Behavioral/Systems/Cognitive

Repeated Cocaine Administration Impairs Group II Metabotropic Glutamate Receptor-Mediated Long-Term Depression in Rat Medial Prefrontal Cortex

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Drug-induced neuroadaptations within the medial prefrontal cortex (mPFC) are thought to underlie the development of cocaine sensitization. Here, we report that repeated cocaine administration *in vivo* impaired the long-term depression (LTD) induced by bath application of group II metabotropic glutamate receptor (mGluR) agonists DCG-IV [$2S, 2'R, 3'R$)-2-($2', 3'$-dicarboxycyclopropyl)glycine] or LY379268 [($1R,4R,5S,6R$)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid] at excitatory synapses onto layer V pyramidal neurons of rat mPFC. In contrast, this impairment was not found in slices from rats treated with saline or a single dose of cocaine. Such effect of cocaine was selectively prevented when cocaine was coadministered with the selective D_1-like receptor antagonist SCH23390 [(R)-(+)-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine]. In slices from control rats, a brief application of either protein kinase C (PKC) activator phorbol-12,13-dibutyrute or adenosine A_3 receptor agonist 2-chloro-N^6-(3-iodobenzyl)-adenosine-5-N-methyluronamide mimicked the effect of repeated cocaine treatment to impair the induction of LTD. Bilateral intra-mPFC infusion of PKC inhibitor bisindolylmaleimide I or adenosine A_3 receptor antagonist MRS1220 (N-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-benzeneacetamide) before cocaine injection prevented cocaine-induced impairment of LTD induction. Furthermore, endogenous adenosine tone is greater in slices from cocaine-treated rats than from the saline-treated controls. When the metabolism of cAMP to adenosine was blocked, the extent of LTD in slices from saline and cocaine-treated rats was similar. These results suggest that cocaine-induced impairment of group II mGluR-mediated LTD is caused, at least in part, by an increase in adenosine subsequent to the rise in cAMP after D_1-like receptor activation, which leads to an adenosine A_3 receptor-mediated upregulation of PKC activity and thereby triggers an inhibition of group II metabotropic glutamate receptor function.

Key words: cocaine; medial prefrontal cortex; metabotropic glutamate receptor; long-term depression; adenosine; addiction

Introduction

Repeated exposure to psychostimulants such as cocaine and amphetamine produces a progressive and enduring increase in their locomotor stimulatory effects. This enhanced behavioral response is referred to as behavioral sensitization and is postulated to contribute, in part, to drug craving and relapse to addiction (Robinson and Berridge, 1993; Wolf, 1998; Carlezon and Nestler, 2002; Hyman et al., 2006). Many studies on the neurobiological basis of behavioral sensitization have focused on the mesolimbic dopaminergic system because cocaine and amphetamine act to block the uptake or stimulate the release, respectively, of dopamine within this system (Reith et al., 1986; Seiden et al., 1993). The mesolimbic system consists of dopamine perikarya located in the ventral tegmental area (VTA) that project to numerous limbic areas including nucleus accumbens (NAc) (Oades and Halliday,

1987). Furthermore, evidence suggests that adaptive changes within the VTA appear to mediate the induction of behavioral sensitization, whereas adaptations in the NAc are involved in its long-term maintenance (Robinson and Berridge, 1993; Wolf, 1998; Carlezon and Nestler, 2002). In addition to the mesolimbic system, previous studies have implicated an important role for the mesocortical system, in particular the medial prefrontal cortex (mPFC), in the development of behavioral sensitization. For example, direct electrical stimulation of the PFC leads to rewarding effects and sensitization to cocaine, whereas lesion of the PFC or impairment of mesocorticolimbic glutamatergic transmission prevents the development of cocaine-induced behavioral sensitization (Li et al., 1997; Tzschentke, 2001). Moreover, evidence is accumulating that the alterations of multiple mPFC neurotransmitter systems, including dopamine, serotonin, glutamate, noradrenalin, acetylcholine, GABA, and peptides, and signaling by receptors activated by these neurotransmitters may also contribute to the development of cocaine sensitization and addiction (Wolf, 1998; Steketee, 2003; Williams and Steketee, 2004, 2005).

Compelling evidence indicates that drug-induced synaptic plasticity may contribute to the reorganization of neural circuitry that underlies behavioral sensitization to drugs of abuse and,

thus, the development of addiction. It has been shown that a single or repeated exposure *in vivo* to addictive drugs induces alterations of synaptic transmission and/or long-term synaptic plasticity at excitatory synapses in *in vitro* slice preparations of the VTA, NAc, periaqueductal gray, hippocampus, and striatum (Ingram et al., 1998; Martin et al., 1999; Thomas et al., 2001; Ungless et al., 2001; Williams et al., 2001; Harrison et al., 2002; Robbe et al., 2002; Hoffman et al., 2003; Borgland et al., 2004; Fourgeaud et al., 2004; Liu et al., 2005; Centonze et al., 2006). Less work has been done on the impact of *in vivo* exposures to drugs of abuse on excitatory synaptic transmission in the mPFC. Our laboratory has demonstrated previously that repeated cocaine administration *in vivo* can promote the induction of long-term potentiation (LTP) in mPFC layer V pyramidal neurons (Huang et al., 2007). However, the effect of repeated cocaine exposure on the induction of long-term depression (LTD) at mPFC excitatory synapses has not yet been explored. Here, we provide the first evidence that repeated cocaine administration, which produces sensitization of locomotor activity, impairs the group II metabotropic glutamate receptor (mGluR)-mediated LTD at these synapses.

Materials and Methods

Animals and treatment. Male Sprague Dawley rats (21–23 d old) were housed under a 12 h light/dark cycle (lights on at 6:00 A.M.) and had *ad libitum* access to food and water. After a 3 d acclamation period, animals were assigned randomly to two groups that received an intraperitoneal injection of saline (1 ml/kg) or cocaine HCl (15 mg/kg) once per day for 5 consecutive days followed by 3 d of withdrawal before the experiments. During this time, they were handled and weighed daily. All comparisons between saline- and cocaine-treated groups were performed by experimenters blind to group assignment. Animal care was consistent with the guidelines set by the Laboratory Animal Center of National Cheng Kung University. All experiments were approved by the National Cheng Kung University Institutional Animal Care and Use Committee governing the participating laboratories.

Cannulation. Rats (21–23 d old) were bilaterally implanted under deep pentobarbital (50 mg/kg, i.p., supplemented as required) anesthesia with 22-gauge double guide cannulas (Plastics One, Roanoke, VA) in the mPFC. Medial PFC coordinates were +2.5 mm anterior to bregma, ±0.5 mm bilateral to midline, and 2.0 mm ventral to brain surface according to the previous studies (Huang et al., 2007) and the stereotaxic atlas of developing rat brain (Sherwood and Timiras, 1970). The cannulas were fixed to the skull with dental cement. After 3–5 d of recovery, rats received 0.5 μl microinjections in each side over a 1 min period using an infusion pump (CAM/100; CAM Microdialysis, Slona, Sweden). The infusion cannulas were kept in place for an additional 2 min to minimize backflow of the injectant. Doses of bisindolylmaleimide I (Bis-I; 0.4 nmol/side), bisindolylmaleimide V (Bis-V; 0.4 nmol/side), or *N*-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-benzeneacetamide (MRS1220; 0.5 nmol/side) were selected on the basis of published studies (Blank et al., 2003; Yang et al., 2004) and pilot experiments in our laboratory. Infusions were administered 30 min before each of the five daily cocaine injections. Rats with injection cannula placements outside the mPFC or with extensive tissue damage at the injection cannula site were excluded from analyses. We never observed any effects of cannulation itself or vehicle artificial CSF (aCSF) [10% dimethylsulfoxide (DMSO)] injection. Only slices from animals with accurate cannula placements, as verified microscopically by the cannula tips located within the mPFC, were taken for electrophysiological recordings. Although infusions spread with a radius of ~1.0 mm (as estimated by pilot experiments using a 0.5 μl solution of 4% methylene blue in saline), we made recordings from neurons within a radius 0.3–0.5 mm from the cannula tips to better get the effects of the infused drugs.

