Distinct Roles of PDE4 and PDE10A in the Regulation of cAMP/PKA Signaling in the Striatum

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Phosphodiesterase (PDE) is a critical regulator of CAMP/protein kinase A (PKA) signaling in cells. Multiple PDEs with different substrate specificities and subcellular localization are expressed in neurons. Dopamine plays a central role in the regulation of motor and cognitive functions. The effect of dopamine is largely mediated through the cAMP/PKA signaling cascade, and therefore controlled by PDE activity. We used in vitro and in vivo biochemical techniques to dissect the roles of PDE4 and PDE10A in dopaminergic neurotransmission in mouse striatum by monitoring the ability of selective PDE inhibitors to regulate phosphorylation of presynaptic [e.g., tyrosine hydroxylase (TH)] and postsynaptic [e.g., dopamine- and CAMP-regulated phosphoprotein of M, 32 kDa (DARPP-32)] PKA substrates. The PDE4 inhibitor, rolipram, induced a large increase in TH Ser40 phosphorylation at dopaminergic terminals that was associated with a commensurate increase in dopamine synthesis and turnover in striatum. In contrast, the PDE10A inhibitor, papaverine, had no effect on TH phosphorylation or dopamine turnover, but instead robustly increased DARPP-32 Thr34 and Glur1 Ser845 phosphorylation in striatal neurons. Inhibition of PDE10A by papaverine activated cAMP/PKA signaling in both striatonigral and striatopallidal neurons, resulting in potentiation of dopamine D1 receptor signaling and inhibition of dopamine D2 receptor signaling. These biochemical results are supported by immunohistochemical data demonstrating differential localization of PDE10A and PDE4 in striatum. These data underscore the importance of individual brain-enriched cyclic-nucleotide PDE isoforms as therapeutic targets for neuropsychiatric and neurodegenerative disorders affecting dopamine neurotransmission.

Key words: phosphodiesterase; DARPP-32; tyrosine hydroxylase; immunohistochemistry; rolipram; papaverine

Introduction

Dopamine plays a central role in the regulation of motor and cognitive functions. The CAMP/protein kinase A (PKA) signaling cascade is essential for dopamine neurotransmission. Dopamine, acting on D1 receptors, stimulates CAMP/PKA signaling via Gs-mediated activation of adenylyl cyclase (Hervé et al., 2001), whereas dopamine, acting on D2 receptors, inhibits CAMP/PKA signaling via Gi-mediated inactivation of adenylyl cyclase (Stoof and Kebabian, 1981). In mammalian striatum, the synthesis of dopamine by tyrosine hydroxylase (TH) (Harada et al., 1996; Dunkley et al., 2004) and the release of dopamine from nigrostriatal dopaminergic terminals (Zhu et al., 2004; Seino and Shibasaki, 2005) are regulated by the CAMP/PKA signaling cascade. In postsynaptic striatal neurons, DARPP-32, a dopamine- and CAMP-regulated phosphoprotein of M, 32 kDa, is a major target for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the CAMP/PKA signaling cascade (Greengard et al., 1999; Svenningsson et al., 2004). DARPP-32 is expressed in both the D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons (Bateup et al., 2008). Phosphorylation at Thr34 for the...
families according to structural and functional properties (Bender and Beavo, 2006). The brain expression and subcellular localization of PDE families are tightly regulated. Multiple PDEs are expressed in neurons, each with distinct roles in cAMP and cyclic GMP (cGMP) signaling. Several PDE families are expressed in striatum (Menniti et al., 2006). For example, PDE1B is abundantly expressed in striatum (Polli and Kincaid, 1994). Mice lacking PDE1B exhibit increased DARPP-32 phosphorylation at Thr34, indicating that PDE1B normally downregulates cAMP/PKA signaling in striatal neurons (Reed et al., 2002). The role of other PDEs, such as PDE4 and PDE10A, in regulating the DARPP-32 signaling pathway is unknown. PDE10A is highly enriched in striatum (Fujishige et al., 1999; Coskran et al., 2006; Xie et al., 2006). Inhibition of PDE10A by papaverine increases phosphorylation of cAMP-dependent substrates, including the cAMP-response element-binding protein (CREB) and extracellular receptor kinase (ERK), by activating cAMP/PKA signaling (Siuciak et al., 2006b). PDE4B, another striatal-enriched PDE, likely plays a regulatory role in dopaminergic neurotransmission because inhibition of PDE4 by rolipram stimulates dopamine synthesis (Keer et al., 1985; Schofflemeer et al., 1985; Yamashita et al., 1997a). However, the precise role of PDE4 in dopaminergic neurotransmission is currently unknown.

Here, we identify distinct roles for PDE4 and PDE10A in cAMP/PKA signaling in striatongral and striatopallidal neurons and at dopaminergic terminals. PDE10A predominantly regulates DARPP-32 phosphorylation in the same direction as a dopamine D2 antagonist in striatopallidal neurons, whereas PDE4 predominantly regulates TH phosphorylation at dopaminergic terminals. Thus, PDE4 and PDE10A have distinct roles in striatal dopaminergic neurotransmission conferred by their discrete cellular localization.

