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D_2 Dopamine Receptors in Striatal Medium Spiny Neurons Reduce L-Type Ca^{2+} Currents and Excitability via a Novel PLCβ1–IP_3–Calcineurin-Signaling Cascade

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In spite of the recognition that striatal D_2 receptors are critical determinants in a variety of psychomotor disorders, the cellular mechanisms by which these receptors shape neuronal activity have remained a mystery. The studies presented here reveal that D_2 receptor stimulation in enkephalin-expressing medium spiny neurons suppresses transmembrane Ca^{2+} currents through L-type Ca^{2+} channels, resulting in diminished excitability. This modulation is mediated by $G_{\beta\gamma}$ activation of phospholipase C, mobilization of intracellular Ca^{2+} stores, and activation of the calcium-dependent phosphatase calcineurin. In addition to providing a unifying mechanism to explain the apparently divergent effects of D_2 receptors in striatal medium spiny neurons, this novel signaling linkage provides a foundation for understanding how this pivotal receptor shapes striatal excitability and gene expression.

Key words: neostriatum; patch clamp; dopamine; neuromodulation; medium spiny neuron; basal ganglia; electrophysiology; single-cell RT-PCR; ion channel; calcium

Disruptions in striatal dopaminergic signaling are thought to underlie a variety of psychomotor disorders including drug abuse, schizophrenia, Tourette's syndrome, and Parkinson's disease (Hornykiewcz, 1973; Meltzer and Stahl, 1976; Sandor, 1993; Nestler and Aghajanian, 1997). In spite of the recognition that alterations in dopaminergic signaling are the basis of these psychomotor disorders, the cellular mechanisms by which dopamine affects striatal function have remained something of a mystery. This is particularly true of D_2 receptors. These receptors are expressed at high levels by several groups of neurons in the striatum, including GABAergic medium spiny neurons that project to the globus pallidus and express enkephalin (Gerfen, 1992; Surmeier et al., 1996).

The prevailing model of the striatum (Gerfen, 1992) suggests that D_2 receptor stimulation suppresses the activity of enkephalin-expressing striatal medium spiny neurons. This inference is based primarily on two indirect observations. First, dopamine-depleting lesions increase striatal expression of enkephalin, a peptide released by medium spiny neurons expressing D_2 receptors (Gerfen, 1992). Second, neuroleptic blockade of D_2 receptors increases striatal expression of immediate early genes and glutamic acid decarboxylase (Chesselet et al., 1998). These changes are taken as evidence of D_2 receptor-mediated suppression of neural activity and gene transcription. However, there are a number of observations that are difficult to reconcile with this model. For example, D_2 receptor stimulation in striatal slices increases the activity of a Ca^{2+}-dependent protein phosphatase (calcineurin) and of Ca^{2+}-dependent mitogen-activated protein (MAP) kinase (Nishi et al., 1997; Yan et al., 1999). D_2 receptor stimulation also is necessary for the induction of synaptic plasticity in the striatum (Calabresi et al., 1992). These studies argue that D_2 receptor stimulation increases, rather than decreases, activity and intracellular Ca^{2+} levels in striatal medium spiny neurons.

Direct measurements of neuronal activity have not provided a means of explaining these seemingly contradictory findings. Because the transcriptional and biochemical events at the heart of the signaling discrepancy are Ca^{2+} dependent, a key question is whether D_2 receptors can directly influence intracellular Ca^{2+} levels. An obvious way this might happen is via the modulation of transmembrane ion channels capable of carrying Ca^{2+} ions into the cytoplasm. One potential target of this type of modulation is the L-type Ca^{2+} channel, a channel that has a privileged association with transcriptional regulators in many neurons (Bading et al., 1993; Graef et al., 1999). Although they can be enhanced by several mechanisms (Viard et al., 1999), in medium spiny neurons L-type Ca^{2+} currents are increased by D_1 receptor stimulation of adenylyl cyclase and protein kinase A (Surmeier et al., 1995). Because the best-described effect of striatal D_2 receptors is inhibition of adenylyl cyclase (Sibley, 1995), D_2 receptor activation should, in principle, reduce L-type currents.

The studies described here were intended to test this hypothesis. They show that indeed D_2 receptor stimulation suppresses L-type Ca^{2+} currents in enkephalin-expressing medium spiny neurons. But, the suppression is not mediated by inhibition of adenylyl cyclase. Rather, D_2 receptor stimulation mobilizes intracellular Ca^{2+} stores via $G_{\beta\gamma}$ activation of a phospholipase Cβ1 pathway, leading to a calcineurin-dependent reduction in L-type currents. This novel signaling linkage establishes a mechanism by which D_2 receptors can suppress spike activity and Ca^{2+}-dependent gene transcription but activate Ca^{2+}-dependent intracellular enzymes.

