D₁ Dopamine Receptor Activation Reduces GABA<sub>A</sub> Receptor Currents in Neostriatal Neurons Through a PKA/DARPP-32/PP1 Signaling Cascade

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Department of Physiology and Institute for Neuroscience, Northwestern University Medical School, Chicago, Illinois 60611; Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, New York City, New York 10021; and Medical Research Council/Laboratory of Molecular Cell Biology and Department of Pharmacology, University College, London WC1E 6BT, United Kingdom

Flores-Hernandez, Jorge, Salvador Hernandez, Gretchen L. Snyder, Zhen Yan, Allen A. Fienberg, Stephen J. Moss, Paul Greenberg, and D. James Surmeier. D₁ dopamine receptor activation reduces GABA<sub>A</sub> receptor currents in neostriatal neurons through a PKA/DARPP-32/PP1 signaling cascade. J. Neurophysiol. 83: 2996–3004, 2000. Dopamine is a critical determinant of neostriatal function, but its impact on intrastriatal GABAergic signaling is poorly understood. The role of D₁ dopamine receptors in the regulation of postsynaptic GABA<sub>A</sub> receptors was characterized using whole cell voltage-clamp recordings in acutely isolated, rat neostriatal medium spiny neurons. Exogenous application of GABA evoked a rapidly desensitizing current that was blocked by bicuculline. Application of the D₁ dopamine receptor agonist SKF 81297 reduced GABA-evoked currents in most medium spiny neurons. The D₁ dopamine receptor antagonist SCH 23390 blocked the effect of SKF 81297. Membrane-permeant cAMP analogues mimicked the effect of D₁ dopamine receptor stimulation, whereas an inhibitor of protein kinase A (PKA; Rp-8-chloroadenosine 3’,5’ cyclic monophosphohosphate) attenuated the response to D₁ dopamine receptor stimulation or cAMP analogues. Inhibitors of protein phosphatase 1/2A potentiated the modulation by cAMP analogues. Single-cell RT-PCR profiling revealed consistent expression of mRNA for the β₁ subunit of the GABA<sub>A</sub> receptor—a known substate of PKA—in medium spiny neurons. Immunoprecipitation assays of radiolabeled proteins revealed that D₁ dopamine receptor stimulation increased phosphorylation of GABA<sub>A</sub> receptor β₁/β₃ subunits. The D₁ dopamine receptor-induced phosphorylation of β₁/β₃ subunits was attenuated significantly in neostriata from DARPP-32 mutants. Voltage-clamp recordings corroborated these results, revealing that the efficacy of the D₁ dopamine receptor modulation of GABA<sub>A</sub> currents was reduced in DARPP-32-deficient medium spiny neurons. These results argue that D₁ dopamine receptor stimulation in neostriatal medium spiny neurons reduces postsynaptic GABA<sub>A</sub> receptor currents by activating a PKA/DARPP-32/protein phosphatase 1 signaling cascade targeting GABA<sub>A</sub> receptor β₁ subunits.

INTRODUCTION

Disordered neostriatal dopaminergic signaling is a central determinant of a variety of psychomotor illnesses including Parkinson’s disease, schizophrenia, and drug abuse (Grace et al. 1998; Hornykiewicz 1973; Koob et al. 1997; Wise 1998). Although dopamine is known to modulate voltage-dependent ion channels in neostriatal neurons (e.g., Surmeier et al. 1992, 1995), its regulation of classical neurotransmission is less clearly defined (Calabresi et al. 1993; Cepeda et al. 1998; Kita et al. 1995; Mercuri et al. 1985; Nicola and Malenka 1998; Yan and Surmeier 1997). This is particularly true of GABAergic signaling. Inhibitory GABAergic synaptic input to neostriatal neurons is thought to be entirely of intrinsic origin, arising either from recurrent collaterals of GABAergic medium spiny projection neurons or from GABAergic interneurons (Kita 1993; Wilson and Groves 1980). Only a handful of studies have attempted to determine how dopamine influences this intrastriatal GABAergic pathway. Most of those studies have focused on dopamine’s actions at D₂ dopamine receptors. For example, D₂ dopamine receptor stimulation increases GABA release (Harsing and Zigmond 1997). However, D₁ dopamine receptor agonists appear to be ineffective in modulating GABAergic synaptic potentials in dorsal neostriatal neurons, in spite of the fact that they inhibit GABAergic signaling in the nucleus accumbens (Nicola and Malenka 1998; cf. Calabresi et al. 1993).

At face value, the inability of D₁ dopamine receptor agonists to modulate GABAergic synaptic potentials in medium spiny neurons is surprising. D₁ dopamine receptors are expressed by the majority of medium spiny neurons (Gerfen 1992; Surmeier et al. 1996). These receptors positively couple to adenylyl cyclase, resulting in the stimulation of protein kinase A (PKA) (Stoof and Kebabian 1984; Walaas and Greengard 1991). PKA has been shown to modulate GABA<sub>A</sub> receptor-mediated currents in both heterologous and native expression systems (Smart 1997).