Materials and Methods
Preparation and incubation of neostriatal slices. Male C57BL/6 mice at 6–8 weeks old were purchased from Japan SLC. All mice used in this study were handled in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and the specific protocols were approved by the Institutional Animal Care and Use Committee of Kurume University School of Medicine. Male C57BL/6 mice were killed by decapitation. The brains were rapidly removed and placed in ice-cold, oxygenated Krebs-HCO3– buffer ([in m)] 124 mM NaCl, 4 KCl, 26 NaHCO3, 1.5 CaCl2, 1.25 KH2PO4, 1.5 MgSO4, and 10 d-glucose, pH 7.4). Coronal slices (350 µm) were prepared using a vibrating blade microtome, VT1000S (Leica Microsystems), as described previously (Nishi et al., 2005). Striata were dissected from the slices in ice-cold Krebs-HCO3– buffer. Each slice was placed in a polypropylene incubation tube with 2 ml of fresh Krebs-HCO3– buffer containing adenosine deaminase (10 µg/ml). The slices were preincubated at 30°C under constant oxygenation with 95% O2/5% CO2 for 60 min. The buffer was replaced with fresh Krebs-HCO3– buffer after 30 min of preincubation. Adenosine deaminase was included during the first 30 min of preincubation. Slices were treated with drugs as specified in each experiment. Drugs were obtained from the following sources: papaverine, 6-choro-2,3,4,5-tetrahydro-1-phenyl-1H-3-benzazepine hydrobromide (SKF81297), R-(-)–7-chloro-oxydans-3-ethyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH23390), and 2- (2-carboxyethyl)phenethylamino-5-N-ethylcarboxamidoadenosine (CGS21680) from Sigma-Aldrich; rolipram, 4-(2-[7-amino-2-(2-furyl)]trizolo[2,3-a][1,3,5]triazin-5-ylamino)ethyl)phenol (2M241385), and 1H- [1,2,4]oxadiazolo[4,3-a]quinolin-1-one (ODQ) from Tocris Bioscience. After drug treatment, slices were transferred to Eppendorf tubes, frozen on dry ice, and stored at –80°C until assayed.

Frozen tissue samples were sonicated in boiling 1% SDS and boiled for an additional 10 min. Small aliquots of the homogenate were retained for protein determination by the BCA protein assay method (Pierce). Equal amounts of protein (40 µg) were loaded onto 4–12% polyacrylamide Bis-Tris gels (#345–0124; Bio-Rad), separated by electrophoresis, and transferred to nitrocellulose membranes (0.2 µm) (Schleicher and Schuell).

Immunoprecipitations of Flag- and Myc-tagged DARPP-32 in neostriatal slices from D1-/DARPP-32–Flag/D2–DARPP-32–Myc mice. D1-/DARPP-32–Flag/D2–DARPP-32–Myc transgenic mice express Flag- and Myc-tagged DARPP-32 under the control of dopamine D1 and D2 receptor promoters, respectively (Bateup et al., 2008). In the striatum, Flag-tagged DARPP-32 was shown to be expressed selectively in D1 receptor-enriched striatonigral neurons, and Myc-tagged DARPP-32 selectively in D2 receptor-enriched striatopallidal neurons. Using antibodies against Flag and Myc tags, we can selectively immunoprecipitate DARPP-32 from D1 receptor- and D2 receptor-expressing neurons and analyze the phosphorylation state of DARPP-32 in a neuronal type-specific manner. In each experiment, six striatal slices were prepared from one mouse, and they were divided into three treatment conditions. In each treatment condition, six slices, collected from three mice (two slices from each mouse), were used for the analysis of DARPP-32 phosphorylation. Six striatal slices were sonicated in 720 µl of immunoprecipitation (IP) lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% SDS, 100 mM okadaic acid, phosphatase inhibitor mixture (#P5726; Sigma-Aldrich), and protease inhibitor mixture (#1187358001; Roche)). After determination of protein concentration, 15 µg of protein was saved for the analysis of DARPP-32 phosphorylation in total striatal homogenate, and the residual homogenates were used for IPs. In each IP from striatal homogenate, 50 µl of washed EZView Red anti-Flag M2 affinity gel (Sigma-Aldrich) and 45 µl of anti-Myc antibody (Novus Biologicals) coupled to magnetic beads (3 µg of Myc antibody for every 5 µl of magnetic beads) (Dynabeads M-280 Tosylactivated; Invitrogen) were added. The homogenate/antibody mixture was gently rotated overnight at 4°C. After the overnight incubation, the Myc magnetic beads were separated from the homogenate/antibody mixture using a magnetic particle concentrator (Invitrogen), and the Flag affinity gels were separated by centrifugation. The Myc magnetic beads and Flag affinity gels were washed with 1× PBS three times. After the final wash, 30 µl of sample buffer was added, and samples were boiled for 2 min.

Flag IP, Myc IP and total striatal samples were loaded onto 4–12% polyacrylamide Bis-Tris gels (Bio-Rad), separated by electrophoresis, and transferred to nitrocellulose membranes (0.2 µm) (Schleicher and Schuell).

Preparation of striatal tissues for analysis of protein phosphorylation in vivo. Mice were injected intraperitoneally with rolipram (10 mg/kg), papaverine (30 mg/kg), or haloperidol (0.1, 0.3, or 1.0 mg/kg) or with saline (5 ml/kg body weight). The vehicle for rolipram contained the following (final concentration): 5% DMSO, 5% Tween 20, 15% polyethylene glycol 400 (PEG 400), and 75% water. The vehicle for papaverine was saline, and the vehicle for haloperidol was acidified saline titrated to pH 5.5. At the indicated time points after injection, the mice were killed by focused microwave cranial irradiation (4.5–5.0 kW for 1.3 s) using a small animal microwave (Muromachi Kikai), which inactivates protein kinases and phosphatases to preserve in vivo levels of protein phosphorylation (O’Callaghan and Sriram, 2004). Striata were dissected from each mouse brain, frozen in liquid nitrogen, and stored at −80°C until assayed.