Slice preparation and electrophysiology. Slice preparation and whole-cell patch-clamp recordings were conducted as reported previously (Huang and Hsu, 2006; Huang et al., 2007). In brief, rats were anesthetized with halothane and decapitated with a guillotine, and coronal slices

(250 μm thick) containing the prelimbic area of the mPFC (2.0–3.5 mm from the bregma) (Sherwood and Timiras, 1970) were prepared using a vibrating microtome (VT1000S; Leica, Nussloch, Germany). The slices were placed in a holding chamber of aCSF oxygenated with 95% O_2-5% CO_2 and kept at room temperature for at least 1 h before recording. The composition of the aCSF solution was as follows (in mM): 117 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 25 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, and 11 glucose at pH 7.3–7.4, and equilibrated with 95% O_2-5% CO_2. For whole-cell patch-clamp recording, one slice was transferred to a recording chamber of standard design and fixed at the glass bottom of the chamber with a nylon grid on a platinum frame. The chamber consisted of a circular well of low volume (1–2 ml) and was perfused constantly at 32.0 ± 0.5°C with a rate of 2–3 ml/min. After a high resistance seal (>2 GΩ before breaking into whole-cell mode) was obtained, suction was applied lightly through the pipette to break through the membrane. The cell was then maintained at −70 mV for several minutes to allow diffusion of the internal solution into the cell body and dendrites. Recordings were made using a patch clamp amplifier (Axopatch 200B; Molecular Devices, Union City, CA) under and infrared-differential interference contrast microscope. Electrical signals were low-pass filtered at 2 kHz and digitized at 10 kHz using a 12 bit analog-to-digital converter (Digidata 1320; Molecular Devices). An Intel (Acer, Taipei, Taiwan) Pentium-based computer with pCLAMP software (version 8.0; Molecular Devices) was used for on-line acquisition and off-line analysis of the data. For measurement of synaptically evoked EPSCs, a bipolar stainless-steel stimulating electrode was placed in layer V, 40–60 μm away the apical dendrites of the recorded neurons to stimulate excitatory afferents at 0.05 Hz, and the superfusate routinely contained bicuculline methiodide (1 μM) to block inhibitory synaptic responses. The strength of synaptic transmission was mostly quantified by measuring the initial rising slope of EPSC (2 ms period from its onset; picoampere/millisecond), which contains only a monosynaptic component. The electrode resistance was typically 3–6 MΩ. The composition of intracellular solution was as follows (mM): 115 K-gluconate, 20 KCl, 10 HEPES, 2 MgCl$_2$, 0.5 EGTA, 3 Na$_2$ATP, 0.3 Na$_3$GTP, 5 QX-314, and sucrose to bring osmolarity to 290–295 mOsM and pH to 7.3. Series resistance and input resistance were monitored on-line throughout the whole-cell recording with a 5 mV depolarizing step given after every afferent stimulus and data were discarded if resistance changed by >20%.

Biochemical measurement of surface-expressed receptors. The procedure was similar to that described previously (Zho et al., 2002). mPFC slices were incubated with aCSF containing 1 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) for 20 min on ice. Unreacted biotinylation reagent was washed once with ice-cold aCSF and quenched by two successive 20 min washes in aCSF containing 100 mM glycine, followed by two washes in ice-cold TBS (50 mM Tris, pH 7.5, 150 mM NaCl). The slices were then lysed in ice-cold homogenate buffer (50 mM Tris-HCl, 100 mM NaCl, 15 mM sodium pyrophosphate, 50 mM sodium fluoride, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 2 mM benzamidine, 60 μg/ml aprotinin, and 60 μg/ml leupeptin) and ground with a pellet pestle. Samples were sonicated and spun down at 14,000 × *g* for 15 min at 4°C. A total of 20 μg of protein was removed to measure total metabotropic glutamate receptor 2 (mGluR$_2$). For surface protein, 150 μg of protein was incubated with 100 μl of 50% Neutravidin agarose (Pierce) for 2 h at 4°C to measure the isolated biotinylated proteins. After the Neutravidin agarose was washed five times with homogenate buffer, bound proteins were eluted with SDS sample buffer by boiling for 15 min. Total protein and isolated biotinylated proteins were analyzed by quantitative immunoblotting with monoclonal anti-mGluR$_2$ C-terminal (1:1500; Abcam, Cambridge, UK). It was then probed with HRP-conjugated secondary antibody for 1 h and developed using the ECL immunoblotting detection system. Immunoblots were quantified by densitometric measurement.

Drug treatment. Cocaine HCl (15 mg/kg), (*R*)-(+)-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH23390, 0.5 mg/kg), raclopride (0.5 mg/kg), and 8-[5-(2,4-dimethoxy-5-(4-trifluoro-methylphenylsulfonamido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4.5] decane-2,4-dione (RS102221, 2 mg/kg) were dissolved in 0.9% NaCl and administered intraperitoneally. Drug doses were selected on the basis of

published studies (Dong et al., 2004, 2005; Conductier et al., 2005; Huang et al., 2007). All drugs used in *in vitro* experiments were applied by manually switching the superfusate. Drugs were diluted from stock solutions just before application. (1*R*,4*R*,5*S*,6*R*)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268), forskolin (FSK), Bis-I, Bis-V, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), phorbol-12,13-dibutyrate (PDBu), 4α-phorbol-12,13-didecanoate (4αPDD), 2-chloro-*N*[6]-(3-Iodobenzyl)-adenosine-5′-*N*-methyluron-amide (Cl-IB-MECA), MRS1220, and 4-(3-butoxy-4-methoxy-phenyl)methyl-2-imidazolidone (RO201724) were dissolved in DMSO stock solutions and stored at −20°C until the day of experiment. Other drugs used in this study were dissolved in distilled water. The concentration of DMSO in the perfusion medium was 0.1%, which alone had no effect on basal synaptic transmission (Huang and Hsu, 2006). LY379268, SCH23390, raclopride, RS102221, 2*S*, 2′*R*, 3′*R*)-2-(2′, 3′-dicarboxycyclopropyl)glycine (DCG-IV), FSK, Bis-I, IB-MECA, MRS1220, and RO201724 were purchased from Tocris Cookson (Bristol, UK); cocaine HCl, probenecid, N6-cyclopentyladenosine (CPA), and bicuculline methiodide were obtained from Sigma (St Louis, MO); adenosine-3′,5′- cyclic monophosphorothioate, 8-bromo-, Sp- isomer (Sp-8-Br-cAMPS), PDBu, 4αPDD, and Bis-V were purchased from Calbiochem (La Jolla, CA).

Statistical analysis. The data for each experiment were normalized relative to baseline, and are presented as mean ± SEM. Numbers of experiments are indicated by *n*. The significance of the difference between the mean was calculated by paired or unpaired Student's *t* test. Probability values (*p*) of <0.05 were considered to represent significant differences.

