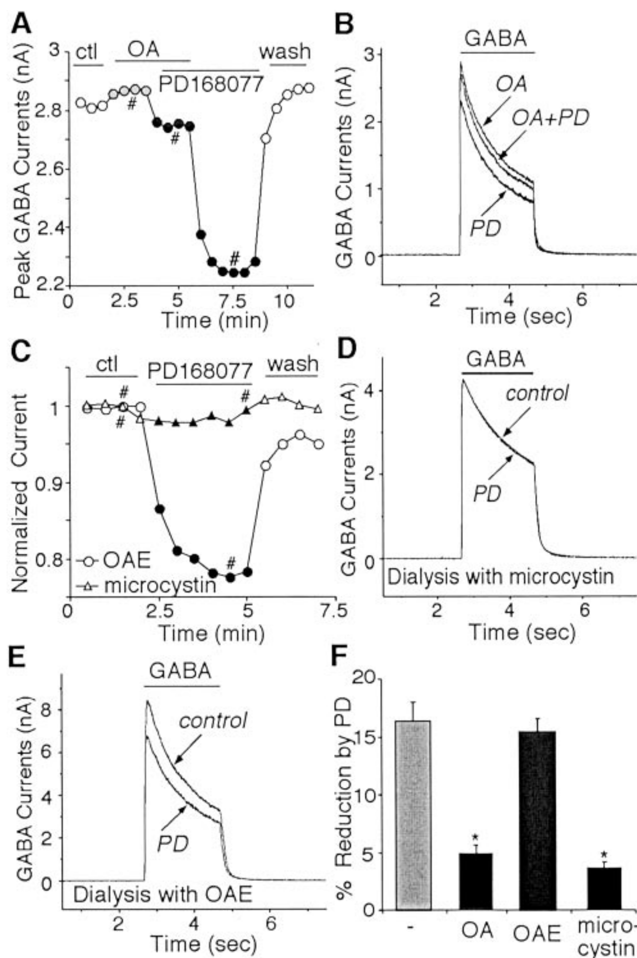

Figure 5. The effect of PD168077 (*PD*) on $GABA_A$ currents was blocked by phosphatase inhibition. *A*, Plot of peak $GABA_A$ currents as a function of time and drug application. In the presence of the membrane-permeable PP1/2A inhibitor OA, PD168077 (20 μM) failed to reduce $GABA_A$ currents. Washing off OA led to recovery of the effect of PD168077. *B*, Representative current *traces* taken from the records used to construct *A* (at time points denoted by #). *C*, Plot of peak $GABA_A$ currents as a function of time and drug application in neurons dialyzed with microcystin (*triangle*) or OAE (*circles*). The PP1/2A inhibitor microcystin (5 μM) but not the inactive agent OAE (1 μM) eliminated the PD168077-induced reduction of $GABA_A$ currents. *D, E*, Representative current *traces* taken from the records used to construct *C* (at time points denoted by #). *F*, Cumulative data (means ± SEM) showing the percentage modulation of $GABA_A$ currents by PD168077 in the absence ($n = 11$) or presence of OA ($n = 13$), OAE ($n = 10$), or microcystin ($n = 9$). *$p < 0.005$; ANOVA. *ctl*, Control.

$p < 0.005$; ANOVA) or in the presence of PKC_{19-36} (14.8 ± 1.6%; $n = 14$; $p < 0.005$; ANOVA). These results indicate that reduction of $GABA_A$ currents by PD168077 depends on the inhibition of PKA.