Frozen samples of brain tissue were processed as described for slices. Equal amounts of protein (15–30 µg) were loaded on 10% polyacrylamide Bis-Tris gels (Bio-Rad), separated by electrophoresis, and transferred to nitrocellulose membranes (0.2 µm) (Schleicher and Schuell).

Immunoblotting. The membranes were immunoblotted using phosphorylation-state-specific antibodies raised against protein phosphotides: phospho-Thr34 DARPP-32, a site phosphorylated by PKA (CC500; 1:4000 dilution); phospho-Ser45 GluR1, a site phosphorylated by PKA (p1:160–845; 1:250 dilution; PhosphoSolutions); phospho-Thr202/Tyr204 ERK (1:2000 dilution; New England BioLabs); phosphoSer40 tyrosine hydroxylase, a site phosphorylated by PKA (AB5935; 1:1000 dilution; Millipore Bioscience Research Reagents); phospho-Ser6 synapsin I, a site phosphorylated by PKA and CaMKI (RU440; 1:6000 dilution; Millipore Bioscience Research Reagents).
dilution). Antibodies generated against DARPP-32 (C24–5a; 1:7500
dilution), GluR1 (E-6; 1:250 dilution; Santa Cruz Biotechnology), ERK
(1:1000 dilution; New England BioLabs), TH (TH-16; 1:10,000 dilution;
Sigma), and synapsin I (G486; 1:5000 dilution), which are not
phosphorylation-state-specific, were used to determine the total amount
of proteins. None of the experimental manipulations used in the present
study altered the total levels of specific phosphoproteins.

The membrane was incubated with a goat anti-mouse or rabbit Alexa
680-linked IgG (1:5000 dilution; Invitrogen) or a goat anti-mouse or
rabbit IRDye800-linked IgG (1:5000 dilution; Rockland). Fluorescence
at infrared wavelengths was detected by the Odyssey infrared imaging
system (LI-COR) and quantified using Odyssey software. In an individ-
ual experiment, samples from control and drug-treated slices were ana-
yzed on the same immunoblot. For each experiment, values obtained for
slices were normalized to values for either the control or the drug-treated
slices, as described in the figure legends. Normalized data from multiple
experiments were averaged and statistical analysis was performed as de-
scribed in the figure legends.

**Immunohistochemistry.** Under deep anesthesia induced with sodium
pentobarbital, male C57BL/6 mice at 6–8 weeks old were perfused rap-
idly through the left ventricle with 50 ml of 4% paraformaldehyde in 0.1
m phosphate buffer, pH 7.2, at room temperature. Serial coronal sections
50 μm in thickness were cut with a vibrating microtome, VT1000S (Leica
Microsystems). Sections were processed for immunohistochemistry us-
using the free-floating method, as described previously (Fukuda et al.,
1996). Sections were incubated with a rabbit anti-PDE4B antibody (sc-
25812; 1:100; Santa Cruz Biotechnology), a rabbit anti-PDE10A anti-
body (101AP; 1:100–500 dilution; FabGennix), a mouse anti-DARPP-32
antibody (C24–5a; 1:20,000 dilution), a mouse anti-flag antibody (M2;
1:2000; Sigma), or a goat anti-Myc antibody (NB600–338; 1:10,000 di-
lution; Novus Biologicals) at 4°C for 7 d. Antibody binding was visual-
ized with a fluorescein isothiocyanate-conjugated donkey anti-rabbit or
mouse IgG (1:100; Jackson ImmunoResearch) and a rhodamine red-
conjugated donkey anti-rabbit or goat IgG (1:100; Jackson ImmunoRe-
search). Sections were mounted in Vectashield (Vector Laboratories)
and examined with a confocal laser-scanning microscope, LSM 5 PASCAL
(Zeiss).

**Analysis of the levels of dopamine and its metabolites in striatal tissue
in vivo.** Mice were injected intraperitoneally with rolipram (10 mg/kg, in
5% DMSO plus 5% Tween 20 plus 15% PEG 400 plus 75% water),
papaverine (30 mg/kg, in saline), or haloperidol (0.3 mg/kg, in acidified
saline titrated to pH 5.5) or with vehicle solution (5 ml/kg body weight).
Thirty minutes after injection, the mice were killed by focused micro-
wave cranial irradiation as described above. Striata were dissected from
each mouse brain, frozen in liquid nitrogen, and stored at −80°C until
assayed.

Dopamine and its metabolites were quantified by high-performance
liquid chromatography with electrochemical detection (HPLC-EC; Wa-
ters). Tissues were homogenized in 300 μl of ice-cold 0.2 m perchloric
acid, containing 1 μm dihydroxybenzylamine as internal standard, and
centrifuged at 10,000 g for 10 min at 4°C. The supernatant was filtered
through a 0.2 μm membrane, and an aliquot (10 μl) was injected from a
temperature-controlled (4°C) automatic sample injector (Waters
717plus Autosampler) connected to a Waters 515 HPLC pump. Cat-
echolamines were separated on a C18 reverse-phase column (LC-18 RP;
Waters SYMMETRY, 25 cm × 4.6 mm; 5 μm), electrochemically de-
tected (Waters 464 Pulsed Electrochemical Detector; range 10 nA, poten-
tial −0.7 V), and analyzed using Millennium software (Waters). The
mobile phase, pH 3.0, for isocratic separation of dopamine consisted of
dibasic sodium phosphate (75 mm), octane sulfonic acid (1.7 mm), ace-
tonitrile (10% v/v), and EDTA (25 μm). Flow rate was maintained at 1
ml/min. Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and ho-
movanillic acid (HVA) standards (0.5–25 pmol) were prepared in 0.2 m
perchloric acid containing dihydroxybenzylamine. Recovery of each ana-
lyte was adjusted with respect to the internal standard and quantified
from a standard curve. The levels of dopamine and its metabolites were
expressed as micrograms per gram of wet tissue.