Results

Repeated cocaine exposure blocks DCG-IV-LTD

In the first series of experiments, we examined whether repeated cocaine administration affects the induction of LTD at mPFC excitatory synapses. The effectiveness of the repeated cocaine treatment on rats was indicated by their heightened locomotor sensitivity to cocaine (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). To induce LTD, we used a brief bath application of DCG-IV protocol that has been shown to effectively induce LTD in rat mPFC *in vitro* (Otani et al., 1999, 2002). As reported previously, application of DCG-IV (0.2 μM) for 10 min reliably induced a robust LTD of EPSCs of layer V pyramidal neurons termed DCG-IV-LTD. The slope of EPSCs was depressed to 76.5 ± 4.9% (*n* = 10; *p* < 0.05) measured 30 min after washout of DCG-IV in slices from rats treated with saline for 5 d (Fig. 1*A*). In an attempt to assign the specificity of the effect of DCG-IV on group II mGluRs at inducing LTD, a more selective group II mGluR agonist LY379268 (Monn et al., 1999) was compared. As DCG-IV did, application of LY379268 (50 nM) for 10 min also resulted in a substantial LTD of EPSCs. The slope of EPSCs was depressed to 53.5 ± 4.5% (*n* = 8; *p* < 0.05) measured 30 min after washout of LY379268 in slices from rats treated with saline for 5 d (supplemental Fig. S2*A*, available at
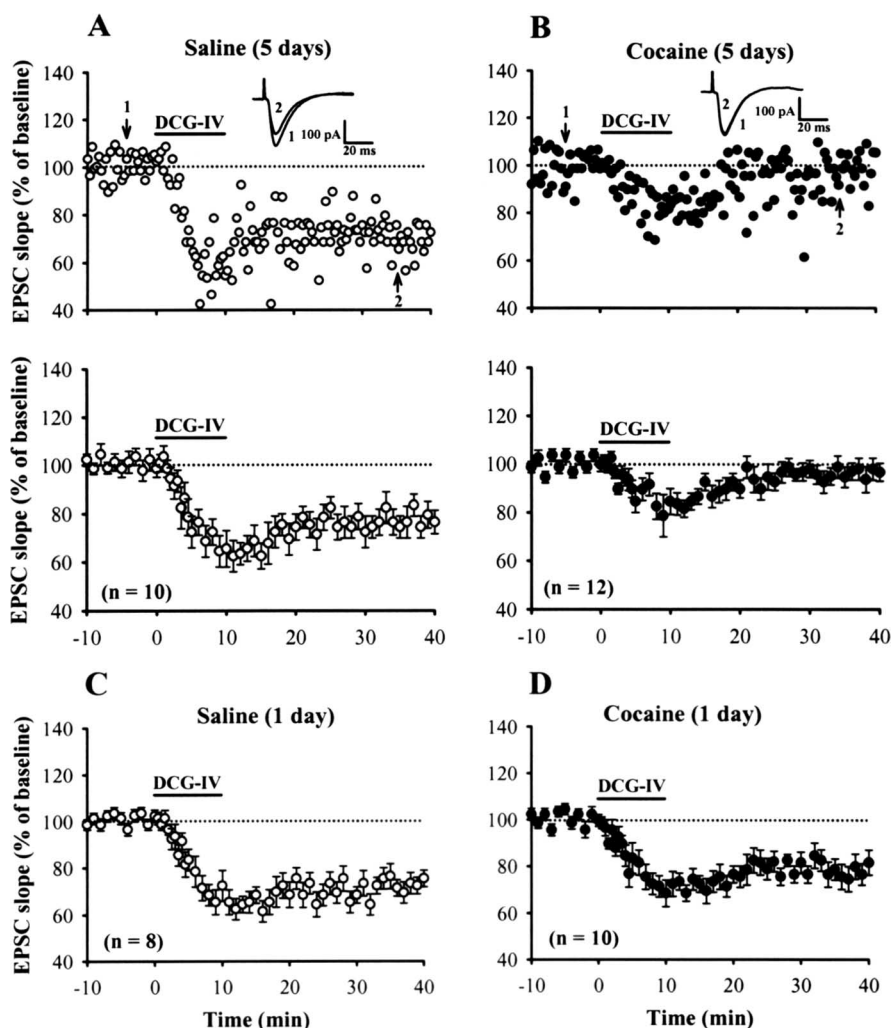
Figure 1. Repeated cocaine administration inhibits the induction of DCG-IV-LTD in mPFC layer V pyramidal neurons. *A*, *B*, Example (top) and summary (bottom) of normalized EPSC slope before and after a brief application of DCG-IV (0.2 μM) for 10 min in slices from rats treated with saline (*A*) or cocaine (*B*) for 5 d. *C*, *D*, Summary of experiments showing the effect of DCG-IV on the EPSC slope in slices from rats treated with a single dose of saline (*C*) or cocaine (*D*) for 1 d. Representative traces of EPSCs were taken at the time indicated by number. The total number of neurons examined is indicated by *n*. Horizontal bar denotes the period of delivery of DCG-IV. The dashed line represents 100%. Error bars indicate SEM.

www.jneurosci.org as supplemental material). Both DCG-IV- and LY379268-induced LTD were completely blocked by bath application of either MCPG (0.5 mM), a nonselective antagonist of group II mGluR, or MSOPPE (100 μM), a more selective antagonist of mGluR₂, suggesting that they are mainly mediated by the activation of group II mGluRs (data not shown). In contrast, the induction of LTD by DCG-IV or LY379268 was impaired in slices from rats treated with cocaine for 5 d (DCG-IV, 96.5 ± 3.6%, *n* = 12; LY379268, 82.7 ± 4.7%, *n* = 8; *p* < 0.05 when compared with slices from 5 d saline-treated rats) (Fig. 1*B*, supplemental Fig. S2*B*, available at www.jneurosci.org as supplemental material). No change in the extent of DCG-IV-LTD was observed in slices from rats that were given a single injection of saline (75.3 ± 3.8%; *n* = 8; *p* < 0.05) or cocaine (79.4 ± 5.3%; *n* = 10; *p* < 0.05). These results suggest that repeated but not single cocaine administration impairs the induction of group II mGluR-mediated LTD in mPFC layer V pyramidal neurons. Because DCG-IV could effectively induce group II mGluR-mediated LTD at mPFC excitatory synapses, we chose this protocol to examine the mechanisms in-
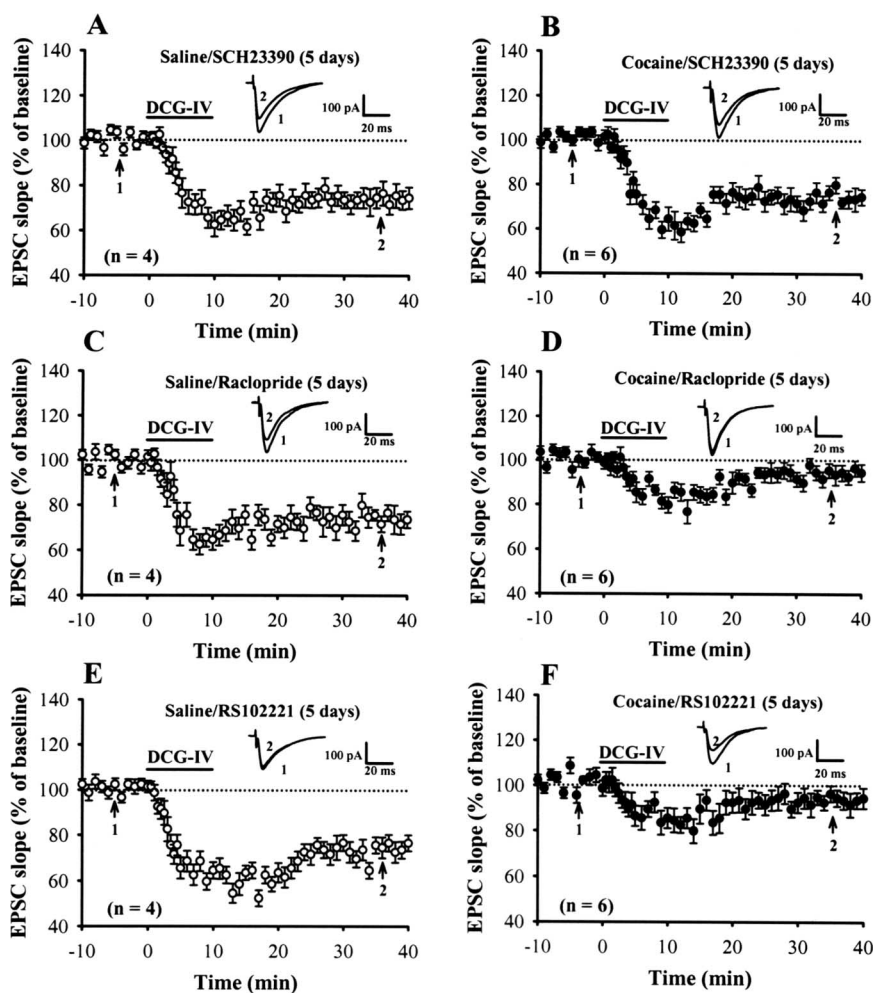
Figure 2. Repeated cocaine administration impairs DCG-IV-LTD induction via the activation of D_1-like dopaminergic receptors. *A*, *B*, Summary of experiments showing the induction of DCG-IV-LTD in slices from rats receiving SCH23390 (0.5 mg/kg) 15 min before saline (*A*) or cocaine (*B*) injection for 5 d. *C*, *D*, Summary of experiments showing the induction of DCG-IV-LTD in slices from rats receiving raclopride (0.5 mg/kg) before the saline (*C*) or cocaine (*D*) injection for 5 d. *E*, *F*, Summary of experiments showing the induction of DCG-IV-LTD in slices from rats receiving RS102221 (2 mg/kg) before the saline (*E*) or cocaine (*F*) injection for 5 d. The superimposed EPSCs in the inset illustrate respective recordings from example experiments taken at time indicated by number. Error bars indicate SEM.

volved in processes by which repeated cocaine treatment impairs the induction of LTD.