The preferred substrate for PKA in the GABA<sub>A</sub> receptor oligomer is the β subunit. But of the three cloned subunits found in neurons (β₁–3), only β₁ and β₃ are efficiently phosphorylated by PKA. Moreover, the functional consequences of β₁ and β₃ subunit phosphorylation are qualitatively different. In heterologous systems, phosphorylation of β₁ subunits results in diminished GABA<sub>A</sub> receptor currents, whereas phosphorylation of β₃ subunits leads to increased currents (McDonald et al. 1998). This suggests that by controlling the expression of β subunits, their assembly into...
functional receptors or their accessibility, neurons can regulate the consequences of PKA activation. An example of how this type of regulation might work has been reported in the hippocampus where PKA diminished GABAergic miniature inhibitory postsynaptic currents (mIPSCs) in CA1 pyramidal neurons that express β1 subunits, whereas PKA had no effect on mIPSCs in granule cells that express primarily β2 subunits (Poisbeau et al. 1999). Although it is clear that medium spiny neurons express GABA\textsubscript{A} receptors, the contribution of β1, β2, and β3 subunits to these channels has not been studied.

To provide a more thorough examination of their regulation by D\textsubscript{1} dopamine receptors, GABA\textsubscript{A} receptors in acutely isolated, voltage-clamped medium spiny neurons were stimulated with exogenous GABA. These experiments revealed a consistent decrement in GABA-evoked currents after D\textsubscript{1} dopamine receptor stimulation. These observations then were pursued with a combination of molecular, biochemical, and electrophysiological techniques to determine the signaling pathway linking D\textsubscript{1} dopamine receptors to GABA\textsubscript{A} receptors.

**METHODS**

**Electrophysiological methods**

**ACUTE-DISSOCIATION PROCEDURE.** Neostriatal neurons from adult (>4 wk) rats were acutely dissociated using procedures similar to those previously described (Song et al. 1998; Surmeier et al. 1996; Yan and Surmeier 1997). In brief, rats were anesthetized with methoxyflurane and decapitated; brains were removed quickly, iced, and then blocked for slicing. The blocked tissue was cut into 400-μm slices with a Vibroslice (Campden Instruments, London) while bathed in a low-Ca\textsuperscript{2+} (100 μM), N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid] (HEPES)-buffered salt solution [containing (in mM) 140 NaCl, 2 KCl, 4 MgCl\textsubscript{2}, 0.1 CaCl\textsubscript{2}, 23 glucose, and 15 HEPES; pH = 7.4, 300–305 mosm/l]. Slices then were incubated for 1–6 h at room temperature (20–22°C) in a NaHCO\textsubscript{3}-buffered saline bubbled with 95% O\textsubscript{2}-5% CO\textsubscript{2} [which contained (in mM) 126 NaCl, 2.5 KCl, 2 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 26 NaHCO\textsubscript{3}, 1.25 NaHPO\textsubscript{4}, 1 pyruvic acid, 0.2 ascorbic acid, 0.1 N\textsuperscript{2}-nitro-l-arginine, 1 kynurenic acid, and 10 glucose; pH = 7.4 with NaOH, 300–305 mosm/l]. All reagents were obtained from Sigma Chemical (St. Louis, MO). Slices then were transferred into the low-Ca\textsuperscript{2+} buffer and regions of the dorsal neostriatum dissected and placed in an oxygenated Cell-Stri chamber (Wheaton, Millville, NJ) containing pronase (Sigma protease Type XIV, 1–3 mg/ml) in HEPES-buffered Hank’s balanced salt solution (HBSS, Sigma Chemical) at 35°C. Dissections were limited to tissue rostral to the anterior commissure. After 20–40 min of enzyme digestion, tissue was rinsed three times in the low-Ca\textsuperscript{2+}, HEPES-buffered saline and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension then was plated into a 35-mm Lux petri dish mounted on the stage of an inverted microscope containing HEPES-buffered HBSS saline.

In some experiments, cultured neostriatal neurons were used. Cultures were generated as previously described from E18 Sprague-Dawley rat pups (Surmeier et al. 1988). After 3 days in vitro, cultures were transferred to defined media consisting of Neurobasal media supplemented with B-27, Pen/Strep, l-glutamine (0.5 mM; Life Technologies), brain derived neurotrophic factor (BDNF, 50 nM; Promega), and glial derived neurotrophic factor (GDNF, 30 nM; Promega). The media was partially replaced (50%) every 4 days thereafter. Cultures were used for recording 10–14 days after plating.