D_4 modulation of $GABA_A$ currents requires the activation of protein phosphatase 1

The D_4-induced inhibition of PKA could directly modulate $GABA_A$ currents through decreased phosphorylation of $GABA_A$ receptor β subunits on the PKA sites (Moss et al., 1992a,b; McDonald et al., 1998; Cai et al., 2002). Alternatively, the inhibition of PKA could cause the disinhibition of PP1 via decreased phosphorylation of the inhibitory protein I-1 (Ingebritsen and Cohen, 1983), leading to the increased dephosphorylation of

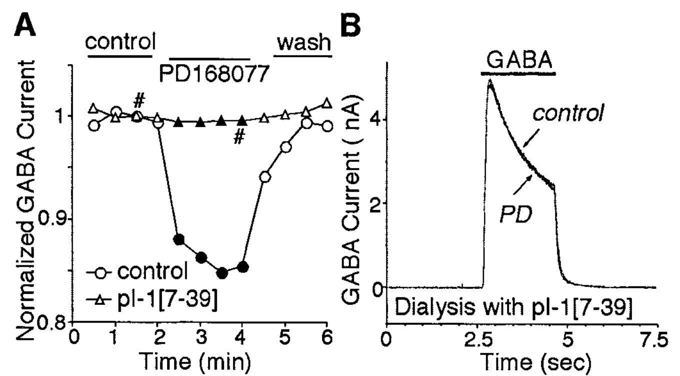

Figure 6. The effect of PD168077 (*PD*) on $GABA_A$ currents was blocked by I-1 inhibition of PP1 activity. *A*, Plot of peak $GABA_A$ currents as a function of time and drug application in neurons dialyzed with the phosphorylated I-1 peptide p^{Thr35}I-1[7–39] or the dephosphorylated I-1[7–39] control peptide. The constitutively active peptide p^{Thr35}I-1[7–39] (40 μM) but not the inactive control peptide I-1[7–39] (40 μM) blocked PD168077 modulation of $GABA_A$ currents. *B*, Representative current *traces* taken from the records used to construct *A* (at time points denoted by #).

$GABA_A$ receptor subunits and downregulation of $GABA_A$ currents. To test which is the potential signaling mechanism, we examined the effect of PD168077 on $GABA_A$ currents in the presence of phosphatase inhibitors.

As shown in Figure 5*A* and *B*, bath application of the PP1/2A inhibitor OA (0.5 μM) eliminated the ability of PD168077 to inhibit $GABA_A$ currents. After washing off OA, the D_4 modulation emerged. To further confirm the involvement of PP1/2A in D_4 modulation of $GABA_A$ currents, we dialyzed cells with microcystin (5 μM), another structurally different and potent PP1/2A inhibitor. In most cells tested, the basal $GABA_A$ currents showed a time-dependent increase (9.5 ± 1.7%; $n = 7$) at the initial dialysis period (~5 min), probably attributable to the inhibition of constitutively active PP1/2A by microcystin. After the basal $GABA_A$ currents became stabilized, subsequent application of PD168077 failed to reduce $GABA_A$ currents (Fig. 5*C,D*). On the contrary, injecting with OAE, a compound with a structure similar to OA but lacking the ability to inhibit PP1/2A, did not affect the PD168077-induced inhibition of $GABA_A$ currents (Fig. 5*C,E*). The effect of PD168077 on $GABA_A$ currents in the presence of phosphatase inhibitors or their inactive analog is summarized in Figure 5*F*. PD168077 caused little reduction of $GABA_A$ currents in the presence of OA (4.8 ± 0.8%; $n = 13$; $p > 0.05$; Mann–Whitney *U* test) or in the presence of microcystin (3.7 ± 0.5%; $n = 9$; $p > 0.05$; Mann–Whitney *U* test), both of which were significantly different from the effect of PD168077 in the presence of OAE (15.4 ± 1.1%; $n = 10$; $p < 0.005$; ANOVA), suggesting the PP1 or PP2A activation is required in the D_4 regulation of $GABA_A$ receptors.