To examine whether repeated cocaine exposure causes a decrease in the sensitivity of group II mGluRs and thereby leads to an apparent impairment of DCG-IV-LTD, the concentration–response relationship for DCG-IV was examined. As shown in supplemental Figure S3 (available at www.jneurosci.org as supplemental material), the concentration–response curves for DCG-IV-induced acute synaptic inhibition and LTD were shifted to the right in slices from cocaine-injected rats compared with saline-injected rats after 5 d of treatment. The maximal inhibitory effect of DCG-IV on EPSCs was also significantly reduced after 5 d of cocaine treatment. These results suggest that repeated cocaine exposure reduces group II mGluR responsiveness at mPFC excitatory synapses.

How might repeated cocaine administration reduce group II mGluR responsiveness in mPFC layer V pyramidal neurons? A straightforward hypothesis is that repeated cocaine administration stimulates postsynaptic endocytotic removal of group II mGluRs from the plasma membrane and thereby leads to an

apparent abolition of DCG-IV-LTD. To test this hypothesis, we made surface biotinylation to measure levels of surface group II mGluRs in mPFC slices. However, we found no significant difference between the 5 d cocaine- and saline-treated rats in the surface and total expression of $mGluR_2$ in the mPFC (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). Thus, durable alterations of the levels of group II mGluRs after *in vivo* exposure to cocaine are unlikely to account for the blockade of DCG-IV-LTD observed in the mPFC of cocaine-treated rats.

Repeated cocaine exposure blocks DCG-IV-LTD via activation of D_1-like receptors

Considering that cocaine blocks the dopamine reuptake transporter and, thus, acutely increases local dopamine concentrations in brain areas receiving dopaminergic inputs (Koe, 1976; Hyman, 1996), it is therefore possible that activation of dopaminergic receptors in critical brain areas is required for the aforementioned cocaine-induced impairment of DCG-IV-LTD induction. Based on gene sequence and pharmacological profile, dopaminergic receptors could be divided into two families, D_1- and D_2-like receptors (Civelli et al., 1993). To examine the role of these dopaminergic receptor subtypes, we administered specific D_1- or D_2-like receptor antagonists before cocaine injection. As shown in Figure 2, *A* and *C*, neither the D_1 receptor antagonist SCH23390 (0.5 mg/kg) nor the D_2 receptor antagonist raclopride (0.5 mg/kg) had a significant effect on DCG-IV-LTD when administered with saline (SCH23390, 74.5 ± 4.8%, $n = 4$; raclopride, 72.5 ± 4.3%, $n = 4$). When coadministered with cocaine, SCH23390, but not raclopride, DCG-IV-LTD was not blocked in slices from 5 d cocaine-treated rats (SCH23390, 72.6 ± 3.9%, $n = 6$; raclopride, 94.5 ± 3.8%, $n = 6$) (Fig. 2*B*,*D*).

It has been shown that administration of SCH23390 into the mPFC prevents the expression of 3,4-methylenedioxymethamphetamine-induced behavioral sensitization through the activation of $5-HT_{2C}$, but not of D_1-like receptors (Ramos et al., 2005). To exclude a role for the $5-HT_{2C}$ receptor activation in the development of cocaine-induced impairment of DCG-IV-LTD, we administered specific $5-HT_{2C}$ receptor antagonist RS102221 before cocaine injection. However, in contrast to SCH23390, RS102221 did not significantly affect the impairment of DCG-IV-LTD in slices from 5 d cocaine-treated rats (93.5 ± 4.1%; $n = 6$) (Fig. 2*F*). RS102221 also had no significant effect on the induction of DCG-IV-LTD when coadministered with saline (76.8 ± 3.7%; $n = 4$) (Fig. 2*E*). Together, these findings indicate that the activation of D_1-like receptors is responsible for the inhibitory effect of repeated cocaine administration on DCG-IV-LTD in the mPFC.

Protein kinase C and adenosine A$_3$ receptor activation mediate the effects of cocaine on DCG-IV-LTD

The best-characterized consequence of D$_1$-like receptor stimulation is the activation of adenylyl cyclases, formation of cAMP, and activation of protein kinase A (PKA) (Stoof and Kebabian, 1981). We reasoned that the D$_1$-like receptor/cAMP/PKA signaling pathway could be necessary to the cocaine-induced impairment of DCG-IV-LTD. Because the induction of DCG-IV-LTD also depends on PKA activation (Otani et al., 2002), it is not appropriate for using the PKA inhibitors to directly test this hypothesis. Therefore, we determined the effects of pharmacologically increasing cAMP levels by FSK on the induction of DCG-IV-LTD in slices from control rats. To potentiate levels of FSK-induced synaptic enhancement, the adenosine A$_1$ receptor antagonist DPCPX was coapplied with FSK to block adenosine-induced synaptic inhibition, as reported in the NAc (Manzoni et al., 1998). As we have shown previously (Huang and Hsu, 2006), bath application of FSK (25 μM) plus DPCPX (0.2 μM) produced a rapid and sustained enhancement of EPSCs (165.5 ± 7.9%; $n = 6$) and also blocked the induction of DCG-IV-LTD (supplemental Fig. S5A, available at www.jneurosci.org as supplemental material). Normalizing the responses immediately before DCG-IV application to 100% reveals that the magnitude of LTD induced in the presence of FSK and DPCPX (8.2 ± 3.2%) was significantly less than control LTD (33.5 ± 4.9%). However, contrary results were obtained after treatment with the slices with Sp-8-Br-cAMPS, a membrane permeable and phosphodiesterase-resistant cAMP analog. As shown in supplemental Figure S5B (available at www.jneurosci.org as supplemental material), bath application of Sp-8-Br-cAMPS (100 μM) plus DPCPX (0.2 μM) caused the expected increase in EPSCs (143.5 ± 6.3%) that was strongly inhibited by application of DCG-IV (106.9 ± 5.7%; $n = 4$). The magnitude of LTD induced by DCG-IV in the presence of Sp-8-Br-cAMPs and DPCPX (36.2 ± 6.1%) was similar to control. These results suggest that the elevated cAMP may impair the induction of DCG-IV-LTD in the mPFC through a PKA-independent mechanism.