**WHOLE CELL RECORDINGS.** Recordings of GABA-activated currents employed standard techniques (Yan and Surmeier 1997). Electrodes were pulled from Corning 7052 glass and fire-polished before use. For acutely isolated neurons, the internal solution consisted of (in mM) 180 N-methyl-D-glucamine (NMG), 40 HEPES, 4 MgCl\textsubscript{2}, 5 1,2 bis-(o-aminophenoxy)-ethane-N,N,N,N’-tetraacetic acid (BAPTA), 12 phosphocreatine, 2 Na\textsubscript{2}ATP, 0.2 Na\textsubscript{2}GTP, and 0.1 leupeptin, pH = 7.2–3 with H\textsubscript{2}SO\textsubscript{4}, 265–270 mosm/l. The external solution consisted of (in mM) 135 NaCl, 20 CsCl, 1 MgCl\textsubscript{2}, 10 HEPES, 0.001 TTX, 0.5 BaCl\textsubscript{2}, and 10 glucose, pH = 7.3 with NaOH, 300–305 mosm/l. For cultured neurons, the internal solution consisted of (in mM) 30 CsCl, 80 K\textsubscript{2}SO\textsubscript{4}, 10 HEPES, 6 MgCl\textsubscript{2}, 3.5 BAPTA, 12 phosphocreatine, 2 Na\textsubscript{2}ATP, 0.2 Na\textsubscript{2}GTP, and 0.1 leupeptin, pH = 7.2–3 with H\textsubscript{2}SO\textsubscript{4}, 265–270 mosm/l. The external solution consisted of (in mM) 140 NaCl, 2.8 KCl, 1 MgCl\textsubscript{2}, 10 HEPES, 0.001 TTX, 0.5 CaCl\textsubscript{2}, and 10 glucose, pH = 7.3 with NaOH, 300–305 mosm/l.

Recordings were obtained with an Axon Instruments 200 patch-clamp amplifier that was controlled and monitored with a PC 486 clone running pCLAMP (v. 6.0) with a DigiData 1200 series interface (Axon Instruments, Foster City, CA). Electrode resistances were typically 2–4 MΩ in the bath. After seal rupture, series resistance (4–10 MΩ) was compensated (70–90%) and periodically monitored. Drugs were applied with a gravity-fed “sewer pipe” system. The array of application capillaries (ca. 150 μm ID) was positioned a few hundred micrometers from the cell under study. Solution changes were made by altering the position of the array with a microprocessor-controlled DC drive system (Newport-Klinger, Irvine, CA). Solution changes were complete within <1 s. The membrane potential was held at 0 mV in recordings from acutely isolated neurons; the membrane potential was held at −80 mV in recordings from cultured neurons. GABA (0.1–2,000 μM) was applied briefly (3–5 s) every minute.

Dopamine receptor ligands—dopamine, SKF-81297 and R(+)-SCH-23390 (RBI, Natick, MA)—were made up as concentrated stocks in deoxygenated water containing 0.1% sodium metabisulfite. Solutions were protected from ambient light. Second-messenger agents Sp-8-chloroadenosine 3',5'-cyclic monophosphothioate (Sp-Ci-cAMPS), Rp-8-chloroadenosine 3',5' cyclic monophosphothioate (Rp-Ci-cAMPS), 3',6' dichloro-1-b-D-ribofuranosylbenzimidazole 3',5' cyclic monophosphothioate (Sp-5,6-DCl-cBIMPS), 8-(4-chlorophenylthio) guanosine-3',5' monophosphate (8-pCPT-cGMP) (Biolog Life Sciences, La Jolla, CA) were made up as concentrated stocks in water or dimethyl sulfoxide and stored at −70°C. Stocks were thawed and diluted immediately before use.

**STATISTICAL METHODS.** Data analyses were performed with Axograph (Axon Instruments, ver. 2.0) and SYSTAT (Chicago, IL). Box plots were used for graphic presentation of the data because of the small sample sizes (Tukey 1977). The box plot represents the distribution as a box with the median as a central line and the hinges as the edges of the box (the hinges divide the upper and lower halves of the distributions in half). The inner fences (shown as a line originating from the edges of the box) run to the limits of the distribution excluding outliers (defined as points that are 1.5 times the interquartile range beyond the interquartiles) (Tukey 1977); outliers are shown as asterisks or circles. Nonparametric statistical tests (Kruskal-Wallis ANOVA, Mann-Whitney U test) were used because of the small sample sizes and the uncertain sampling distributions.