We then tried to determine the identity of the phosphatase involved in the D_4 regulation of $GABA_A$ currents. I-1, once it is phosphorylated by PKA at Thr^{35}, acts as a specific inhibitor of PP1 (Ingebritsen and Cohen, 1983). To test the role of PP1 in D_4 modulation of $GABA_A$ currents, we dialyzed PFC pyramidal neurons with the phosphorylated I-1 peptide p^{Thr35}I-1[7–39], derived from the PP1 interaction region. Biochemical analysis demonstrated that the phospho-I-1 peptide p^{Thr35}I-1[7–39] potently inhibited PP1 catalytic activity with an IC_{50} at the nanomolar range, whereas the dephospho-I-1 peptide I-1[7–39], was

much less effective (Hemmings et al., 1990; Kwon et al., 1997). As shown in Figure 6*A* and *B*, dialysis with the active p^{Thr35}I-1[7–39] peptide (40 μM) but not the inactive control peptide I-1[7–39] (40 μM) abolished the ability of PD168077 to modulate $GABA_A$ currents. In summary, in cells dialyzed with the p^{Thr35}I-1[7–39] peptide, PD168077 reduced $GABA_A$ currents by 2.3 ± 0.8% (n = 18; $p > 0.05$; Mann–Whitney U test), which was significantly smaller than the effect of PD168077 in cells dialyzed with the inactive I-1[7–39] control peptide (13.2 ± 2.5%; n = 7; $p < 0.005$; ANOVA). These results indicate direct involvement of PP1, which links D_4 receptor activation to a reduction of $GABA_A$ currents.

D_4 modulation of $GABA_A$ currents requires the Yotiao-mediated anchoring of protein kinase A/ protein phosphatase 1 complex

Emerging evidence has shown that signaling enzymes with broad substrate selectivity, such as PKA and PP1, achieve the efficacy and specificity of signal transduction through anchoring protein-mediated subcellular targeting to their substrates in central neurons (Colledge and Scott, 1999). Previous studies have found that the multivalent scaffold protein Yotiao binds PP1 and PKA, allowing the two enzymes to regulate their substrates dynamically, like NMDA receptors (Westphal et al., 1999). We subsequently examined whether Yotiao is involved in D_4 modulation of $GABA_A$ channels in PFC pyramidal neurons. If Yotiao is responsible for targeting the PKA/PP1 complex to $GABA_A$ receptors and allowing the kinase and phosphatase to regulate the phosphorylation state of these substrates effectively, then disrupting the complex should lead to the removal of PKA/PP1 from $GABA_A$ receptors, thereby attenuating the regulation of these channels.

Previous biochemical studies have found that a peptide encompassing residues 1440–1457 of Yotiao blocked PKA binding *in vitro* (Westphal et al., 1999). Based on this result, we synthesized a peptide, Yotiao[1440–1457], dialyzed neurons with it, and then examined the effects of D_4 on $GABA_A$ currents. As shown in Figure 7*A* and *B*, dialysis with Yotiao[1440–1457] (10 μM) significantly attenuated the ability of PD168077 to modulate $GABA_A$ currents. In contrast, a control peptide with a scrambled amino acid sequence, sYotiao[1440–1457], had no effect on D_4 regulation of $GABA_A$ currents. As summarized in Figure 7*C*, in a sample of PFC neurons dialyzed with the peptide Yotiao[1440–1457], PD168077 reduced $GABA_A$ currents by 5.9 ± 0.6% (n = 12; $p > 0.05$; Mann–Whitney U test), which was significantly smaller than the effect of PD168077 in neurons dialyzed with the control peptide sYotiao[1440–1457] (17.2 ± 1.1%; n = 7; $p < 0.005$; ANOVA).