MATERIALS AND METHODS

Electrophysiology. Whole-cell recordings from acutely isolated rat striatal neurons were obtained using previously published techniques (Surmeier et al., 1995; Mermelstein et al., 1999). The pipette solution consisted of (in mM): 180 *N*-methyl-D-glucamine (NMG), 40 HEPES, 4 $MgCl_2$, 0–20 BAPTA, 12 phosphocreatine, 2 Na_2ATP, 0.2 Na_2GTP, and 0.1 leupeptin, pH 7.2–7.3 with H_2SO_4, 265–270 mOsm/l. The external solution consisted of (in mM): 135 NaCl, 20 CsCl, 1 $MgCl_2$, 10 HEPES, 0.001 TTX, 5 $BaCl_2$, and 10 glucose, pH 7.4 with NaOH, 300–305 mOsm/l. All reagents were obtained from Sigma (St. Louis, MO) except ATP and GTP (Boehringer Mannheim, Indianapolis, IN) and BAPTA, calcineurin autoinhibitory peptide, and leupeptin (Calbiochem, La Jolla, CA). All drugs were prepared according to the manufacturer's specifications and applied with a

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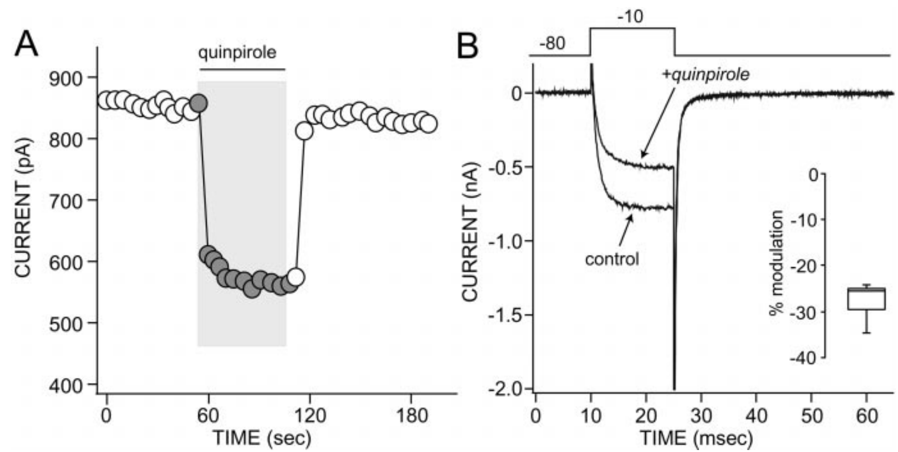

Figure 1. D_2-class receptor agonists decrease whole-cell Ba^{2+} current through Ca^{2+} channels in acutely isolated striatal neurons. *A*, Plot of peak Ba^{2+} currents evoked by a step to −10 mV from a holding potential of −80 mV. Quinpirole (10 μM) reversibly decreased peak currents. *B*, Representative currents used to construct *A*. Voltage protocol is shown at the *top*. *Inset*, A box plot summary of the percent reduction in peak current produced by quinpirole ($n = 5$). The *central line* of the *box* is the median of the distribution. The *edges* of the *box* are the interquartiles. The *lines* running from the *edge* of the *box* show the extremes of the distribution, excluding outliers.

"sewer pipe" capillary array (Surmeier et al., 1995; Mermelstein et al., 1999). C terminus of β adrenergic receptor kinase 1 (βARK-C) peptide (βARK-Cp) is comprised of residues 548–671 of the rat homolog of βARK. βARK-Cp (4.9 mg/ml) was dialyzed against the recording internal solution. This solution was diluted in the recording internal solution for a final concentration of 1 mg/ml.

Intracellular recordings were performed on rat dorsal neostriatal slices maintained *in vitro* as reported previously (Hernandez-Lopez et al., 1997). Recording was done in a submerged-type chamber superfused with saline of the same composition (34–36°C). Sharp microelectrodes filled with 3 M K-acetate and 1% biocytin were used. Rectangular current pulses of varying strengths and durations were used to evoke spike activity. Records were obtained with an active bridge electrometer (Neuro Data, Cygnus Technology, Inc., Delaware Water Gap, PA), digitized, and saved on video tapes (40 kHz) for off-line analysis with a personal computer. Neurons were injected with biocytin as described previously. All neurons were medium spiny projection neurons. Experiments were paired, so that records in the presence and absence of bath-applied drugs were compared in the same neuron.

Fluorometry. For combined patch clamp and fluorometry, neurons were loaded with fura-2 pentapotassium salt (100 μM; Molecular Probes, Eugene, OR) through the patch pipette in a chelator-free recording internal solution (see above). Concomitant fluorometry and patch-clamp recording used Ba^{2+} as the charge carrier to eliminate transmembrane flux as a contributor to the fluorometric signal. For fluorometry without patch recording, neurons were incubated in buffer containing fura-2 AM (5 μM; Molecular Probes) for 25 min at 37°C in the dark. After loading, neurons were rinsed with saline and equilibrated for 20 min at room temperature. Changes in cytoplasmic Ca^{2+} concentration were determined by measuring the fluorescence ratio (510 nm) after excitation with 340 and 380 nm wavelength light. Emission ratios were corrected for background fluorescence. Measurements were obtained with a Nikon Diaphot equipped with a DeltaScan fluorometry system (Photon Technology International) running proprietary software.

Single-cell reverse transcription-PCR protocol. Protocols similar to those described previously were used (Baranauskas et al., 1999; Mermelstein et al., 1999). The PCR primers were developed from GenBank sequences using OLIGO software (National Biosciences). The primers used for enkephalin and substance P cDNA amplification have been published previously (Surmeier et al., 1996). The primers for phospholipase C β1 (PLCβ1) cDNA (GenBank accession number M20636) were 5′-AAA GGG AAG GTT AGT GAG GAC AG-3′ and 5′-TTC AGG CTA AGG GAT GTT TCT C-3′. The predicted product length was 253 bp. The primers for PLCβ2 cDNA (GenBank accession number AJ011035) were 5′-ATC CAA GCC ATG ACC AAA GTC-3′ and 5′-GTC TCC CAT TTC TGC CTT ATG TG-3′. The predicted product length was 547 bp. The primers for PLCβ3 cDNA (GenBank accession number M99567) were 5′-AGC GCA ACA ACA GCA TCT CAG A-3′ and 5′-CTC TTG CTC CGC CAG TTC AAA G-3′. The predicted product length was 420 bp. The primers for PLCβ4 cDNA (GenBank accession number L15556) were 5′-GGC AAT GAA GCA GTC GAA AGA-3′ and 5′-GGC GTG ATC CTC TGG TGT TCT AT. The predicted product lengths were 209 and 246 bp.