Because activation of protein kinase C (PKC) has been shown to reduce dramatically the presynaptic inhibitory function of group II mGluRs at many excitatory synapses (Swartz et al., 1993; Tyler and Lovinger, 1995; Kamiya and Yamamoto,
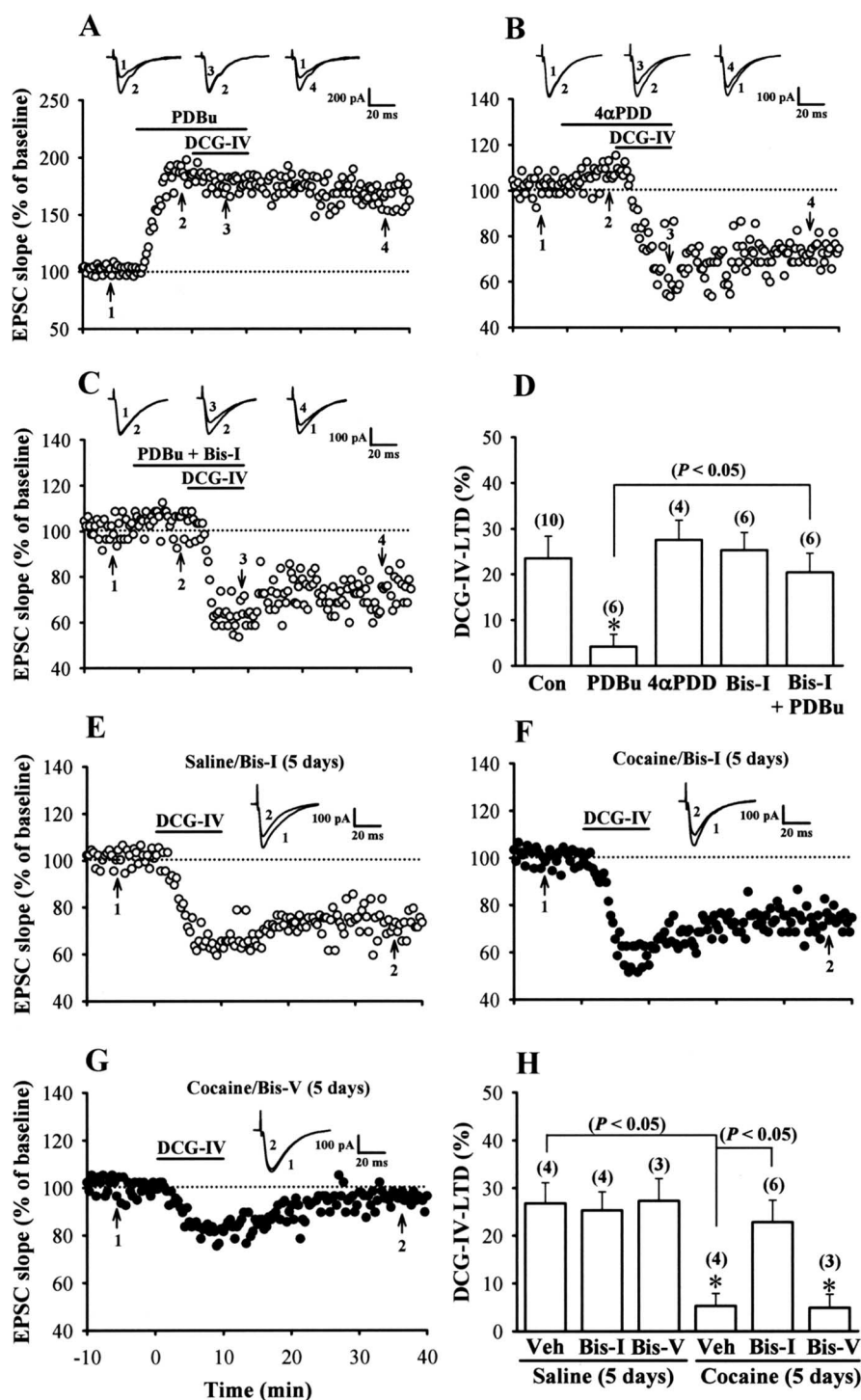
Figure 3. A role for PKC in cocaine-induced impairment of DCG-IV-LTD induction in the mPFC. **A**, Example of experiments in which PDBu (10 μM) was applied before the induction of LTD by application of DCG-IV (0.2 μM) in slices from control rats. **B**, Example of experiments in which 4αPDD (10 μM) was applied before application of DCG-IV. **C**, Example of experiments depicting that coapplication of Bis-I (1 μM) blocked the inhibitory effect of PDBu on DCG-IV-LTD. **D**, Summary of experiments showing the effects of phorbol esters on DCG-IV-LTD induction. The total number of neurons examined is shown in parentheses. The asterisk indicates significant difference ($p < 0.05$) compared with the control group. **E**, **F**, Example of normalized EPSC slope before and after a brief application of DCG-IV (0.2 μM) for 10 min in slices from rats receiving bilateral intra-mPFC infusion of Bis-I (0.4 nmol/side) before each of the five daily saline (**E**) or cocaine (**F**) injections. **G**, Example of normalized EPSC slope before and after application of DCG-IV (0.2 μM) for 10 min in slices from rats receiving bilateral intra-mPFC infusion of Bis-V (0.4 nmol/side) before the cocaine injection for 5 d. **H**, Summary of experiments showing the effects of intra-mPFC infusion of PKC inhibitor before cocaine injection on the development of cocaine-induced impairment of DCG-IV-LTD. Asterisks indicate significant difference ($p < 0.05$) compared with the slices from rats receiving bilateral intra-mPFC infusion of vehicle aCSF (10% DMSO) before the saline injection for 5 d. Error bars indicate SEM.
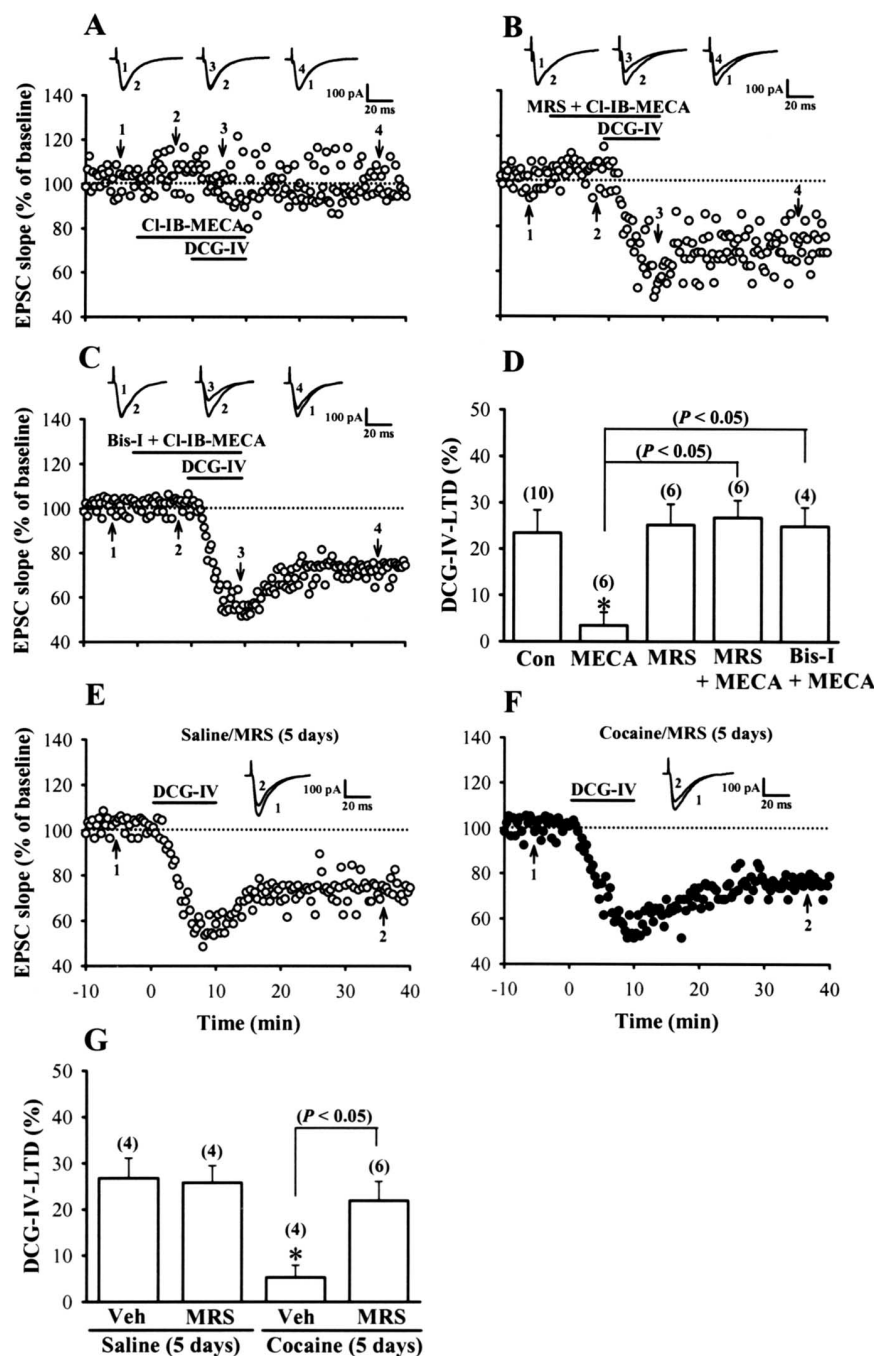
Figure 4. Adenosine A_3 receptor activation mediates the effect of repeated cocaine exposure to impair the induction of DCG-IV-LTD. **A**, Example of experiments in which Cl-IB-MECA (1 μM) was applied before and together with DCG-IV (0.2 μM) in slices from control rats. **B**, Example of experiments showing coapplication of MRS1220 (MRS; 10 μM) blocked the effect of Cl-IB-MECA on DCG-IV-LTD. **C**, Example of experiments showing coapplication of Bis-I (1 μM) blocked the effect of Cl-IB-MECA on DCG-IV-LTD. **D**, Summary of experiments showing the effect of adenosine A_3 receptor agonist and antagonist on the induction of DCG-IV-LTD. The total number of neurons examined is shown in parentheses. The asterisk indicates significant difference ($p < 0.05$) compared with the control (Con) group. **E**, **F**, Example of normalized EPSC slope before and after a brief application of DCG-IV (0.2 μM) for 10 min in slices from rats receiving bilateral intra-mPFC infusion of MRS1220 (0.5 nmol/side) before each of the five daily saline (**E**) or cocaine (**F**) injections. **G**, Summary of experiments showing the effects of intra-mPFC infusion of MRS1220 before cocaine injection on the development of cocaine-induced impairment of DCG-IV-LTD. The asterisk indicates significant difference ($p < 0.05$) compared with the slices from rats receiving bilateral intra-mPFC infusion of vehicle aCSF (10% DMSO) before the saline injection for 5 d. Error bars indicate SEM.