**Results**

**Effect of a PDE10A inhibitor, papaverine, and a PDE4 inhibitor, rolipram, on DARPP-32 Thr34, GluR1 Ser845, and ERK2 Thr202/Tyr204 phosphorylation in neostriatal slices**

The phosphorylation of DARPP-32 at Thr34 (PKA site), GluR1 at Ser845 (PKA site), and ERK2 at Thr202/Tyr204 is known to play important roles in striatal neurons (Svenningsson et al., 2004; Girault et al., 2007). We therefore examined the effects of rolip-
ram and papaverine on the phosphorylation of these substrates in
neostriatal slices. Treatment of mouse neostriatal slices with pa-
vference for 60 min increased the levels of phospho-Thr34
DARPP-32, phospho-Ser845 GluR1, and phospho-Thr202/
Tyr204 ERK2 maximally at a concentration of 10 μM by seven-
fold, fivefold, and twofold, respectively, with a half-maximal ef-
fect at ~300 nM (Fig. 1A–C, left and center panels). Papaverine at 1
μM induced maximal changes in DARPP-32, GluR1, and ERK2
phosphorylation by 60 min of incubation (Fig. 1A–C, right panels).

Treatment of neostriatal slices with rolipram for 60 min in-
creased the levels of phospho-Thr34 DARPP-32 and phospho-
Ser845 GluR1 at a high concentration of 100 μM by approxi-
mately twofold, but not significantly at lower concentrations
(Fig. 1A, B, left and center panels). In the analysis of the time
course, rolipram at 10 μM slightly increased DARPP-32 Thr34
phosphorylation at 2 min of incubation and GluR1 Ser845 phos-
phorylation at 30 and 60 min. Treatment with rolipram increased
ERK2 Thr202/Tyr204 phosphorylation in a dose- and time-
dependent manner similar to that seen with papaverine (Fig. 1C).

These results clearly demonstrate that the effects of the
PDE10A inhibitor, papaverine, on the phosphorylation of
postsynaptic PKA substrates in striatal neurons, DARPP-32 (p <
0.01 for both dose–response and time course, two-way ANOVA)
and GluR1 (p < 0.01 for both dose–response and time course,
two-way ANOVA), were much larger than those of the PDE4
inhibitor, rolipram.

**Effect of papaverine and rolipram on TH Ser40 and synapsin I
Ser9 phosphorylation in neostriatal slices**

We next examined the effect of papaverine and rolipram on the
phosphorylation of presynaptic PKA substrates, TH at Ser40 and
synapsin I at Ser9, which are known to play an important role in
the synthesis of dopamine (Harada et al., 1996; Dunkley et al.,
2004) and the release of neurotransmitters (Fiumara et al.,
2007). We therefore examined the effects of rolip-
ram and papaverine on the phosphorylation of these substrates in
neostriatal slices. Treatment of mouse neostriatal slices with pa-
vference for 60 min increased the levels of phospho-Ser40
TH and phospho-Ser9 synapsin I maximally at a concentration of 10 μM by threefold and fourfold, respectively, with a half-maximal ef-
fect of ~100 nM (Fig. 2A, B, left and center panels). Rolipram at 10 μM in-
cuced a maximal effect on TH Ser40 and synapsin I Ser9 phosp-
phorylation within 10 min of incubation (Fig. 2A, B, right panels).

Treatment with papaverine at a high concentration of 10 μM
for 60 min increased the levels of phospho-Ser40 TH and phospho-
Ser9 synapsin I by approximately twofold, but not at lower concentrations (Fig. 2A, B, left and center panels). Papave-
rine at 1 μM did not affect TH Ser40 and synapsin I Ser9 phos-
phorylation at any incubation time (Fig. 2A, B, right panels). The
effects of the PDE4 inhibitor, rolipram, on the phosphorylation
of TH (p < 0.01 for both dose–response and time course, two-
way ANOVA) and synapsin I (p < 0.01 for both dose–response and
time course, two-way ANOVA) were much larger than those of the
PDE10A inhibitor, papaverine.

These results suggest that the inhibition of PDE10A by papave-
rine induces activation of cAMP/PKA signaling in medium
spiny neurons, leading to the phosphorylation of DARPP-32 and GluR1, and that the inhibition of PDE4 by rolipram induces activation of cAMP/PKA signaling mainly at presynaptic, dopaminergic terminals, leading to the phosphorylation of TH and synapsin I. ERK2 is likely expressed both in medium spiny neurons and at dopaminergic terminals, and therefore, ERK2 phosphorylation is similarly affected by papaverine and rolipram.

PDE10A regulates dopamine D₁ receptor, dopamine D₂ receptor, and adenosine A₂A receptor signaling in neostriatal neurons
Dopamine D₁ and adenosine A₂A receptors couple to Gₛₐ₉ and stimulate cAMP synthesis by adenylyl cyclase in striatonigral and striatopallidal neurons, respectively (Nishi et al., 1997; Hervé et al., 2001; Yabuuchi et al., 2006). To evaluate the contribution of...
dopamine D₁ and adenosine A₂A receptor signaling to the papaverine-induced increase in DARPP-32 Thr34 phosphorylation, the effect of papaverine was examined in the presence of a dopamine D₁ receptor antagonist, SCH23390, or an adenosine A₂A receptor antagonist, ZM241385 (Fig. 3A). The papaverine-induced increase in DARPP-32 Thr34 phosphorylation was attenuated both by SCH23390 and by ZM241385. However, the papaverine-induced increase was not affected by an inhibitor of soluble guanylyl cyclase, ODQ, indicating that papaverine did not activate cGMP/protein kinase G (PKG) signaling (Siuciak et al., 2006b) coupled to DARPP-32 Thr34 phosphorylation in this slice preparation (Nishi et al., 2005).