**Single-neuron RNA harvest and RT-PCR analysis**

Methods similar to those previously described were used (Song et al. 1998; Tkatch et al. 1998; Yan and Surmeier 1997). Briefly, after recording, neostriatal neurons were lifted up into a stream of control solution and aspirated into the electrode by negative pressure. Electrodes contained ~5 μl of sterile recording solution (see preceding text). The capillary glass used for making electrodes had been autoclaved and heated to 150°C for 2 h. Sterile gloves were worn during the procedure to minimize RNase contamination. After aspiration, the electrode was broken and contents ejected into a 0.5-ml Eppendorf tube containing 5 μl diethyl pyrocarbonate (DEPC)-treated water, 1
\[ \mu l \text{RNasin (28 U/\mu l), 1 \mu l \text{dithiothreitol (DTT)} (0.5 \mu g/\mu l), and 1 \mu l \text{of oligo(dT)} (0.5 \mu g/\mu l) \text{primer. The mixture was heated to 70^\circ C for 10 min and incubated on ice for 1 min. Single-strand cDNA was synthesized from the cellular mRNA by adding SuperScript II RT (1 \mu l, 200 U/\mu l) and buffer (4 \mu l, 5 \times \text{first strand buffer: 250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl}_2, \text{DTT (1 \mu l, 0.1 M) and mixed dNTPs (1 \mu l, 10 mM). The mixture (20 \mu l) was incubated for 50 min in a 42^\circ C \text{water bath. The reaction was terminated by heating the mixture to 70^\circ C for 15 min and then icing. The RNA strand in the RNA-DNA hybrid then was removed by adding 1 \mu l \text{RNase H (2 U/\mu l), and the solution was incubated for 20 min at 37^\circ C. All reagents except for RNasin (Promega, Madison, WI) were obtained from Life Technologies (Grand Island, NY). The cDNA from the reverse transcription (RT) of RNA in single neostriatal neurons was subjected to polymerase chain reactions (PCR) to detect the expression of various mRNAs.}

PCR amplification was carried out with a thermal cycler (MJ Research, Watertown, MA) with thin-walled plastic tubes. Conventional 45-cycle PCR amplification was used for the detection of ChaT mRNA. Reaction mixtures contained 2–2.5 mM MgCl\textsubscript{2}, 0.5 mM of each of the dNTPs, 0.8–1 \mu M primers, 2.5 U Taq DNA polymerase (Promega), 5 \mu l 10X buffer (Promega), and 1–2 \mu l cDNA template made from the single cell RT reaction (see preceding text). The thermal cycling program for these PCR amplifications was: 94°C for 1 min, 58°C for 1 min, and 74°C for 1.5 min. To detect GABA\textsubscript{A} receptor subunit mRNAs (\alpha 1–4, \beta 1–3) in single cells, degenerate and “nested” primers were employed. In the first step, degenerate primers targeting the conserved regions of all the \alpha subunits were mixed with one-fourth of the single cell cDNA template and 30 rounds of amplification were performed. The same procedure was used to amplify \beta subunit cDNA. In the second step, an aliquot (2 \mu l) of diluted (1:10) first-round PCR product was used as the template for 40-cycle PCR with subunit-specific nested primers. These primers were positioned inside the region spanned by the degenerate primers. The primer sets used have been published previously (Surmeier et al. 1996; Yan and Surmeier 1997).

Products were visualized by staining with ethidium bromide and analyzed by electrophoresis in 2% agarose gels. All products were sequenced using a dye termination procedure and found to match the published sequences. Care was taken to ensure that the PCR signal arose from cellular mRNA. In addition to the controls noted above (e.g., primers that span splice sites), negative controls for contamination from extraneous and genomic DNA were run for every batch of neurons. To ensure that genomic DNA did not contribute to the PCR products, neurons were aspirated and processed in the normal manner except that the reverse transcriptase was omitted. Contamination from extraneous sources was checked by replacing the cellular template with water. Both controls were consistently negative in these experiments.

**Preparation, radioactive labeling, and treatment of neostriatal slices**

Male C57BL/6 mice (8–12 wk of age) were killed by decapitation. The brain was removed rapidly from the skull and transferred to an ice-cold surface where it was blocked then mounted to the cutting surface of a Vibratome (TPI). Coronal sections (400 \mu m) of the brain were cut and pooled in 10 ml of ice-cold, oxygenated calcium-free, phosphate-free Krebs bicarbonate buffer containing the following components (in mM): 125 NaCl, 4 KCl, 26 NaHCO\textsubscript{3}, 1.5 MgSO\textsubscript{4}, 0.5 EGTA, and 10 glucose (pH 7.4). Slices of neostriatum were dissected from these coronal sections under a dissecting microscope. The slices were pooled in a dish of cold buffer and transferred individually to 4-ml polypropylene centrifuge tubes containing 2 ml of fresh buffer at 4°C. The Krebs bicarbonate buffer then was replaced with fresh solution. The tubes were connected to an oxygenation manifold supplying a 95%O\textsubscript{2}-5%CO\textsubscript{2} mix and maintained in a 30°C water bath. After 15 min the buffer was replaced with a fresh phosphate-free, oxygenated Krebs bicarbonate buffer containing 1.5 mM CaCl\textsubscript{2} and lacking EGTA. A 2.0-mCi aliquot of [\textsuperscript{32}P]orthophosphoric acid (DuPont NEN; specific activity 8,500–9,120 Ci/mmol) was added to each tube, and the tissue was preincubated for 60 min. The radioactive buffer then was removed, and tissue sections were rinsed twice with 2 ml of fresh buffer. The tissue was incubated in the absence or presence of test substances, as indicated. At the end of the incubation, the buffer was rapidly aspirated, and the tissue slices were immediately frozen in liquid nitrogen and stored at −80°C until assayed.