Statistical procedures. Data analysis was performed with SYSTAT (version 5.2; SPSS, Inc., Chicago, IL). Sample statistics are given as means ± SEs. Box plots were used for graphic presentation of the data because of the small sample sizes.

RESULTS

D_2 receptor activation reduces Ca^{2+} currents

Whole-cell Ba^{2+} currents through Ca^{2+} channels were reversibly inhibited by the D_2-class receptor agonists (−)-quinpirole (Fig. 1) and R(−)-propylnorapomorphine (NPA) in ~65% of the acutely isolated medium-sized striatal neurons tested. At saturating agonist concentrations (10 μM), the mean reduction in peak current evoked by a voltage step to −10 mV was 28 ± 2% ($n = 5$) for quinpirole and 26 ± 3% for NPA ($n = 4$). Lower agonist concentrations produced smaller, qualitatively similar modulations (0.50–5 μM; $n = 6$). Previous studies have shown that D_2 receptors, like other $G_{i/o}$-coupled receptors, inhibit N- and P/Q-type Ca^{2+} channels but typically do not modulate L-type Ca^{2+} channels (Yan et al., 1997). However, in medium spiny neurons, the L-type channel antagonist nifedipine significantly reduced the modulation produced by quinpirole, suggesting that L-type channels were a major target of the D_2 receptor pathway (Fig. 2*A,B*). The mean modulation in the absence of nifedipine was 29% ($n = 8$), whereas it was only 10% ($n = 6$) in the presence of nifedipine ($p < 0.05$, Kruskal–Wallis).

Another way of testing the involvement of L-type Ca^{2+} channels is via use of the dihydropyridine agonists such as (−)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester (BAYK) 8644 and 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1H-pyrrole-3-carboxylic acid methyl ester (FPL) 64176 (Rampe et al., 1993). These agonists slow the deactivation of L-type Ca^{2+} channels during repolarization of the membrane; this selective slowing provides a convenient way of isolating currents through L-type channels. NPA reversibly reduced the slowly deactivating tail current attributable to L-type Ca^{2+} channels (see Fig. 2*C,D*). As shown in Figure 2*C*, *inset* box plot, the median reduction in the amplitude of the slow tail current by NPA (10 μM) was just >20% in responsive neurons ($n = 13$).

To verify the involvement of D_2-class receptors in the response, the ability of (−)-sulpiride to antagonize the response was examined. Sulpiride (5 μM) (Weiss et al., 1985) had no effect of its own on the BAYK-enhanced L-type currents but blocked the effect of NPA (10 μM) on both step and tail currents; the effect of NPA reemerged when sulpiride was washed off the cell (Fig. 3*A,B*). In six neurons, the median NPA-induced modulation of the slow tail current was 22% in the absence of sulpiride and 2% in its presence (see Fig. 3*A*, *inset*; $p < 0.05$, Kruskal–Wallis). The modulation of the current evoked during the depolarizing step also was antagonized by sulpiride ($n = 6$; median modulation = 4%; $p < 0.05$, Kruskal–Wallis).

There are three D_2-class receptors (D_2, D_3, or D_4) with a high affinity for NPA, quinpirole, and sulpiride. Although the D_2 receptor is the predominant striatal isoform, previous studies have identified a substantial subset of medium spiny neurons that express D_3 receptors (Surmeier et al., 1996). To determine which of these D_2-class receptors was responsible for the modulation, whole-cell recordings were followed by a single-cell reverse transcription (scRT)-PCR analysis. Because the dopamine receptor mRNAs appear to be of relatively low abundance and difficult to detect after whole-cell recording, the scRT-PCR experiments focused on two high-abundance peptide mRNAs that are strongly correlated with