We next examined the effect of a dopamine D₁ receptor agonist, SKF81297 (1 μM), and an adenosine A₂A receptor agonist, CGS21680 (5 μM), on DARPP-32 Thr34 phosphorylation in the absence or presence of papaverine (Fig. 3B). The effects of SKF81297 and CGS21680 on DARPP-32 Thr34 phosphorylation were enhanced by papaverine. These results suggest that PDE10A regulates both dopamine D₁ receptor-stimulated cAMP/PKA signaling in striatonigral neurons and adenosine A₂A receptor-stimulated cAMP/PKA signaling in striatopallidal neurons.

Dopamine D₁ receptors are expressed in striatopallidal neurons, couple to Gα, and thereby inhibit cAMP/PKA signaling in striatopallidal neurons (Nishi et al., 1997). Because dopamine D₂ receptors play a central role in the regulation of psychomotor functions by dopamine, the role of PDE10A in dopamine D₂ receptor signaling was examined (Fig. 3C). Treatment with a dopamine D₂ receptor agonist, quinpirole, decreased the level of phospho-Thr34 DARPP-32 to 50% of control, whereas treatment with a dopamine D₂ receptor antagonist, raclopride, did not affect DARPP-32 Thr34 phosphorylation. Treatment with papaverine (10 μM) increased DARPP-32 Thr34 phosphorylation by 14-fold. In the presence of papaverine, quinpirole failed to decrease the level of phospho-Thr34 DARPP-32. These results suggest that the PDE10A inhibitor, papaverine, attenuates the effect of a dopamine D₂ receptor agonist in striatopallidal neurons. The inhibitory effect of quinpirole on TH Ser40 phosphorylation was not attenuated by papaverine at dopaminergic terminals (data not shown), where papaverine has little effect.

PDE4 regulates adenosine A₂A receptor signaling in striatopallidal neurons

A possible role of PDE4 in dopamine D₁ receptor and adenosine A₂A receptor signaling in striatal neurons was examined. Treatment of slices with rolipram (100 μM) slightly increased the level of phospho-Thr34 DARPP-32, but the effect was not statistically significant in this series of experiments (Fig. 4). Both CGS21680 (5 μM) and SKF81297 (1 μM) increased DARPP-32 Thr34 phos-
Phosphorylation by approximately fourfold. Rolipram enhanced the stimulatory effect of CGS21680 on DARPP-32 Thr34 phosphorylation, but not that of SKF81297. These results suggest that PDE4 preferentially regulates adenosine A2A receptor-stimulated cAMP/PKA signaling in striatopallidal neurons, in addition to dopaminergic terminals (Fig. 2).

Expression patterns of PDE10A and PDE4B in striatal neurons

The expression patterns of PDE10A and PDE4B in striatal neurons were analyzed by immunohistochemistry. DARPP-32 was used as a marker of medium spiny neurons (Ouimet et al., 1992). The expression of PDE10A was detected in all DARPP-32-positive striatal neurons (Fig. 5A), and a punctate pattern of PDE10A staining was observed. Strong immunoreactivity of PDE4B was detected in a subset of DARPP-32-positive neurons (Fig. 5B).

To determine the subset of medium spiny neurons that expresses PDE4B, PDE4B expression was analyzed using neostriatal tissues from D1-DARPP-32-Flag/D2-DARPP-32-Myc mice. In these mice, Flag-tagged DARPP-32 is expressed in striatonigral neurons, and Myc-tagged DARPP-32 is expressed in striatopallidal neurons (Bateup et al., 2008). In confirmation of a previous report (Bateup et al., 2008), there was no overlap of the expression of Flag-tagged and Myc-tagged DARPP-32 (Fig. 6A). In this study, we found that the expression of PDE4B was higher in Myc-positive striatopallidal neurons than in Flag-positive striatonigral neurons (Fig. 6B, C).

Regulation of DARPP-32 Thr34 phosphorylation in striatonigral and striatopallidal neurons by papaverine and rolipram

Neuronal type-specific regulation of DARPP-32 Thr34 phosphorylation by papaverine and rolipram was investigated using neostriatal slices from D1-DARPP-32-Flag/D2-DARPP-32-Myc mice. Flag- and Myc-tagged DARPP-32 (D1-Flag and D2-Myc) was immunoprecipitated from D1 receptor-enriched striatonigral and D2 receptor-enriched striatopallidal neurons, respectively, and the phosphorylation states of DARPP-32 at Thr34 in the two types of neurons were analyzed (Fig. 7). Treatment of neostriatal slices from D1-DARPP-32-Flag/D2-DARPP-32-Myc mice with papaverine (10 μM) increased the level of phospho-Thr34 DARPP-32 by approximately sevenfold in total striatal homogenate. Papaverine increased the levels of phospho-Thr34 Flag- and Myc-tagged DARPP-32 by twofold and sixfold, respectively. Treatment of slices with rolipram (100 μM) increased the level of phospho-Thr34 DARPP-32 by ~1.9-fold in total striatal homogenate. Rolipram increased the levels of phospho-Thr34 Flag- and Myc-tagged DARPP-32 by 2.2-fold and 2.6-fold, respectively. We analyzed the relative stoichiometry of the phosphorylation of Flag- and Myc-tagged DARPP-32 at Thr34 in slice preparations under basal conditions, and found that the Thr34 phosphorylation of Myc-tagged DARPP-32 was ~4.5-fold higher than that of Flag-tagged DARPP-32. Taking this stoichiometry into consideration, the phosphorylated level of DARPP-32 at Thr34 is 13.5-fold higher after papaverine treatment, and 5.3-fold higher after rolipram treatment, in the striatopallidal compared with the striatonigral neurons.
Effect of papaverine and rolipram on TH phosphorylation at presynaptic dopaminergic terminals in vivo