**Immunoprecipitation and analysis of [\textsuperscript{32}P]phosphate-labeled GABA\textsubscript{A} receptor \beta subunit**

[\textsuperscript{32}P]phosphate-labeled tissue slices were sonicated in 150 \mu l of 1% sodium dodecyl sulfate (SDS) containing NaF (50 mM) and 1 mM EGTA added as phosphatase inhibitors, and a cocktail of protease inhibitors, including 25 mM benzamidine, 100 \mu M phenylmethylsulfoxide, 20 \mu g/ml chymostatin, 20 \mu g/ml pepstatin A, 5 \mu g/ml leupeptin, and 5 \mu g/ml antipain (Peptide International). To this homogenate were added 5 volumes of Buffer A, composed of 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% bovine serum albumen (BSA) and the cocktail of phosphatase and protease inhibitors described above. Aliquots of the homogenate (10 \mu l) were retained for the determination of total [\textsuperscript{32}P]phosphate incorporation into trichloroacetic acid-precipitated protein. A 10 mg aliquot of preswollen Protein A-Sepharose CL-4B (Pharmacia Biochem) was added to each sample and the mixture agitated for 30 min at 4°C. The Sepharose beads were pelleted by centrifugation for 15 s at 2,000 rpm in a tabletop microcentrifuge. The supernatant was transferred to tubes containing 2.5 \mu g of an antisera generated against a peptide sequence contained in both the \beta1 and \beta3 subunits of the GABA\textsubscript{A} receptor. The samples were mixed for 2 h at 4°C, then transferred to fresh 1.5-ml Eppendorf tubes containing 10 mg preswollen Protein A-Sepharose Cl-4B beads and mixed for 1 h at 4°C. The beads were pelleted by centrifugation and washed once with 1 ml of Buffer A; three times with 1 ml of a buffer containing 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, and 0.2% BSA; three times with a buffer containing 20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 0.5% Triton X-100, and 0.2% BSA; and once with 1 ml of a buffer containing 50 mM Tris/HCl, pH 8.0. After the final wash the beads were resuspended in 50 \mu l of a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer composed of 50 mM Tris/HCl (pH 6.7), 10% glycerol, 2% SDS, 10% 2-mercaptoethanol, and 0.01% bromphenol blue. The tubes were vortexed vigorously and the beads were centrifuged. The recovered proteins were separated on 10% acrylamide gels. The gels were dried, and [\textsuperscript{32}P]phosphate incorporation was quantified using a PhosphoImager 400B and ImageQuant software from Molecular Dynamics. Values for [\textsuperscript{32}P]phosphate content were normalized for the total [\textsuperscript{32}P]phosphate incorporated into TCA-precipitable protein.

**RESULTS**

\( D_1 \) dopamine receptor stimulation reduces GABA-evoked currents

The application of GABA (100 \mu M) evoked a rapidly desensitizing current in medium spiny neurons voltage clamped at 0 mV. The current was blocked by bicuculline (100 \mu M) and reversed near the Cl\textsuperscript{−} reversal potential (data not shown), implicating GABA\textsubscript{A} receptors. As shown in Fig. 1A, the \( D_1 \) dopamine receptor agonist SKF 81297 (1 \mu M) reversibly decreased GABA-evoked currents in the majority of medium spiny neurons (20/28) initially sampled (\( n = 20, P < 0.05, \text{Kruskal-Wallis ANOVA} \)). On average, peak whole cell currents evoked by GABA (100 \mu M) were reduced by 18% by...
SKF 81297 (0.1–1 μM). The kinetics of the evoked currents were not noticeably altered by D1 dopamine receptor agonists. Coapplication of the D1 dopamine receptor antagonist SCH 23390 (1 μM) blocked the reduction in evoked currents produced by SKF 81297 (n = 9, P < 0.05, Kruskal-Wallis ANOVA; Fig. 1A). To verify that the D1 receptor-mediated modulation was not a consequence of dissociation, cultured neostriatal neurons also were studied. In these recordings, the Cl− reversal potential was near 0 mV, and the cell was held at −80 mV. As shown in Fig. 1B, the D1 receptor agonist SKF 81297 (1 μM) also reversibly reduced the inward currents evoked by GABA (100 μM) in these neurons. More than one-half of the cultured neurons responded to SKF 81297 (11/17). In the responsive subset, SKF 81297 reduced the bicuculline-sensitive GABA-evoked currents by an average of 22 ± 4% (mean ± SD).