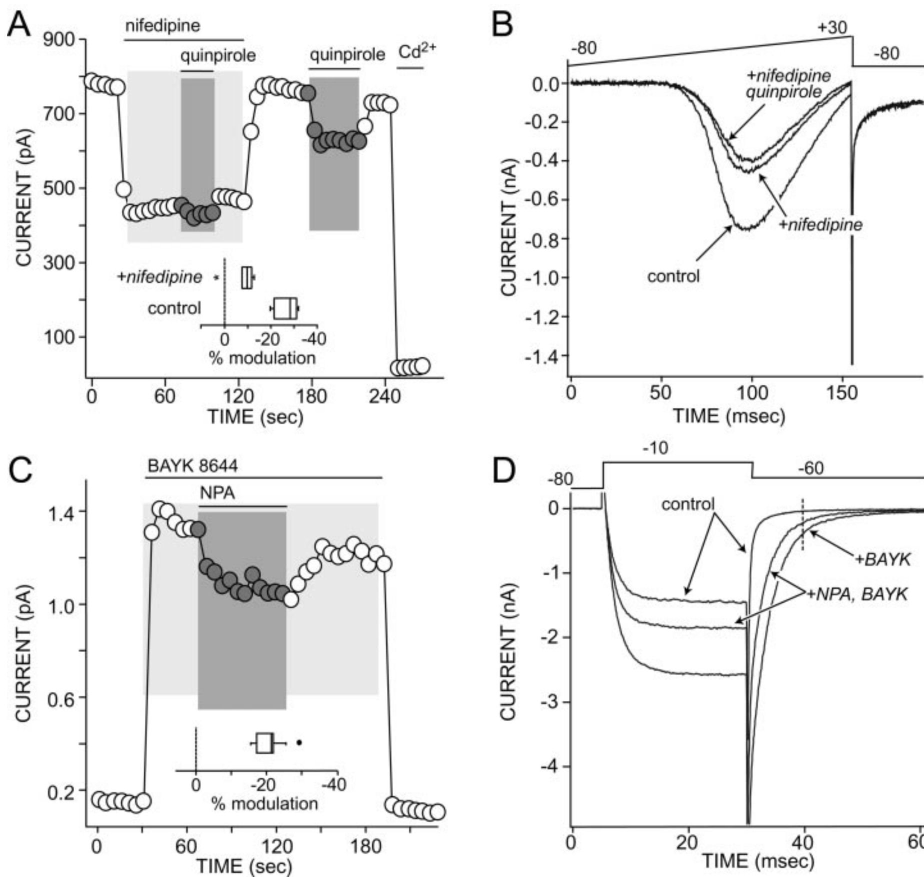

Figure 2. D_2-class receptor agonists decrease currents through L-type Ca^{2+} channels. *A*, Plot of peak Ba^{2+} current evoked by a voltage ramp (see *B*). Nifedipine (5 μM) reduced evoked currents and occluded the effects of quinpirole (10 μM); washing nifedipine off restored the quinpirole modulation. *Inset*, A box plot summary of the modulation in the presence and absence (control) of nifedipine ($n = 6$). The *asterisk* is an outlier, defined as a point that is either greater than three halves the interquartile range above the upper interquartile or less than three halves the interquartile range below the lower interquartile (Tukey, 1977). *B*, Representative currents used to construct *A*. Voltage protocol is shown at the *top*. *C*, Plot of tail current amplitude evoked by the protocol shown in *D* and measured at the *dashed vertical line* in *D*. BAYK 8644 increased tail amplitudes; NPA reversibly reduced the amplitude. *Inset*, A box plot summary of the percent reduction in tail current amplitude produced by NPA ($n = 13$). The *filled circle* is an outlier. *D*, Representative current *traces* used to construct *C*.

receptor expression. D_2 receptor expression is limited to medium spiny neurons expressing the releasable peptide enkephalin (Gerfen, 1992; Surmeier et al., 1996). On the other hand, D_3 receptor expression is limited to a subpopulation of medium spiny neurons expressing substance P in the dorsal striatum (Surmeier et al., 1996). In neurons expressing enkephalin, the modulation of L-type Ca^{2+} channels was robust (Fig. 3*C*; $n = 6$; median modulation = 19%), whereas neurons that only expressed substance P failed to exhibit a significant response (Fig. 3*D*; $n = 3$; median modulation = 0%), clearly implicating D_2 receptors in the modulation.

The D_2 receptor modulation is not dependent on inhibition of adenylyl cyclase

The activation of D_2 receptors inhibits adenylyl cyclase activity, reducing cytosolic cAMP levels and protein kinase A (PKA) activity (Sibley, 1995). PKA can enhance L-type Ca^{2+} channel currents in medium spiny neurons (Surmeier et al., 1995). To test directly whether D_2 receptors reduced L-type currents by inhibiting adenylyl cyclase, three experiments were performed. First, adenylyl cyclase was stimulated by incubating neurons in forskolin (1 μM) before NPA exposure. If inhibition of adenylyl cyclase were a key element in the signaling mechanism, forskolin stimulation should increase the absolute magnitude of the NPA modulation (Battaglia et al., 1985). It did not. Although forskolin significantly enhanced tail currents in the absence of BAYK 8644 (control median = 45 pA; $n = 13$; forskolin median = 74 pA; $n = 6$; $p < 0.05$, Kruskal–Wallis), the absolute modulation of BAYK 8644-enhanced tail currents was indistinguishable from that seen in control neurons (Fig. 4*A*; $n = 5$; median reduction = 20%; $p > 0.05$, Kruskal–Wallis). The D_2 receptor modulation of currents evoked by the test step were unaltered as well ($n = 5$; median reduction = 20%; $p > 0.05$, Kruskal–Wallis). A cyclase-dependent mechanism also predicts that blocking the degradation of cAMP should attenuate the D_2 modulation. But, the phosphodiesterase inhibitor IBMX (5 μM) did not affect the D_2 modulation of slow tail currents ($n = 6$; median modulation = 19%; $p > 0.05$, Kruskal–Wallis) or step currents ($n = 6$; median modulation = 19%; $p > 0.05$, Kruskal–Wallis). Lastly, blocking the access of cAMP to PKA should blunt the D_2 modulation. However, as shown in Figure 4*B*, dialysis with a competitive inhibitor of cAMP, the Rp isomer of cyclic adenosine monophosphothioate (Rp-cAMPS; 10 μM), did not affect the ability of D_2 receptors to modulate the slow tail current ($n = 4$; median modulation = 22%; $p > 0.05$, Kruskal–Wallis) or step currents ($n = 4$; median modulation = 20%; $p > 0.05$, Kruskal–Wallis). These observations, taken together with the fact that D_2 receptor activation effectively modulated currents in the absence of receptor-mediated stimulation of adenylyl cyclase, clearly suggest that D_2 receptors were working by another mechanism.