Because the PD168077-induced reduction of $GABA_A$ currents requires the activation of PP1, we also examined whether blocking the binding of PP1 to Yotiao affected the D_4 modulation of $GABA_A$ currents. Previous studies have found that the PP1 targeting inhibitor peptide Gm[63–75] (Egloff et al., 1997) could disrupt PP1 binding to Yotiao (Westphal et al., 1999), so we dialyzed PFC pyramidal neurons with the Gm[63–75] peptide. As shown in Figure 7*D,E*, dialysis with Gm[63–75] (10 μM) almost eliminated the ability of PD168077 to modulate $GABA_A$ currents. On the contrary, a control peptide with a scrambled amino acid sequence, sGm[63–75], had no effect on the D_4 regulation of $GABA_A$ currents. As summarized in Figure 7*F*, in a sample of PFC neurons dialyzed with the peptide Gm[63–75], PD168077 reduced $GABA_A$ currents by 4.3 ± 0.7% (n = 14; $p > 0.05$; Mann–Whitney U test), which was significantly smaller than the effect of PD168077 in neurons dialyzed with the control peptide sGm[63–75] (16.5 ± 1.2%; n = 8; $p < 0.005$; ANOVA). Collectively, these data suggest that D_4 reduction of $GABA_A$ currents requires the Yotiao-mediated anchoring of the PKA/PP1 complex.

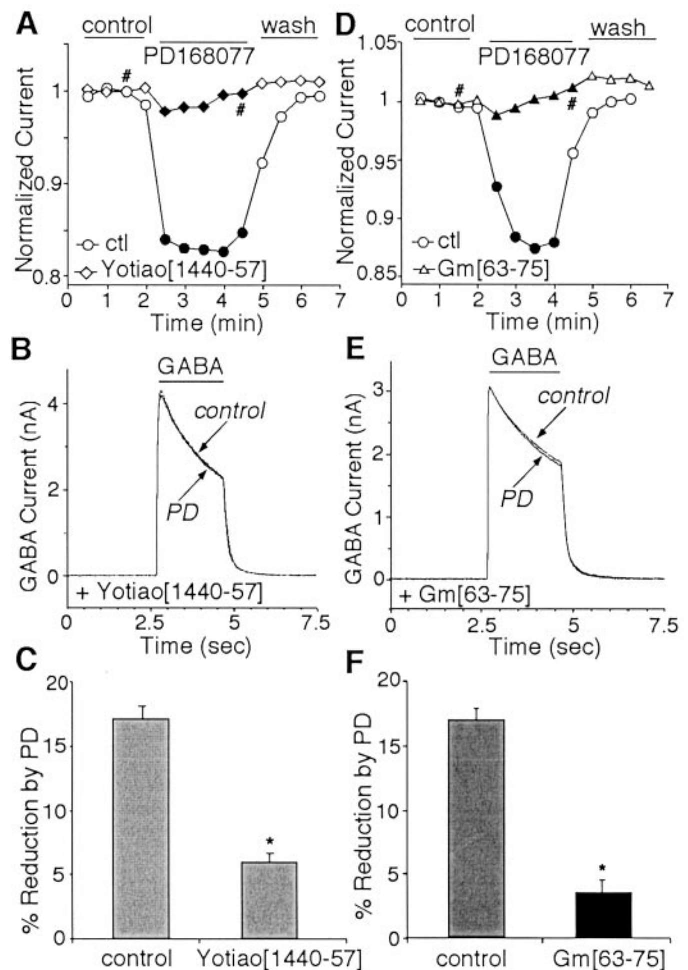

Figure 7. The D_4 modulation of $GABA_A$ receptor currents required anchoring of the PKA/PP1 complex to the channel by Yotiao. *A*, Plot of peak $GABA_A$ current as a function of time and agonist application with the PKA anchoring inhibitory peptide Yotiao[1440–1457] (10 μM, *diamonds*) or the control (*ctl*) peptide sYotiao[1440–1457] (10 μM, *circles*) in the recording pipette. *B*, Representative current *traces* taken from the records used to construct *A* (at time points denoted by #). *C*, Cumulative data (means ± SEM) showing the percentage modulation of $GABA_A$ currents by PD168077 (*PD*; 20 μM) in the presence of Yotiao[1440–1457] peptide (n = 12) or the scrambled control peptide sYotiao[1440–1457] (n = 7). *$p < 0.005$; ANOVA. *D*, Plot of peak $GABA_A$ current as a function of time and agonist application with the PP1 anchoring inhibitory peptide Gm[63–75] (10 μM, *triangles*) or the control peptide sGm[63–75] (10 μM, *circles*) in the recording pipette. *E*, Representative current *traces* taken from the records used to construct *D* (at time points denoted by #). *F*, Cumulative data (means ± SEM) showing the percentage modulation of $GABA_A$ currents by PD168077 (20 μM) in the presence of Gm[63–75] peptide (n = 14) or the scrambled control peptide sGm[63–75] (n = 8). *$p < 0.005$; ANOVA.