Activation of D1 dopamine receptors in medium spiny neurons leads to the stimulation of adenyl cyclase and the elevation of cytosolic cyclic AMP (cAMP) (Stoof and Kebarian 1984). If the modulation of GABA receptors by D1 dopamine receptors was mediated by stimulation of adenyl cyclase, exogenous application of membrane permeant cAMP analogues should mimic the receptor-driven modulation. To test this hypothesis, Sp-Cl-cAMPS (100 nM) was perfused during whole cell recording. As shown in Fig. 2A, Sp-Cl-cAMPS decreased GABA-evoked currents in a manner similar to the D1 dopamine receptor agonist (n = 12, P < 0.05, Kruskal-Wallis ANOVA). The median decrease in peak evoked current was similar (22%) to that of SKF 81297 (Fig. 2B). The membrane permeant cGMP analogue 8-pCPT-cGMP (100 μM) had no effect on GABA-evoked currents, arguing that the modulation was PKA specific (n = 7, P > 0.05, Kruskal-Wallis ANOVA).
A major cellular target of cAMP is the regulatory subunit of PKA. To determine whether the cellular effects of the D1 agonist and cAMP analogues were mediated by PKA, the membrane permeant PKA inhibitor Rp-Cl-cAMPS was employed. Application of Rp-Cl-cAMPS (5 μM) significantly reduced the response to SKF 81297 (median response = 8%, n = 4, P < 0.05, Kruskal-Wallis ANOVA) and to Sp-Cl-cAMPS (median response = 12%, n = 4, P < 0.05, Kruskal-Wallis ANOVA).

To test for the involvement of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), the membrane permeant inhibitor calyculin A (100 nM) was applied. In the presence of the cAMP analogue Sp-5,6-DCI-cBIMPS (100 nM), calyculin A further reduced the GABA-evoked currents (Fig. 2C), increasing the median modulation to near 38% (n = 4, P < 0.05, Kruskal-Wallis ANOVA, Fig. 2D).

### Medium spiny neurons express β1 subunit mRNA

In the GABA<sub>Δ</sub> receptor oligomer, the preferred targets of PKA are β subunits. In heterologous systems, PKA has been shown to reduce GABA<sub>Δ</sub> receptor-mediated currents by phosphorylating a serine residue (S409) in the β1 subunit (McDonald et al. 1998). On the other hand, phosphorylation of β3 subunits by PKA increases GABA-evoked currents. β2 subunits do not appear to be regulated by PKA in situ (McDonald et al. 1998). In light of these findings, our results predict that medium spiny neurons that are responsive to D1 dopamine receptor agonists should express β1 subunits. To test this hypothesis, single-cell RT-PCR experiments were performed (Yan and Surmeier 1997). Neurons expressing mRNA for the releasable peptide substance P (SP) previously have been shown to express D<sub>1</sub> dopamine receptor mRNA (Gerfen 1992; Surmeier et al. 1996). β1 mRNA was detected in all 11 SP-expressing neurons tested, β3 mRNA was found in 7 of these 11. In contrast, β2 mRNA was not detected in medium spiny neurons. In Fig. 3A, the PCR amplicons from one of these neurons are shown.

D<sub>1</sub> dopamine receptor stimulation increases phosphorylation of GABA<sub>Δ</sub> β1/β3 subunits

To determine whether PKA phosphorylated β subunits in situ, immunoprecipitation experiments were performed. Neostriatal slices from C57BL/6 mice were preincubated with [32P]orthophosphate, washed, and incubated with the adenyl cyclase activator, forskolin (50 μM). GABA<sub>Δ</sub> receptor β subunits were immunoprecipitated using an antibody that recognized both the β1 and β3 subunits. Forskolin treatment increased the phosphorylation of a protein band of ~M<sub>r</sub> 55 kDa by 843 ± 143% (n = 5, P < 0.05 vs. control, Mann-Whitney U test). This protein band was verified to be a β subunit because its appearance was selectively blocked by preabsorption of the antibody with a fusion protein representing a sequence common to both β1 and β3 subunits (Fig. 3B). Incubation of neostriatal slices with the D<sub>1</sub> receptor agonist, SKF81297 increased receptor phosphorylation by 250% (Fig. 3C).