D_2 receptors mobilize intracellular Ca^{2+} via a phospholipase C pathway

If D_2 receptors were not acting via adenylyl cyclase and PKA, then how were they working? A number of studies have shown that L-type Ca^{2+} currents can be suppressed by elevations of the intracellular Ca^{2+} concentration (Chad and Eckert, 1986; Armstrong et al., 1991; Lukyanetz et al., 1998). In cells dialyzed with high concentrations of the fast Ca^{2+} chelator BAPTA (20 mM), a concentration sufficient to "clamp" the free Ca^{2+} concentration at a low nanomolar level, quinpirole had little or no effect on the BAYK-enhanced tail currents (Fig. 5*A*). Although quinpirole failed to modulate the slow tails in these neurons, it continued to reduce the peak current (although to a lesser extent), suggesting that the modulation of non-L-type channels was intact ($n = 5$; median modulation = 11%). Because BAPTA can have effects unrelated to Ca^{2+} buffering (Bernheim et al., 1991), cytosolic $[Ca^{2+}]$ was measured directly with fluorometric techniques in voltage-clamped neurons dialyzed with fura-2 (100 μM). These experiments revealed that in neurons in which the slow BAYK tail currents were modulated, NPA also induced a rapid and reversible elevation in cytosolic Ca^{2+}. This elevation occurred in the absence of external Ca^{2+} and with the cell's membrane held at −80 mV ($n = 4$) (Fig. 5*B*), implicating release from intracellular stores.

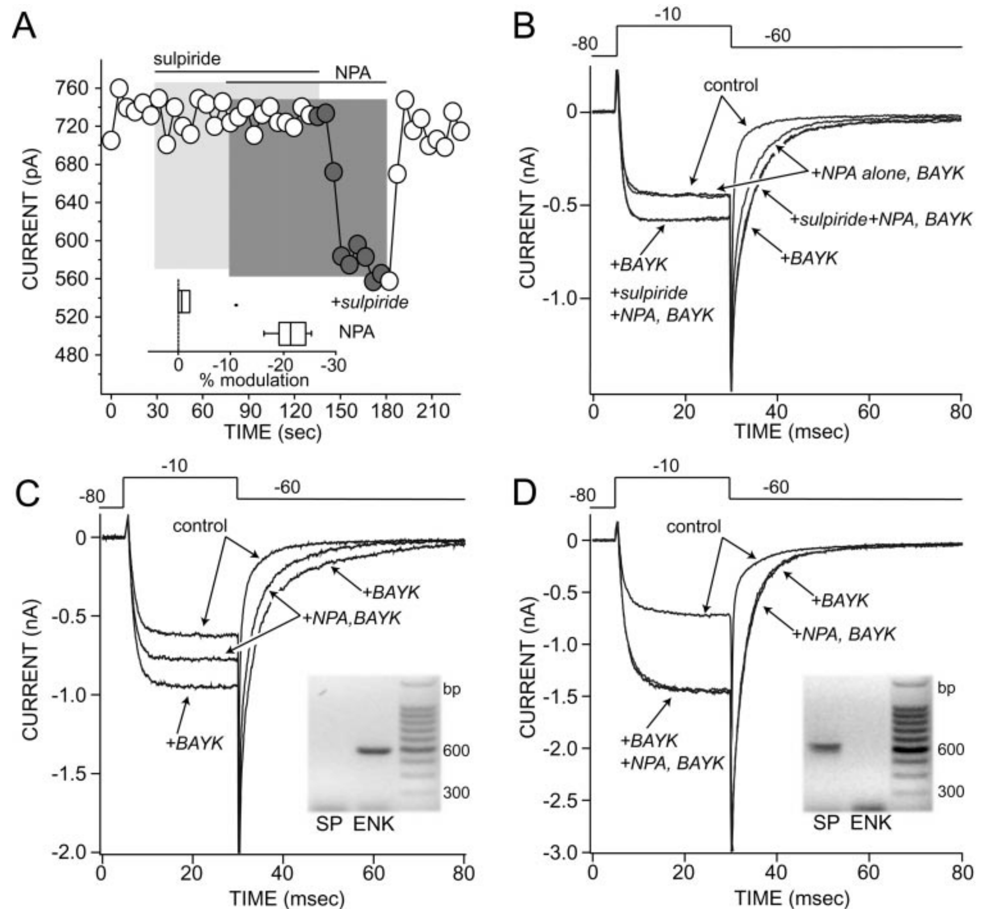

Figure 3. The modulation is dependent on D_2 receptors. *A*, A plot of Ba^{2+} current tail amplitudes as a function of time (see Fig. 2*D*). Sulpiride (5 μM) blocked the effects of NPA (10 μM); washing sulpiride off restored the NPA modulation. *Inset*, A box plot summary of the modulation in the presence and absence (NPA) of sulpiride (n = 6) is shown. The *filled circle* is an outlier. *B*, Representative currents used to construct *A* are presented. Voltage protocol is shown at the *top*. *C*, NPA modulated Ba^{2+} currents only in neurons shown by scRT-PCR to express enkephalin (n = 6). *Inset*, The gel shows the presence of enkephalin (*ENK*) and absence of substance P (*SP*) amplicons in this cell. *D*, Neurons expressing substance P, but not enkephalin, did not respond to NPA (n = 3). *Inset*, The gel shows the *SP* amplicon derived from this neuron.