DISCUSSION

Despite the well recognized association of D_4 receptors with schizophrenia, attention deficit hyperactivity disorder, and other mental disorders (Oak et al., 2000), the cellular mechanisms by

which D_4 receptors modulate PFC neuronal functions remain elusive. Anatomical studies have found that D_4 receptors are enriched in PFC (Mrzljak et al., 1996; Ariano et al., 1997). Unlike D_1 receptors that are concentrated at the dendritic spines of pyramidal neurons (Smiley et al., 1994), D_4 receptors are localized predominantly on the periphery of the cell body and dendritic processes (Wedzony et al., 2000). Because $GABA_A$ receptors exhibit a compartmentalized distribution on postsynaptic domains of GABAergic synapses on the soma and proximal dendrites (Nusser et al., 1996), it suggests that most D_4 receptors may be localized in the vicinity of $GABA_A$ receptors in PFC pyramidal neurons.

In this study, we demonstrated that activation of D_4 receptors in PFC pyramidal neurons significantly reduced $GABA_A$ receptor-mediated currents, indicating that the postsynaptic $GABA_A$ receptor is one of the key cellular substrates of D_4 receptors in the PFC. Because GABAergic inhibition in the frontal cortex is critical for controlling the timing of neuronal activities during the thought process, which is fundamental for processing ongoing information and planning appropriate actions at a future time (Constantinidis et al., 2002), the D_4 modulation of GABAergic signaling could be one of the mechanisms underlying the involvement of D_4 receptors in PFC cognitive functions. It is conceivable that dysregulation of GABAergic inhibition by D_4 receptors could contribute to the PFC cognitive deficits associated with schizophrenia. This notion is supported by the discovery that, in addition to elevated D_4 receptors (Seeman et al., 1993), selective alterations in $GABA_A$ receptors, GABA content, and GABAergic local circuit neurons have been discovered in the PFC of patients with neuropsychiatric disorders (Benes et al., 1996; Dean et al., 1999; Ohnuma et al., 1999; Lewis, 2000). Although it has been suggested that D_4 receptors function as an inhibitory modulator of glutamate activity in the PFC (Rubinstein et al., 2001), D_4 receptors could exert both excitatory and inhibitory influences on the activity of neurons in the PFC by targeting different channels (Werner et al., 1996; Wilke et al., 1998), similar to the multifunctional feature of D_1 receptors (for review, see Nicola et al., 2000).

The effect of dopamine on GABAergic synaptic transmission in the PFC is complex and dependent on the receptors activated. Recent studies have shown that dopamine produces temporally biphasic effects on GABAergic IPSCs, which are mediated by D_1 and D_2 receptors (Seamans et al., 2001). Changes in the excitability of GABAergic interneurons, the probability of release at GABAergic terminals, as well as the properties of postsynaptic $GABA_A$ receptors, have been suggested as the underlying mechanisms for the D_1 and D_2 modulation of GABAergic transmission (Seamans et al., 2001). The present study has revealed that D_4 receptors also produce a significant reduction of the mIPSC amplitude in pyramidal neurons of PFC slices, suggestive of a D_4 receptor-mediated downregulation of postsynaptic $GABA_A$ receptor sensitivity or conductance. This is consistent with the D_4 receptor-mediated reduction of whole-cell $GABA_A$ receptor currents found in acutely dissociated PFC pyramidal neurons.