**Disruption of DARPP-32 diminishes the efficacy of D<sub>1</sub> stimulation**

DARPP-32 is a key inhibitor of PP1 in medium spiny neurons that express D<sub>1</sub> dopamine receptors (Greengard et al. 1999). Phosphorylation of DARPP-32 by PKA increases its inhibition of PP1 activity. Deletion mutations of DARPP-32 have been shown to blunt PKA-mediated phosphorylation of...
other cellular proteins (Fienberg et al. 1998). As shown in Fig. 3, D₁ dopamine receptor agonists significantly increased radiolabeled phosphate incorporation into β₁/β₃ subunits in slices from wild-type mice but failed to do so in DARPP-32 knockout mice.

Functional assays then were performed to determine whether the DARPP-32 mutation altered the ability of D₁ agonists to modulate GABA-evoked currents. In wild-type mouse neostriatal neurons, as in the rat neurons described in the preceding text, SKF 81297 (1 μM) decreased GABAₐ receptor-mediated currents. In neostriatal neurons from DARPP-32 knockout mice, 1 μM SKF 81297 reduced currents by a similar amount. However, at lower concentrations of SKF 81297 (200 nM), the modulation of GABA-evoked currents was reduced dramatically in medium spiny neurons from DARPP-32 knockout mice compared with that seen in wild-type neurons (Fig. 4, A and B). A statistical summary of these experiments is shown in Fig. 4C. These results clearly indicate that DARPP-32 increases the efficacy of D₁ dopamine receptor stimulation in modulating GABAₐ receptors.

**DISCUSSION**

D₁ dopamine receptor activation triggers a PKA-dependent modulation of GABAₐ receptors

The data presented demonstrate that D₁ dopamine receptor stimulation reduces GABAₐ receptor-evoked currents in neostriatal medium spiny neurons. This reversible modulation was effectively antagonized by SCH 23390 at D₁ dopamine receptor-specific concentrations. As expected of a Gₛ/olf-linked D₁ dopamine receptor signaling pathway (Stoof and Kebabian 1984), the modulation was mimicked by cAMP analogues. A primary cellular target of cAMP is the regulatory subunit of the PKA holoenzyme. An inhibitor of PKA (Rp-Cl-cAMPS) effectively reduced the consequences of receptor stimulation as well as bath application of cAMP analogues, implicating PKA-mediated protein phosphorylation in the modulation. The enhancement of the D₁ dopamine receptor-mediated modulation by inhibition of PP1/PP2A with calyculin A added strength to the inference that protein phosphorylation was involved. These observations are consistent with previous studies showing PKA-mediated reductions in recombinant and native GABAₐ receptor currents (Heuschneider and Schwartz 1989; Moss et al. 1992; Poisbeau et al. 1999; Porter et al. 1990; Schwartz et al. 1991). As expected from recombinant studies showing that PKA phosphorylation of β₁ subunits reduces evoked currents (McDonald et al. 1998), single-cell RT-PCR experiments revealed that essentially all medium spiny neurons expressed GABAₐ β₁ subunit mRNA. Radiolabeling/immunoprecipitation studies confirmed that β₁/β₃ subunit protein was phosphorylated in striatal neurons after D₁ dopamine receptor stimulation, suggesting that the β₁ subunit was the obligate target of D₁ dopamine receptor-stimulated PKA.

In addition to β₁ subunit mRNA, neostriatal medium spiny neurons frequently had detectable levels of β₃ subunit mRNA. The lower detection probability of β₃ subunit mRNA suggests that it was present in lower copy number than β₁ subunit mRNA (Song et al. 1998; Tkatch et al. 1998). If this difference was preserved at the protein level, β₁-containing GABAₐ receptors would be the predominant oligomer. This inference is in agreement with the reduction
in current amplitudes produced by D₁ dopamine receptor agonists. However, phosphorylation of β3 subunits may be evident in other circumstances. Preliminary experiments using higher concentrations of the D₁ dopamine receptor agonist SKF 81297 (10 μM) or cAMP analogues (e.g., Sp-Cl-cAMPS, 100 μM) have revealed an enhancement of GABA-evoked currents in medium spiny neurons. It is possible that in this circumstance, β3 subunits are phosphorylated by PKA, leading to the increment in current amplitude (McDonald et al. 1998). Subunit-specific phosphoantibodies will be required to test this hypothesis.