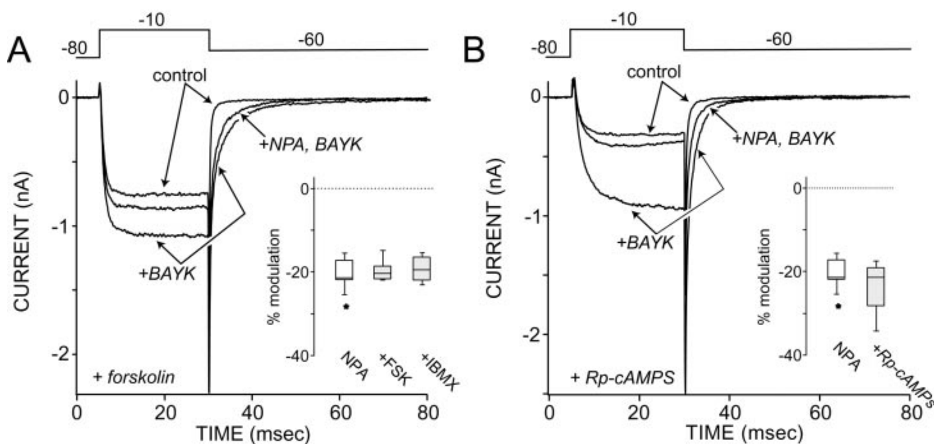

Figure 4. The D_2 receptor modulation is independent of alterations in adenylyl cyclase activity. *A*, Preincubation of cells in forskolin (1 μM) failed to alter the NPA (5 μM) modulation of BAYK 8644-enhanced tail currents or the modulation of currents evoked by the test step to −10 mV. Voltage protocol is shown at the *top*. *Inset*, Box plots of the tail modulation in control (NPA; n = 10), forskolin (*FSK*; n = 5), and IBMX (n = 6) solutions are shown. The *asterisk* is an outlier. These data were not significantly different. *B*, Cellular dialysis with the cAMP antagonist Rp-cAMPS also failed to alter the NPA modulation of BAYK 8644-enhanced tail currents. *Inset*, A box plot summary of the tail modulation in control (n = 10) and Rp-cAMPS (n = 4)-dialyzed neurons is shown. These data were not significantly different.

NPA failed to alter intracellular Ca^{2+} levels in those neurons in which the slow BAYK tail currents were unmodulated (n = 3). To test this linkage further, medium spiny neurons were loaded with fura-2 AM and D_2 agonists applied in the presence and absence of extracellular Ca^{2+}. Fluorometric measurements were taken in these neurons without concomitant patch-clamp recording. NPA evoked a calcium transient in 70% of these neurons regardless of whether external Ca^{2+} was present or not (14/20; data not shown).

The best-described mechanism for receptor-mediated mobilization of intracellular Ca^{2+} stores is via activation of PLC isoforms (Sternweis and Smrcka, 1992). PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate, yielding 1,2-diacylglycerol and inositol 1,4,5-triphosphate (IP_3). Cytosolic IP_3 binds to its cognate receptor, releasing Ca^{2+} from intracellular pools. To determine whether D_2 receptors relied on a similar mechanism, neurons were dialyzed with the PLC inhibitor U-73122 (10 μM) before D_2 receptor stimulation. U-73122 blocked the ability of NPA to reduce the slow, BAYK-enhanced tail currents in enkephalin-expressing neurons (Fig. 5*C*; n = 9; median modulation = 0%; $p < 0.05$, Kruskal–Wallis). In contrast, non-L-type currents evoked by the depolarizing voltage step continued to be reduced by NPA (Fig. 5*C*; n = 9; median modulation = 19%; $p < 0.05$, Kruskal–Wallis). Dialysis with the inactive analog U-73343 (10 μM) failed to alter the D_2 modulation of the tail currents (n = 4; median modulation = 21%; $p > 0.05$, Kruskal–Wallis). PLCβ isoforms are generally thought to mediate receptor-driven responses like the ones observed here (Sternweis and Smrcka, 1992). The involvement of other PLC isoforms, like PLCγ, or tyrosine kinase itself (Diverse-Pierluissi et al., 1997) seems unlikely because of the inability of the tyrosine kinase inhibitor genistein (50 μM) to reduce the D_2 receptor modulation (n = 3; $p > 0.05$, Kruskal–Wallis) (Lajiness et al., 1993; Rhee and Bae, 1997). There are four