The role of PDE10A and PDE4 in the regulation of the phosphorylation state of the presynaptic PKA substrate, TH at Ser40, was examined in intact animals. A single injection of papaverine (30 mg/kg, i.p.) or rolipram (10 mg/kg, i.p.) did not affect the basal level of phospho-Ser40 phosphorylation (Fig. 8A) up to 60 min after intraperitoneal injection. We examined whether either of these phosphodiesterase inhibitors might potentiate the ability of a neuroleptic compound to elevate TH phosphorylation state at Ser40. A dose–response study shown in Figure 8A (inset) revealed that the neuroleptic drug, haloperidol, induced a submaximal increase in TH Ser40 phosphorylation at a concentration of 0.3 mg/kg, i.p. Rolipram (Fig. 8C), but not papaverine (Fig. 8B), enhanced the haloperidol-induced increase in TH Ser40 phosphorylation.

Effect of papaverine and rolipram on GluR1 phosphorylation in striatal neurons in vivo

The role of PDE10A and PDE4 in the regulation of the phosphorylation state of the postsynaptic PKA substrate, GluR1 at Ser845, was examined in intact animals. The basal level of phospho-Ser845 GluR1 was increased maximally by 2.3-fold at 15 min after a single injection of papaverine (30 mg/kg, i.p.). Rolipram (10 mg/kg, i.p.), given as a single injection, induced a much smaller effect on the level of phospho-Ser845 GluR1 at 30 min after injection (Fig. 8D). The effects of papaverine and rolipram were examined in combination with haloperidol. Haloperidol (0.3 mg/kg, i.p.) alone increased GluR1 Ser845 phosphorylation by ~1.5-fold. Papaverine clearly potentiated the haloperidol-induced increase in GluR1 Ser845 phosphorylation, increasing levels to ~3.4-fold (Fig. 8E). In contrast to the effect of papaverine, rolipram induced only a small additional effect on the GluR1 Ser845 phosphorylation, enhancing the haloperidol-induced increase from ~1.4-fold to ~1.7-fold (Fig. 8F). Phosphorylation of DARPP-32 was also analyzed, but changes in DARPP-32 Thr34 phosphorylation were not detected in response to papaverine, rolipram and haloperidol alone, or in combination (data not shown).

These results in vivo demonstrate that the inhibition of PDE4 by rolipram predominantly enhances haloperidol-induced activation of cAMP/PKA signaling at dopaminergic terminals, leading to TH Ser40 phosphorylation, and that the inhibition of
followed by Newman–Keuls test.

slices from D1-DARPP-32-Flag/D2-DARPP-32-Myc homogenate (Homog), Flag-tagged DARPP-32 in striatonigral neurons (D1-Flag), and Myc-tagged DARPP-32, expressed in D2 receptor-enriched striatopallidal neurons, were immunoprecipitated. The figure shows data from total striatal

Effect of papaverine and rolipram on dopamine metabolism in the striatum in vivo

We examined whether the enhancement of haloperidol-induced TH phosphorylation by rolipram reflects a functional effect of the inhibitor on dopamine synthesis and turnover in the striatum in vivo. The effect of the drug on the rate of dopamine metabolism was quantitated by measuring the relative tissue concentrations of dopamine and its major metabolites, DOPAC and HVA in the striatum (Table 1). An increase in the ratio of DOPAC/dopamine and/or HVA/dopamine indicates an increase in striatal dopamine biosynthesis. A single injection of rolipram (10 mg/kg, i.p.) or papaverine (30 mg/kg, i.p.) per se did not affect the tissue content of dopamine, DOPAC, or HVA as determined in untreated slices. Alternatively, there may be functional differences in PDE10A inhibition because immunocytochemical data clearly show PDE10A to be abundantly expressed in their respective neuronal subtypes in the striatum and dopaminergic terminals in the striatum. The inhibition of PDE10A by papaverine activates cAMP/PKA signaling in medium spiny neurons comprising striatopallidal and striatonigral neurons. Both PDE10A and PDE4 have been proposed as therapeutic targets for psychotic disorders (Menniti et al., 2006; Hebb and Robertson, 2007). Elucidation of the distinct roles of PDE10A and PDE4 in dopaminergic neurotransmission reveals the mechanisms by which selective PDE10A and PDE4 inhibitors may exert antipsychotic activity.

Role of PDE10A in striatal neurons

Using both pharmacological and genetic tools, we have shown that PDE10A regulates cAMP/PKA signaling in both striatopallidal and striatonigral neurons. In striatopallidal neurons, PDE10A inhibition by papaverine activated cAMP/PKA signaling by simultaneously potentiating adenosine A2A receptor signaling and inhibiting dopamine D2 receptor signaling. In striatonigral neurons, PDE10A inhibition by papaverine also activated cAMP/PKA signaling, leading to the potentiation of dopamine D3 receptor signaling in striatopallidal neurons and the potentiation of dopamine D1 receptor signaling in striatonigral neurons. Both PDE10A and PDE4 have been proposed as therapeutic targets for psychotic disorders (Menniti et al., 2006; Hebb and Robertson, 2007). Elucidation of the distinct roles of PDE10A and PDE4 in dopaminergic neurotransmission reveals the mechanisms by which selective PDE10A and PDE4 inhibitors may exert antipsychotic activity.