The mechanism underlying the D_4 receptor-mediated reduction of $GABA_A$ receptor currents has been investigated in this study. Several lines of evidence show that the D_4 receptor-mediated suppression of $GABA_A$ receptor currents is through a signaling pathway mediated by the inhibition of PKA and subsequent activation of PP1. The activity of PP1 is controlled by PKA through the regulatory protein I-1. I-1, after phosphorylation by PKA at Thr^{35}, becomes a potent inhibitor of PP1 (Ingebritsen and Cohen, 1983). Previous studies have found that $GABA_A$ receptor currents are enhanced in response to elevated PKA activation in hippocampal dentate granule cells and neostriatal cholinergic interneurons (Kapur and Macdonald, 1996; Yan and Surmeier, 1997), presumably because of the increased PKA phosphorylation of $GABA_A$ receptor $\beta3$ subunits (McDonald et al., 1998). In the present study, tonic PKA activity, along with the inhibited PP1, may keep $GABA_A$ receptors in PFC pyramidal neurons at a relatively high phosphorylation state, in which many of the $\beta3$ subunits are phosphorylated. The D_4 receptor-mediated suppression of PKA activity, along with the disinhibited PP1, switches $GABA_A$ receptors to a lower phosphorylation state, in which $\beta3$ subunits are dephosphorylated, therefore leading to the reduction of $GABA_A$ receptor currents.

Because both PKA and PP1 have broad substrate selectivity, a crucial issue in channel regulation is to control the specificity of their actions. Subcellular targeting through association with anchoring proteins has emerged as an important mechanism by which signaling enzymes achieve precise substrate recognition and enhanced efficacy of signal transduction (Rosenmund et al., 1994; Pawson and Scott, 1997; Yan et al., 1999; Feng et al., 2001). Yotiao, an NMDA receptor-associated protein (Lin et al., 1998), binds both PKA and PP1 (Feliciello et al., 1999; Westphal et al., 1999). This targeting protein is present in the cortex and is localized at somatodendritic regions (Lin et al., 1998). Recent studies have revealed the key role played by Yotiao in mediating the assembly of a macromolecular signaling complex and the dynamic regulation of NMDA receptor channels and the slow outward potassium channels (Westphal et al., 1999; Marx et al., 2002). To test whether the targeting of activated PKA/PP1 to $GABA_A$ receptors via Yotiao may allow these enzymes to regulate the phosphorylation state of $GABA_A$ receptors effectively *in vivo*, we examined the involvement of Yotiao in D_4 modulation of $GABA_A$ currents in PFC neurons. Dialysis with a Yotiao-derived peptide that can specifically disrupt the interaction between Yotiao and PKA (Westphal et al., 1999) significantly attenuated the D_4 modulation of $GABA_A$ currents. The same was true when a peptide that can disrupt the interaction between Yotiao and PP1 was dialyzed. These data suggest that the D_4 regulation of $GABA_A$ receptors requires the Yotiao-anchored pool of the PKA/PP1 complex, and that changing the subcellular targeting of these signaling enzymes leads to disruption of this regulation. It remains to be determined whether Yotiao is directly associated with $GABA_A$ receptor subunits or acts only to recruit the PKA/PP1 signaling complex to the proximity of $GABA_A$ receptors and thus facilitate the compartmentalized regulation of these substrates.

Together, our results show that the activation of D_4 receptors decreased the postsynaptic $GABA_A$ receptor function in PFC pyramidal neurons via the regulation of the Yotiao-anchored PKA/PP1 signaling complex. Key signaling components engaged in D_4 modulation of $GABA_A$ receptors provide the potential targets for novel pharmacological agents with greater therapeutic potential and fewer side effects in the treatment of neuropsychiatric disorders in which D_4 receptors are highly involved.

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