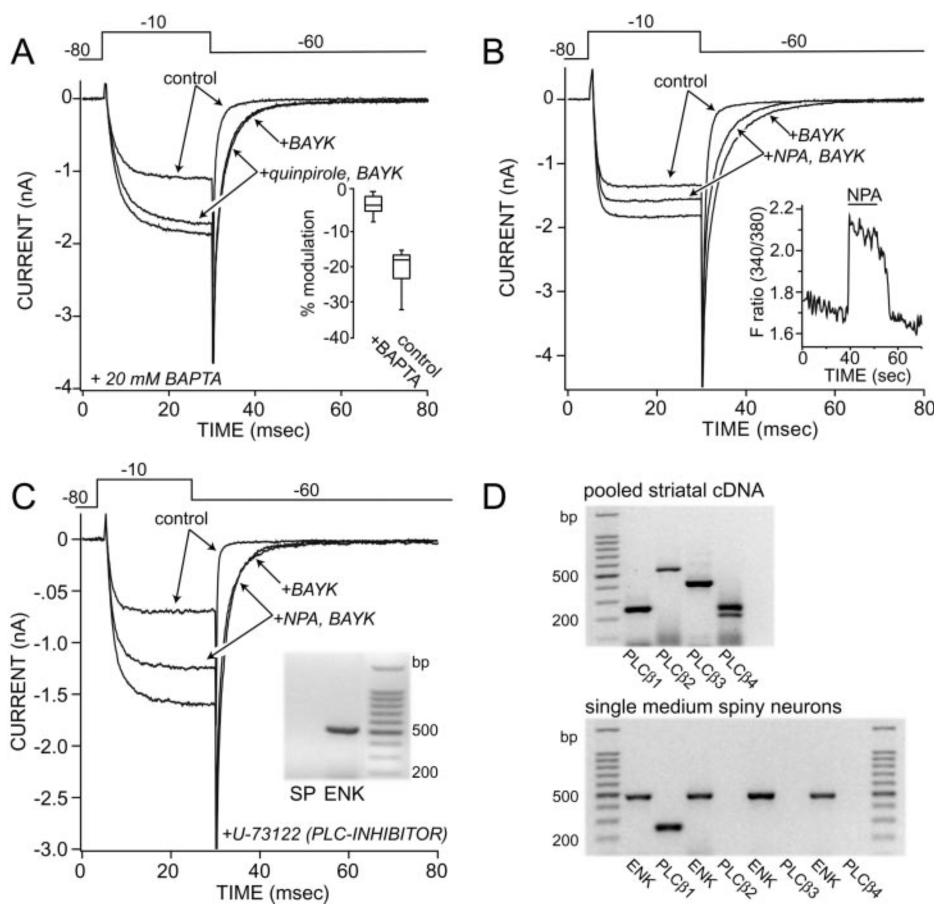

Figure 5. D_2 receptor modulation depends on the release of intracellular Ca^{2+} via a PLC-dependent mechanism. *A*, Dialysis with BAPTA (20 mM) blocked the D_2 modulation of tail currents but not peak currents (n = 5). Voltage protocol is shown at the *top*. *Inset*, A box plot summarizes the modulations seen with BAPTA internals (n = 5) and matched controls (n = 10). *B*, NPA (10 μM) reduced BAYK tail currents and increased intracellular Ca^{2+} levels in the same cells. *Inset*, The ratio of 510 nm fura-2 emission after excitation at 340 and 380 nm in the same neuron is shown. Measurements were taken while the cell was clamped at −80 mV and in the absence of external Ca^{2+}. *C*, NPA failed to modulate slow tail currents in enkephalin-expressing neurons dialyzed with the PLC inhibitor U-73122 (n = 9). *Inset*, The recorded neuron expressed enkephalin but not substance P. *D, Top*, The gel shows RT-PCR amplicons for PLCβ1–4 in pooled striatal mRNA. *Bottom*, The gel shows representative amplicons from four *ENK*-positive medium spiny neurons. Only PLCβ1 mRNA was detected in *ENK* neurons (n = 20).

isoforms of PLCβ (1–4) that have been cloned (Exton, 1997). All four isoforms were expressed in pooled striatal tissue (Fig. 5*D*, *top*); however, when the RT-PCR analysis was limited to neurons expressing enkephalin mRNA, only the PLCβ1 isoform was detected (Fig. 5*D*, *bottom*).

These findings are consistent with the hypothesis that D_2 receptors activate PLCβ1. PLCβ1, like the other PLCβ isoforms, is capable of being activated by $G_{\beta\gamma}$ subunits (Exton, 1997; Morris and Scarlata, 1997). To test whether D_2 receptors activated PLCβ1 in this way, neurons were dialyzed with an inhibitor of $G_{\beta\gamma}$ signaling (βARK-C peptide; 1 mg/ml) (Koch et al., 1994). βARK-Cp effectively inhibited the NPA modulation of the BAYK-enhanced, L-type tail currents, as well as the peak current, in enkephalin-expressing neurons (Fig. 6*A*). The median modulation of the slow tail currents in the presence of βARK-Cp was 6% (Fig. 6*B*, *inset*; n = 5; $p < 0.05$, Kruskal–Wallis). The NPA modulation of the currents evoked by the depolarizing voltage step was also 6% (n = 5; $p < 0.05$, Kruskal–Wallis). In the same βARK-Cp-dialyzed neurons, $G_{q\alpha}$-linked M1 muscarinic receptors continued to reduce both peak and slow tail currents (Fig. 6*B*, *inset*) (Howe and Surmeier, 1995).

Inhibition of calcineurin blocks the D_2 receptor modulation of L-type Ca^{2+} currents

PLCβ isoforms regulate intracellular Ca^{2+} levels via the production of IP_3. Dialysis with competitive antagonists of IP_3 (heparin, 10 mg/ml; n = 6; xestospongin, 1 μM; n = 13) (Simpson et al., 1995; Gafni et al., 1997) antagonized NPA effects on L-type channels in enkephalin-expressing neurons ($p < 0.05$, Kruskal–Wallis). Lastly, caffeine (10 mM; externally applied), which is known to promote the release from ryanodine-sensitive Ca^{2+} stores and inhibit IP_3-mediated Ca^{2+} release (Simpson et al., 1995), induced an elevation in cytosolic Ca^{2+} levels and occluded the effects of quinpirole on FPL 64176-enhanced tail currents (n = 10; $p < 0.05$, Kruskal–Wallis). One potential means by which elevations in cytosolic Ca^{2+} levels could suppress L-type Ca^{2+} currents is via the Ca^{2+}-dependent phosphatase calcineurin (Chad and Eckert, 1986). To test this possibility, neurons were dialyzed with a peptide inhibitor of calcineurin (25 μM) (Hashimoto et al., 1990). As shown in Figure 6*C*, the calcineurin inhibitor significantly reduced the NPA modulation of the BAYK-enhanced tail currents in enkephalin-expressing neurons (n = 4; median modulation = 5%; $p < 0.05$, Kruskal–Wallis) without blocking the modulation of non-L-type currents (median modulation = 15%; $p < 0.05$, Kruskal–Wallis). In contrast, inhibition of protein phosphatase 1 and 2A with okadaic acid (1 μM) had no effect on the ability of D_2 agonists to suppress the BAYK-enhanced tail current (n = 2; median modulation = 20%; $p > 0.05$, Kruskal–Wallis).