1997; Macek et al., 1998), we explored the possibility that cocaine-induced impairment of DCG-IV-LTD is caused by an increase in PKC activity. As an initial test of this hypothesis, we examined the effects of direct pharmacological activation of PKC

with phorbol esters on the induction of DCG-IV-LTD in slices from control rats. The PKC activator PDBu (10 μM) caused a large and sustained increase in EPSCs (182.3 $\pm$ 8.7%; $n = 6$) and completely abolished the inhibitory effect of DCG-IV at mPFC excitatory synapses (Fig. 3 A,D). To exclude the nonspecific actions of phorbol esters, we also examined the effect of DCG-IV after exposure to an inactive phorbol ester, 4αPDD. Application of 4αPDD (10 μM) for 20 min had no significant effect on EPSCs (105.6 $\pm$ 3.2%; $n = 4$) or the LTD induced by DCG-IV (72.5 $\pm$ 4.3%) (Fig. 3 B,D). Furthermore, the response to PDBu was blocked completely by Bis-I (1 μM), a selective membrane-permeable PKC inhibitor, suggesting that this effect is indeed specific to the activation of PKC (Fig. 3 C,D). If an increase in PKC activity is required for cocaine-induced impairment of DCG-IV-LTD, then preventing PKC activation should reverse the ability of DCG-IV to induce LTD in slices from rats treated with cocaine. To test this prediction, Bis-I (0.4 nmol/side) was bilateral infusion into the mPFC before each of the five daily cocaine injections. We found that Bis-I prevents the cocaine-induced impairment of DCG-IV-LTD (Fig. 3 F). DCG-IV induced a robust LTD in mPFC layer V pyramidal neurons in slices from rats treated with cocaine plus Bis-I for 5 d to a similar extent as that observed for rats treated with saline plus Bis-I for 5 d (Fig. 3 E,F,H). In contrast, the inactive analog Bis-V (0.4 nmol/side) did not significantly affect the effects of cocaine (Fig. 3 G,H). These data are consistent with the hypothesis that the inhibitory effects of repeated cocaine treatment on group II mGluR functions in the mPFC are mediated, at least in part, through a PKC-dependent mechanism.

What is the cellular mechanism that couples repeated cocaine exposure to PKC activation in the mPFC? Because a role for adenosine in the long-lasting changes of synaptic transmission in response to repeated cocaine exposure has been described in the VTA and NAc (Manzoni et al., 1997, 1998) and there is evidence that activation of adenosine A_3 receptor can elicit a PKC-dependent inhibition of mGluR function at the Schaffer collateral–CA1 synapses (Macek et al., 1998), the potential involvement of adenosine A_3 receptor activation in the development of cocaine-induced impairment of DCG-IV-

LTD was investigated. As reported previously (Macek et al., 1998), application of specific adenosine A_3 receptor agonist Cl-IB-MECA (1 μM) for 20 min had no significant effect on baseline synaptic transmission at mPFC excitatory synapses but signifi-

cantly blocked the inhibitory effects of DCG-IV on EPSCs (96.5 ± 3.8%; $n = 6$) in slices from control rats (Fig. 4A,D). The action of Cl-IB-MECA was blocked completely by either MRS1220 (10 μM), a selective antagonist of adenosine A_3 receptor (Fig. 4B,D), or Bis-I (1 μM) (C,D), suggesting this effect is mediated by the activation of adenosine A_3 receptors and PKC-dependent signaling pathway. To better characterize the role of adenosine A_3 receptors in the development of cocaine-induced impairment of DCG-IV-LTD, MRS1220 (0.5 nmol/side) was bilateral intra-mPFC infusion before each of the five daily cocaine injections. We found that MRS1220 prevents the cocaine-induced impairment of DCG-IV-LTD induction (Fig. 4F). DCG-IV induced a stable LTD in mPFC layer V pyramidal neurons in slices from rats treated with cocaine plus MRS1220 for 5 d to a similar extent as that observed for rats treated with saline plus MRS1220 for 5 d (Fig. 4E–G). These results suggest a role of adenosine A_3 receptor-mediated PKC activation in triggering the inhibitory effect of repeated cocaine treatment on the induction of DCG-IV-LTD.

The activation of adenosine A_3 receptors could be mediated via an increase in adenosine subsequent to the rise in cAMP after D_1-like receptor activation. To determine whether the endogenous adenosine tone in the mPFC is upregulated after repeated cocaine administration, we examined the extent of the increased baseline EPSCs induced by the adenosine A_1 receptor antagonist DPCPX (0.5 μM), a widely used protocol to measure endogenous adenosine tone in the hippocampus (Fredholm and Dunwiddie, 1988). The EPSCs were augmented by DPCPX (0.5 μM), an effect that was significantly larger in slices from cocaine-injected rats compared with those from saline-injected rats after 5 d of treatment (saline, 12.3 ± 2.1%, $n = 5$; cocaine, 24.8 ± 3.7%, $n = 7$; $p < 0.05$) (Fig. 5A), but not from rats treated with a single injection of cocaine (1 d) (saline, 9.8 ± 2.3%, $n = 4$; cocaine, 10.6 ± 2.7%, $n = 5$; $p > 0.05$) (Fig. 5B). An increase in the responsiveness of adenosine A_1 receptors is not responsible for the augmented response to DPCPX because the inhibition of EPSCs induced by exogenously applied adenosine A_1 receptor agonist N6-CPA was not changed (supplemental Fig. S6, available at www.jneurosci.org as supplemental material). The EC_{50} for inhibition of the EPSCs was 0.17 μM in slices from cocaine-treated rats and was not significantly different in slices from saline-treated rats (0.16 μM).

The preceding results point to an involvement of the upregulation in the endogenous adenosine tone in mediating cocaine-induced impairment of DCG-IV-LTD. We next wanted to know further the source of endogenous adenosine. There are multiple mechanisms that can lead to an increase in the levels of endogenous adenosine, including an augmented metabolism of cAMP to adenosine or an inhibition of adenosine uptake (Rosenberg et al., 1994). To determine whether the metabolism of cAMP is one primary source of adenosine to mediate the impairment of DCG-IV-LTD after repeated cocaine administration, we examined the induction of DCG-IV-LTD in the presence of the cAMP transporter inhibitor, probenecid, or cAMP-dependent phosphodiesterase inhibitor, RO201724 (Beavo and Reifsnyder, 1990). We found that, in the presence of either probenecid (200 μM) or RO201724 (200 μM), DCG-IV normally induced LTD in mPFC pyramidal neurons in slices from rats treated with cocaine for 5 d (probenecid, 76.5 ± 3.7%, $n = 6$; RO201724, 81.4 ± 4.2%, $n = 6$), to a similar extent as that observed for rats treated with saline for 5 d (probenecid, 72.8 ± 5.1%, $n = 5$; RO201724, 73.6 ± 5.3%, $n = 5$) (Fig. 6A--D). Likewise, application of RO201726 also prevented the forskolin-induced blockade of DCG-IV-LTD (Fig.
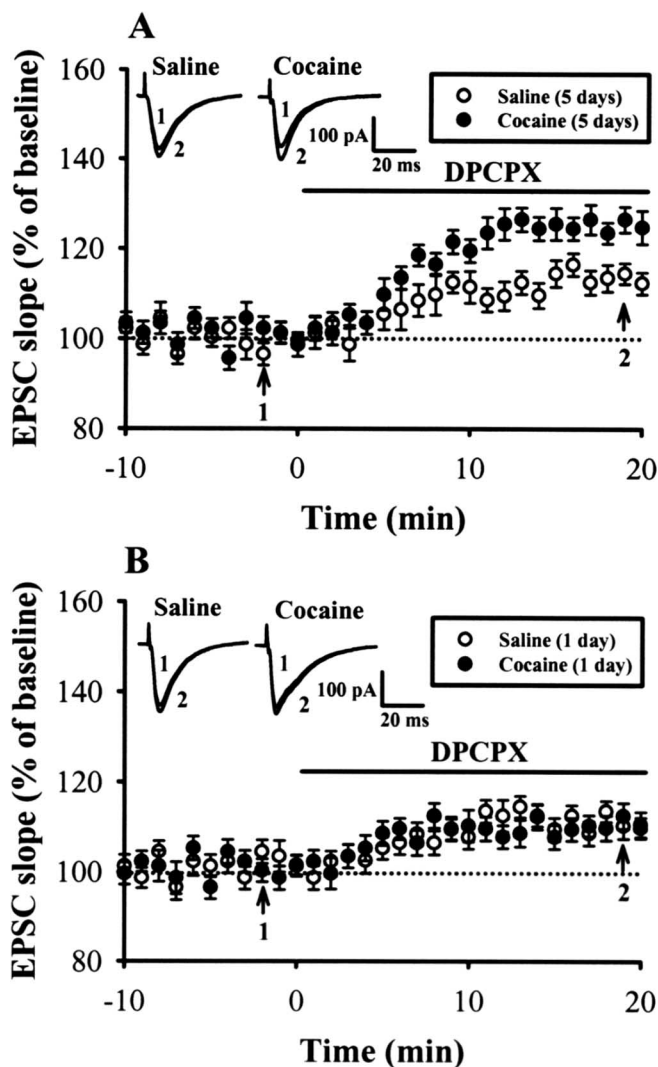