PDE10A by papaverine selectively enhances haloperidol-induced activation of cAMP/PKA signaling in striatal neurons, leading to GluR1 Ser845 phosphorylation.

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The present study suggests that PDE10A exerts preferential effects on cAMP-mediated, relative to cGMP-mediated, signaling in striatal neurons. For example, although PDE10A is reported to hydrolyze cAMP and cGMP with near equal affinity (Bender and Beavo, 2006; Siuciak et al., 2006b), papaverine had no effect on the modulation of cGMP/PKG/phospho-Thr34 DARPP-32 signaling (Fig. 3A). Thus, the PDE10A inhibitor appears to effectively counteract dopamine D2 receptor signaling in striatopallidal neurons and potentiate dopamine D1 receptor signaling in striatonigral neurons mainly via cAMP-mediated effects.

Under in vivo conditions, papaverine stimulates cAMP/PKA signaling, leading to the phosphorylation of GluR1 at Ser845 in striatal neurons (Fig. 8D), as observed in slice preparations. It is
likely that the ability of papaverine to activate cAMP/PKA signaling is suppressed by high dopamine D₂ tone, because the effect of papaverine was enhanced in the presence of a typical antipsychotic, haloperidol (Fig. 8E). Haloperidol treatment has recently been shown to activate cAMP/PKA signaling selectively in striatopallidal neurons by inhibiting dopamine D₂ receptors (Bateup et al., 2008). It is noteworthy that the PDE10A inhibitor and the antipsychotic drug inhibit dopamine D₂ receptor signaling by different mechanisms and act synergistically to affect phosphoproteins.

In agreement with our results, inhibition of PDE10A by papaverine was previously shown to increase the phosphorylation of CREB and ERK by activating cAMP/PKA signaling in the striatum (Siuciak et al., 2006b). In a behavioral analysis, papaverine treatment (Siuciak et al., 2006b; Becker and Grecksch, 2008) or disruption of the PDE10A gene (Siuciak et al., 2006a) reduced spontaneous locomotor activity, phencyclidine-stimulated locomotor activity, and conditioned avoidance responding. Because inhibition of conditioned avoidance responding serves as a sensitive measure of antipsychotic activity of drugs (Wadenberg and Hicks, 1999), PDE10A inhibitors have been proposed as therapeutic reagents for schizophrenia (Hebb and Robertson, 2007; Menniti et al., 2007). The pharmacological profile of the PDE10A inhibitor, papaverine, to counteract dopamine D₂ receptor signaling and potentiate dopamine D₂ receptor signaling, resembles that of atypical antipsychotics, and therefore supports the idea that PDE10A inhibition would be expected to address the symptoms and cognitive deficits of psychosis.

Role of PDE4 at dopaminergic terminals

This study demonstrates an important role for PDE4 in the regulation of cAMP/PKA signaling at dopaminergic terminals in the striatum. Inhibition of PDE4 activity by rolipram increased the state of phosphorylation of TH at Ser40 in neostriatal slices. In contrast, under in vivo conditions, inhibition of PDE4 by rolipram alone was insufficient to induce the phosphorylation of TH at Ser40. Rolipram did elicit a significant induction of TH phosphorylation when coadministered with the antipsychotic agent, haloperidol. The lack of effect of rolipram alone on TH phosphorylation in vivo may be attributable to high dopamine D₂ tone, which would be likely to exist in the intact animal but not in the isolated striatal slice preparation.

The PKA-dependent phosphorylation of TH at Ser40 increases the catalytic activity of TH (Harada et al., 1996; Dunkley et al., 2004), the rate-limiting step in dopamine biosynthesis (Nagatsu et al., 1964). The inhibition of PDE4 and consequent enhancement of TH phosphorylation at Ser40 in the presence of haloperidol was accompanied by an increase in DOPAC/dopamine ratio in the striatum in vivo. This increase in relative DOPAC concentration reflects an increased metabolism of dopa.
Table 1. Effect of rolipram and papaverine on basal and haloperidol-induced levels of dopamine and dopamine metabolites DOPAC and HVA

| DA     | DOPAC | HVA | DOPAC/DA | HVA/DA |
|--------|-------|-----|----------|--------|
| Control | 15.63 ± 0.45 | 0.77 ± 0.05 | 1.12 ± 0.08 | 7.72 ± 0.32 |
| Haloperidol | 12.80 ± 0.89 | 2.90 ± 0.20** | 2.94 ± 0.24** | 22.67 ± 0.52** |
| Rolipram | 18.22 ± 1.33 | 1.05 ± 0.04 | 1.25 ± 0.10 | 6.86 ± 0.24 |
| Papaverine | 15.31 ± 1.38 | 1.05 ± 0.13 | 1.38 ± 0.12 | 9.01 ± 0.18 |
| Haloperidol plus rolipram | 12.72 ± 0.37 | 4.06 ± 0.16***,**,†† & | 2.81 ± 0.14***,**,†† & | 31.96 ± 1.14***,**,†† & |
| Haloperidol plus papaverine | 11.69 ± 1.20 | 2.82 ± 0.23**,§§ & | 2.27 ± 0.24**,§§ & | 24.52 ± 1.04**,§§ & |

Mice were treated with vehicle, rolipram (10 mg/kg) alone, papaverine (30 mg/kg) alone, or in combination with haloperidol (0.3 mg/kg) and killed 30 min later by focused microwave irradiation. Striatum was dissected and analyzed for dopamine (DA) and its metabolites, DOPAC and HVA. Data represent means ± SEM for 4–5 experiments. **p < 0.01 compared with control; ††p < 0.01 compared with rolipram alone; §§p < 0.01 compared with papaverine alone; §§,¶¶p < 0.01 compared with haloperidol alone; one-way ANOVA followed by Newman–Keuls test.