**Disruption of DARPP-32 diminishes the efficacy of D₁ dopamine receptor coupling to GABAₐ receptors**

Null mutation of DARPP-32 significantly attenuated the phosphorylation of presumptive β1 subunits after D₁ dopamine receptor stimulation. This observation is in accord with previous studies showing a functional attenuation of D₁ dopamine receptor signaling in DARPP-32 mutants (Fienberg et al. 1998). Phosphorylation of DARPP-32 by PKA effectively inhibits PP1 and dephosphorylation of phosphoproteins targeted by PKA (Greengard et al. 1999). The physiological studies presented here argue that the loss of DARPP-32 lowered the efficacy of the D₁ dopamine receptor agonist but did not reduce the maximum functional effect. These results suggest that at low levels of D₁ dopamine receptor stimulation, phospho-DARPP-32 inhibition of PP1 effectively enhances PKA-mediated phosphorylation of GABAₐ receptor β1 subunits. However, at higher levels of receptor stimulation and PKA activation, PP1 must compete less effectively with PKA at the β1 subunit, making phosphoDARPP-32 inhibition of PP1 a less significant factor. This shift in balance would explain the relatively modest consequences of exogenous PP1/PP2 inhibitors at higher agonist or cAMP analogue concentrations. It recently was demonstrated that cdk5-induced phosphorylation of DARPP-32 at thr-75 converts DARPP-32 into a PKA inhibitor (Bibb et al. 1999). It will be interesting to determine whether this phenomenon contributes to the phenotype of the DARPP-32 knockout mice seen in the present study. The ability of elevated doses of D₁ receptor agonist to overcome the signaling deficit in DARPP-32-deficient mice has been observed with other targets (Fienberg et al. 1998; Greengard et al. 1999). It is unclear whether this feature will generalize to other D₁ dopamine receptor/PKA signaling targets such as L-type Ca²⁺ channels (Surmeier et al. 1995), AMPA receptors (Yan et al. 1999), or N-methyl-d-aspartate receptors (Snyder et al. 1998).

**Reconciliation with previous studies**

How can our results be reconciled with the reported inability of D₁ dopamine receptor stimulation to modulate inhibitory postsynaptic potentials in dorsal striatal neurons (Nicola and Malenka 1998)? There are several potential explanations. One is that the modulation described here depends on a reduction in the affinity of GABAₐ receptors for GABA. If this was the case, the D₁ receptor modulation may not be evident at the saturating concentrations of GABA thought to be achieved at synapses (Jones and Westbrook 1995). Another possibility is that the small sample size in the previously reported study did not include medium spiny neurons that express D₁ dopamine receptors. There were no controls for this possibility, and the D₁ receptor expressing group constitutes only ~60% of all medium spiny neurons (Gerfen 1992; Surmeier et al. 1996). A third possibility is that GABAₐ receptors modulated by the D₁ dopamine receptor signaling cascade are at specific GABAergic synapses or are extrajunctional. In contrast to local synaptic stimulation, bath application of GABA would effectively activate all GABAₐ receptors, providing a more robust (if less physiological) test. In support of this thesis, there is compelling evidence that the subunit composition of GABAₐ receptors can be site specific (Nusser et al. 1996–1998; Somogyi et al. 1996) and that β subunits can serve a targeting function (Connolly et al. 1996). It is also true that studies using different means of activating GABAergic input to medium spiny neurons have led to the conclusion that D₁ dopamine receptors are capable of modulating GABAergic responsiveness at synaptic sites (Calabresi et al. 1993; Kita et al. 1995; Mercuri et al. 1985).

**Functional implications**

The attenuation of intrastriatal GABAergic inhibition of medium spiny neurons could significantly alter their activity patterns and signal processing. In vivo, blockade of ongoing GABAergic inhibition of medium spiny neurons significantly elevates basal activity (Nisenbaum and Berger 1992). D₁ dopamine receptor-mediated suppression of GABAergic inhibition arising from GABAergic interneurons (Kita 1993; Koos and Tepper 1999) would substantially enhance the ability of cortical activity to evoke spiking in medium spiny neurons. D₁ dopamine receptor-mediated modulation of intrinsic voltage-dependent conductances suppresses the responses to cortical inputs at negative membrane potentials but enhances the ability of glutamatergic inputs to evoke activity during sojourns in the depolarized up-state (Calabresi et al. 1987; Galarraga et al. 1997; Hernandez-Lopez et al. 1997; Schiﬀmann et al. 1995; Surmeier and Kitai 1997; Surmeier et al. 1992, 1995). In the depolarized up-state, near spike threshold, D₁ receptor-mediated alterations in perisomatic GABAergic efficacy would have a profound impact on spike generation and timing (Koos and Tepper 1999).

The dopaminergic suppression of GABAₐ receptor currents also may help unravel the paradoxical role of recurrent axon collaterals of medium spiny neurons. These recurrent collaterals form a dense intrastriatal network that is known from anatomic studies to target medium spiny neurons (Wilson and Groves 1980). This network often has been postulated to provide a functionally important feedback inhibition within the striatum (Beiser and Houk 1998; Wicke ns et al. 1995). However, a direct test of this hypothesis has failed to find any evidence of recurrent inhibition mediated by these collaterals (Jaeger et al. 1994). These observations could be reconciled if the response to GABA at these sites was suppressed by ambient D₁ dopamine receptor tone and was only functional in the absence of substantial dopaminergic tone as found in Parkinson’s disease.
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