Figure 5. Endogenous adenosine tone is greater in slices from repeated cocaine-treated rats. **A**, Summary of normalized EPSC slope before and after application of adenosine A_1 receptor antagonist DPCPX (0.5 μM) in slices from rats treated with saline or cocaine for 5 d. DPCPX increased EPSC to a significantly greater extent in slices from cocaine-treated rats than from saline-treated rats for 5 d. **B**, Summary of experiments showing the effect of DPCPX on the EPSC slope in slices from rats treated with a single dose of saline or cocaine for 1 d. Error bars indicate SEM.

6E). Together, these data indicate that phosphodiesterase-mediated degradation of cAMP is a major source of adenosine to account for the cocaine or forskolin-induced impairment of DCG-IV-LTD.

Discussion

In the present study, we have demonstrated that repeated *in vivo* exposure to cocaine impairs the induction of group II mGluR-mediated LTD in layer V pyramidal neurons of the mPFC *in vitro*. The molecular mechanism mediating this change may involve an increase of endogenous adenosine tone subsequent to the rise in cAMP after D_1-like receptor activation, which leads to an enhancement of adenosine A_3 receptor-mediated PKC activation and thereby triggers an inhibition of group II mGluR function (Fig. 7). This is the first study, to our knowledge, to correlate the development of behavioral sensitization to cocaine with alterations in LTD of excitatory synaptic transmission within the mPFC.
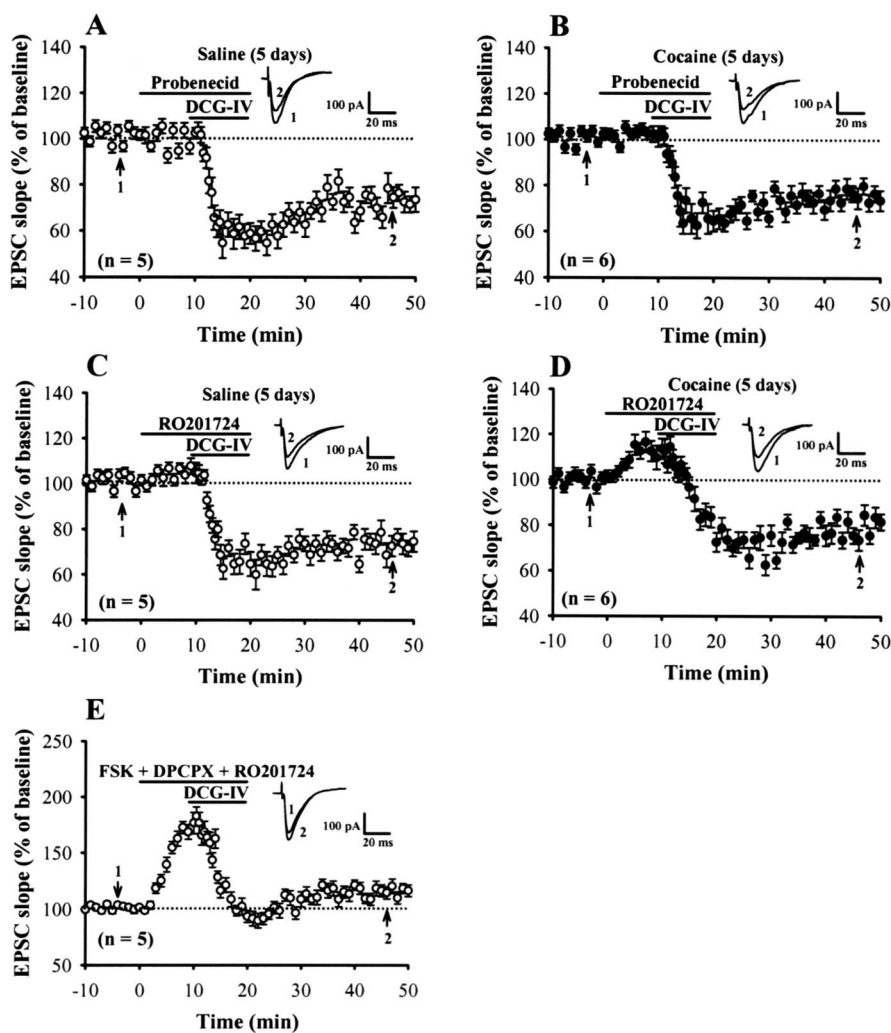
Figure 6. Probenecid and RO201724 have the same effect to reverse the cocaine-induced impairment of DCG-IV-LTD. ***A***, ***B***, Summary of experiments in which probenecid (200 μM) was applied before the induction of LTD by application of DCG-IV (0.2 μM) in slices from rats treated with saline (***A***) or cocaine (***B***) for 5 d. ***C***, ***D***, Summary of experiments in which RO201724 (200 μM) applied before application of DCG-IV in slices from rats treated with saline (***C***) or cocaine (***D***) for 5 d. ***E***, Summary of experiments showing coapplication of RO201724 (200 μM) blocked the inhibitory action of FSK (25 μM) and DPCPX (0.2 μM) on the induction of DCG-IV-LTD. Error bars indicate SEM.

2007). A previous study showed that administration of SCH23390 into the mPFC prevents the expression of 3,4-methylenedioxymethamphetamine-induced behavioral sensitization through the activation of 5-HT$_{2C}$, but not of D$_1$-like receptors (Ramos et al., 2005). Given that SCH23390 also exhibits a high affinity to 5-HT$_{2C}$ receptors ($K_i \approx 0.3$ nM for D$_1$ receptor vs $K_i \approx 6.3$ nM for 5-HT$_{2C}$ receptors) (Millan et al., 2001), it might be thought that the antagonizing effect of SCH23390 on cocaine seen in the present study is in part through its action on 5-HT$_{2C}$ receptors. However, this possibility appears unlikely because the selective 5-HT$_{2C}$ receptor antagonist RS102221 did not significantly affect the cocaine-induced impairment of DCG-IV-LTD.