Figure 9. Differential role of PDE10A and PDE4 in striatal neurons and at dopaminergic terminals. This study provides evidence for differential expression and action of PDE10A and PDE4 in the striatum. PDE10A is expressed in two types of striatal neurons: D1 receptor-enriched striatopallidal and D2 receptor-enriched striatonigral neurons. The inhibition of PDE10A by papaverine potentiates the adenosine A2A receptor-induced increase in DARPP-32 phosphorylation, counteracts the dopamine D2 receptor-induced decrease in DARPP-32 phosphorylation in striatopallidal neurons, and potentiates the dopamine D1 receptor-induced increase in DARPP-32 phosphorylation in striatonigral neurons. PDE4 predominantly functions at dopaminergic terminals, and an inhibition of PDE4 by rolipram results in an increase in TH phosphorylation and dopamine synthesis. The inhibition of PDE4 also increases DARPP-32 phosphorylation in striatopallidal neurons, and potentiates the dopamine D1 receptor-induced increase in DARPP-32 phosphorylation in these neurons.

The PDE4 isoform responsible for effects at dopaminergic terminals has yet to be characterized. PDE4A and PDE4D mRNA have been detected in situ hybridization in the substantia nigra (Pérez-Torres et al., 2000). However, a separate immunohistochemical study reported moderate expression of PDE4B and low expression of PDE4D in the same brain region (Cherry and Davis, 1999). Biochemical analysis of TH phosphorylation and dopamine turnover in the striatum from PDE4A, PDE4B, and PDE4D knock-out mice may be required to identify the PDE4 subtype responsible for the regulation of dopamine biosynthesis at dopaminergic terminals observed here.

Role of PDE4 in striatal neurons

PDE4 plays a major role in regulating dopamine synthesis at dopaminergic terminals, and also regulates cAMP/PKA signaling in striatal neurons. The expression of PDE4B at mRNA and protein levels has previously been reported in caudate–putamen (Cherry and Davis, 1999; Pérez-Torres et al., 2000). We used transgenic mice expressing Flag and Myc under the control of dopamine D1 and D2 receptor promoters, respectively, to compare the levels of PDE4B expression in the two major, functionally distinct subpopulations of medium spiny neurons by immunohistochemistry. The expression level of PDE4B was higher in striatopallidal neurons than that in striatonigral neurons.

Consistent with this observation, we found that PDE4 inhibition selectively potentiated cAMP/PKA signaling in striatopallidal neurons. Rolipram treatment augmented phosphorylation of Thr34 DARPP-32 in response to an adenosine A2A receptor agonist, but had no effect on phosphorylation mediated by a dopamine D1 receptor agonist. These results suggest that PDE4 preferentially regulates cAMP/PKA signaling in striatopallidal neurons. Thus, cAMP/PKA signaling in striatopallidal neurons is regulated by at least two PDEs, PDE10A and PDE4, although the impact of PDE4 inhibition is less robust than that of PDE10A inhibition. The increase in cAMP/PKA signaling in striatopallidal neurons elicited by the PDE4 inhibitor, rolipram, would be expected to oppose dopamine D2 receptor signaling in these cells, similar to the effects of the PDE10A inhibitor, papaverine.

The PDE4 inhibitor, rolipram, like the PDE10A inhibitor, papaverine, inhibits dopamine D2 receptor signaling consistent...
with the pharmacological profile of an antipsychotic medication. Interestingly, at the same time, rolipram stimulates dopamine synthesis, indicating that PDE4 inhibition raises dopaminergic tone with mild inhibition of dopamine D2 receptor signaling in the striatum. This pharmacological profile is similar to that of some atypical antipsychotic medications, and may explain the antipsychotic activity of rolipram observed in animal models of schizophrenia, in which the compound rescued amphetamine-induced reductions in auditory-evoked potentials (Maxwell et al., 2004), MK801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate]-induced deficits in latent inhibition (Davis and Gould, 2005), and amphetamine-induced deficits in prepulse inhibition (Kanes et al., 2007). Among PDE4 subtypes, PDE4B is a possible target for rolipram, because the inhibition of conditioned avoidance responding by rolipram was attenuated in PDE4B knock-out mice (Siuciak et al., 2007, 2008). Because the nonselective PDE4 inhibitors including rolipram are known to induce the side effect of nausea and vomiting possibly via inhibition of PDE4D (Robichaud et al., 2002), the development of a PDE4B-selective inhibitor may be necessary for clinical trials in the treatment of psychiatric disorders.

In conclusion, the present study employs pharmacological, genetic, and neuroanatomical approaches to dissect the contribution of PDE4 and PDE10A to cAMP/PKA signaling in the striatum. Our studies reveal that PDE10A predominantly regulates distribution of PDE4 and PDE10A to cAMP/PKA signaling in the striatum, and neuroanatomical approaches to dissect the contribution in the treatment of psychiatric disorders.

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