Although adenosine A$_3$ receptor is expressed in brain neurons (Lopes et al., 2003), the physiological role of this receptor is still poorly understood. The present study has demonstrated that adenosine A$_3$ receptor is responsible for cocaine-induced impairment of DCG-IV-LTD in the mPFC. Results presented here support a link between adenosine A$_3$ receptors and the development of cocaine effects. We found that bilateral infusion of adenosine A$_3$ receptor antagonist MRS1220 into the mPFC before cocaine injection prevents the expression of cocaine-induced impairment of DCG-IV-LTD induction (Fig. 4*F*). This observation adds a novel role of this receptor in the brain. The finding that Cl-IB-MECA disrupted the normal inhibitory response to DCG-IV is consistent with a previous report showing that activation of A$_3$ adenosine receptor induces a PKC-mediated inhibition of mGluR function at the Schaffer collateral–CA1 synapses (Macek et al., 1998). Our findings that Bis-I, the PKC inhibitor, completely prevented Cl-IB-MECA-induced suppression of DCG-IV-LTD in slices from control rats also indicate that adenosine A$_3$ receptors mainly use PKC signaling pathway to mediate the actions of cocaine in the mPFC. In accordance with this notion, we found that bilateral infusion of Bis-I, but not Bis-V, into the mPFC before cocaine injection almost completely blocks the development of cocaine-induced impairment of DCG-IV-LTD (Fig. 3*F*). It is also note worthy that a similar cocaine treatment as the one used in this study produces an increase in the levels of PKCβ1 in the mPFC (Steketee et al., 1998). The mechanism by which PKC inhibits group II mGluR function remains unclear. Given that PKC activation has been reported previously to promote endocytosis of several neurotransmitter receptors including AMPA (Chung et al., 2000), somatostatin $_{2A}$ (Elberg et al., 2002) and GABA$_A$ receptors (Herring et al., 2005), it is possible that PKC activation promotes group II mGluR endocytosis and decreases cell surface expression of the receptor. This seems unlikely, however, because the levels of surface mGluR$_2$, a major receptor sub-
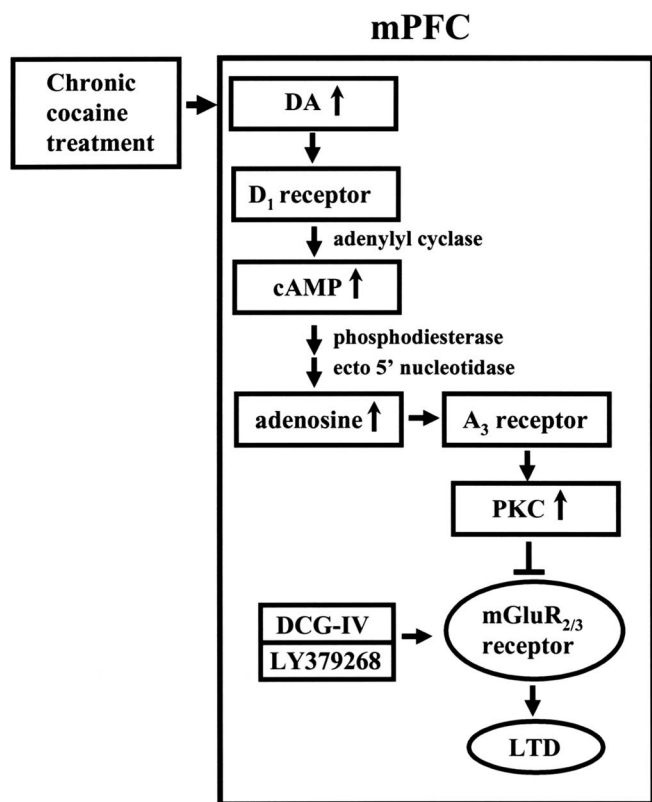
Cocaine blocks the reuptake of dopamine, norepinephrine, and serotonin (Koe, 1976; Heikkila et al., 1979; Hyman, 1996). There is substantial evidence revealing a fundamental role for mesolimbic and mesocortical dopamine systems in the development and expression of cocaine-induced behavioral sensitization, although norepinephrine and serotonin systems have also been proposed to be involved (Cunningham et al., 1992; Drouin et al., 2002). Here, we further show that the effect of repeated cocaine administration on the induction of DCG-IV-LTD in the mPFC is likely mediated by the activation of D$_1$-like receptors. This conclusion is mainly based on the observation that the cocaine-induced impairment of DCG-IV-LTD was prevented after pharmacological blockade of D$_1$-like, but not of D$_2$-like receptors. Although these experiments do not allow us to determine where in the brain D$_1$-like receptors are required to trigger the effects of cocaine, these data are entirely consistent with the our current findings showing that the activation of D$_1$-like receptors contributes to the facilitatory effect of repeated exposure to cocaine on LTP induction in mPFC layer V pyramidal neurons (Huang et al.,

mPFC

Figure 7. Schematic model illustrating the proposed mechanism for the impairment of group II mGluR-mediated LTD induction in the mPFC after repeated cocaine administration. According to this model, repeated cocaine administration increases levels of dopamine (DA) in the mPFC and then leads to increased formation of cAMP via the activation of D_1 receptors. Extracellular cAMP is then metabolized to adenosine by phosphodiesterase and ecto 5′-nucleotidase and acts on the adenosine A_3 receptors. The activation of adenosine A_3 receptors would increase PKC activity and thereby triggers an impairment of group II mGluR function, resulting in an impairment of LTD induced by DCG-IV or LY379268.

type of group II mGluR to mediate the action of DCG-IV seen in this study, remained unaffected after repeated exposure to cocaine. Macek et al. (1998) have proposed another way in which PKC can inhibit mGluR signaling. They found that activation of PKC inhibits presynaptic inhibitory functions of mGluR at several synapses in the hippocampus through a mechanism by an inhibition of coupling of receptors to G-proteins. This could occur by phosphorylation of a site on either receptors or the G-protein. Furthermore, previous work has demonstrated that elevated activator of G-protein signaling 3 (AGS3) in the PFC contributes to the reduced $G_{i\alpha}$-coupled receptor (e.g., mGluR$_{2/3}$ and D$_{2/3}$) signaling during withdrawal from repeated cocaine administration (Bowers et al., 2004). It has been unclear whether the increased content of AGS3 in the PFC after repeated cocaine administration requires the activation of adenosine A_3 receptors and PKC-coupling signaling cascade. Additional biochemical and molecular studies are needed to delineate whether these mechanisms may account for the changes in group II mGluR function observed in the mPFC of repeated cocaine-treated rats.

The estimated affinity of rat adenosine A_3 receptors for the endogenous adenosine ($K_i = 1 \mu M$) is considerably lower compared with that of A_1 ($K_i = 10$ nM) or adenosine A_{2A} receptors ($K_i = 30$ nM) (Jacobson et al., 1995). Under normal conditions, the concentrations of endogenous adenosine in the brain are es-

timated to be 150–200 nM (Dunwiddie and Diao, 1994), which are unlikely to activate a significant fraction of adenosine A_3 receptors. Thus, the cellular processes linked to adenosine A_3 receptor activation may require an elevation in endogenous adenosine levels. In support of this idea, we found that the endogenous adenosine level is augmented after repeated cocaine treatment. The observation that inhibition of cAMP transport or phosphodiesterase-mediated degradation prevented the cocaine-induced impairment of DCG-IV-LTD suggests that cAMP is the main source for the increased adenosine tone in cocaine-treated rats. The mechanisms underlying the increased level of cAMP and its subsequent conversion to adenosine remain unknown. A putative upregulation of D_1-like receptor-driven adenylyl cyclase activity after repeated cocaine exposure, at least in part, could account for the observed result. Indeed, we found that coadministration of D_1-like receptor antagonist SCH23390 with cocaine not only prevented the cocaine-induced impairment of DCG-IV-LTD, but also blocked the augmentation of endogenous adenosine levels observed in the drug-treated rats (C.-C. Huang and K.-S. Hsu, unpublished observations).

We found that pharmacologically increasing cAMP levels with adenylyl cyclase activator forskolin caused a large increase in the synaptic responses and also blocked the induction of DCG-IV-LTD. However, activation of PKA by a nonhydrolyzable cAMP analog Sp-8-Br-cAMPS did not affect transient synaptic depression and LTD induced by DCG-IV. Because inhibition of the conversion of cAMP to adenosine with a phosphodiesterase inhibitor RO201724 prevented the forskolin-induced blockade of DCG-IV-LTD, it seems likely that the impairment of DCG-IV-LTD by forskolin was not caused by a direct effect of cAMP, but to an elevation of extracellular concentration of adenosine.

A previous study has reported that the induction of DCG-IV-LTD in mPFC layer V pyramidal neurons requires postsynaptic activation of PKC (Otani et al., 2002). However, we found that DCG-IV still induced a stable LTD in the presence of PKC inhibitor Bis-I in slices from control rats. The basis for the difference in the present results and those of Otani et al. (2002) is uncertain but could be attributable partly to the use of different stimulation sites to elicit synaptic response (stimulating electrode was placed on layer V vs layer I-II of the prelimbic area), resulting in the activation of different synapses that may vary in their mode of synaptic plasticity. It remains to be determined whether this technical difference has a role in explaining the conflicting results. Furthermore, the lack of effect of Bis-I on DCG-IV-LTD induction is unlikely to be attributable to the invalidation of our treatment protocol to inhibit PKC activity, because the same manipulation can effectively prevent the PDBu-induced blockade of DCG-IV-LTD.

In conclusion, our data indicate that repeated cocaine exposure *in vivo* may impair the induction of group II mGluR-mediated LTD in the mPFC by a PKC-dependent mechanism. Our data also indicate that higher levels of adenosine subsequent to the rise in cAMP after D_1-like receptor activation may activate adenosine A_3 receptors, increase PKC activation, and thereby block group II mGluR function. Such a change may underlie some of the previously reported changes in glutamatergic transmission in the mPFC that occur after repeated cocaine administration (Williams and Steketee, 2004) and could also be involved in behavioral neuroadaptations associated with cocaine sensitization and craving. Targeting of this drug-induced alteration of synaptic plasticity might be a potential therapeutic strategy for the treatment of drug abuse and addiction.

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