D_2 receptor activation suppresses spike activity evoked from depolarized membrane potentials

L-type Ca^{2+} currents are important determinants of evoked spike activity in medium spiny neurons (Hernandez-Lopez et al., 1997). The influence of these currents can be seen by holding medium spiny neurons at a depolarized level, close to that seen in the upstate *in vivo*. At this potential, a brief current pulse is capable of triggering a prolonged depolarization (hundreds of milliseconds) that occasionally results in spike generation. This type of response was seen in approximately two-thirds of all trials with a given neuron; in the other trials, the membrane potential decayed passively back to the "resting" potential. In the presence of the L-type channel agonist BAYK 8644, this quasibistable response was enhanced in duration and probability (Fig. 7*A*). Spikes become a much more common event in this situation as well. On the other hand, in the presence of the L-type channel antagonist nicardipine (5 μM), the bistable behavior was almost entirely abolished, resulting in passive membrane responses in the vast majority of trials (Hernandez-Lopez et al., 1997). D_2 receptor stimulation also suppressed the bistable behavior. In the presence of quinpirole (10 μM), the probability of evoking a sustained depolarization dropped

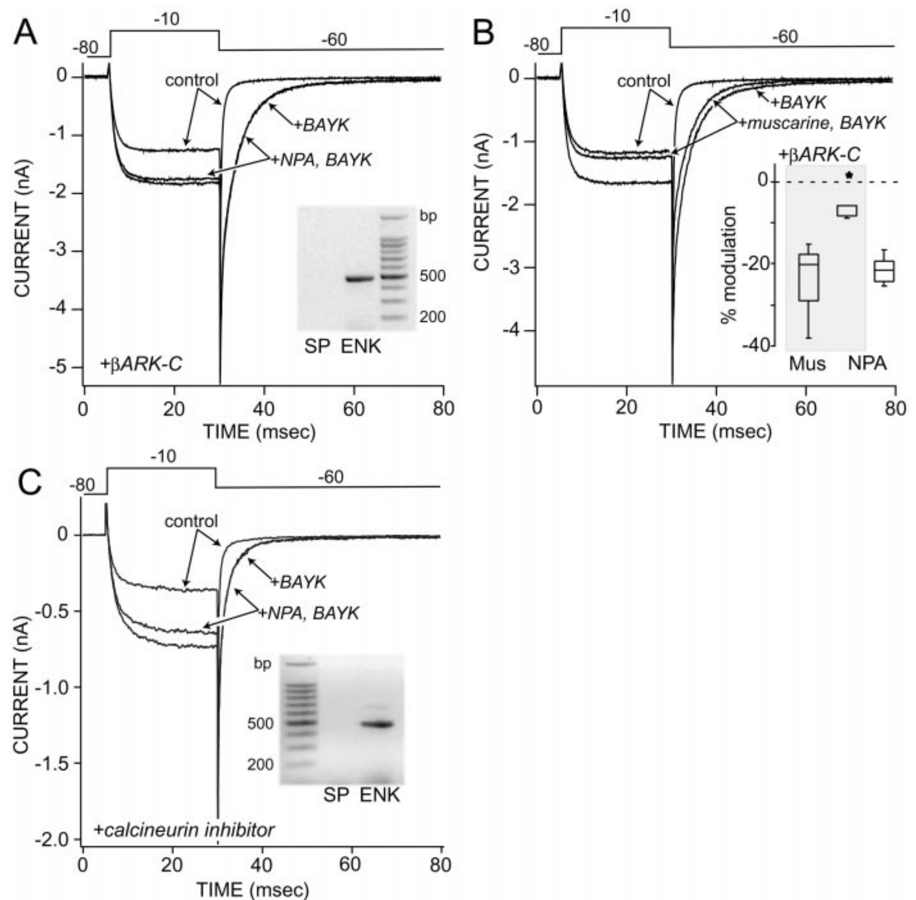

Figure 6. Inhibitors of $G_{\beta\gamma}$ and calcineurin signaling attenuate the D_2 receptor modulation of L-type channels. *A*, Dialysis with βARK-C peptide (20 μM) blocked the NPA (10 μM) modulation of BAYK (1 μM)-enhanced tail currents as well as peak evoked currents in enkephalin-expressing medium spiny neurons. *Inset*, The gel from the scRT-PCR profile is shown. The median modulation of the tail currents in enkephalin-expressing neurons ($n = 4$) was 4% (see *B*, *inset* box plot). *B*, In the same neuron depicted in *A*, muscarine (*Mus*; 1 μM) continued to modulate both currents evoked by the step to −10 mV and slow tail currents. *Inset*, The median muscarinic modulation of the tail currents was 20% in the presence of βARK-C. This was very similar to that seen previously (Howe and Surmeier, 1995) and indistinguishable from the D_2 modulation (see box plot; the *asterisk* is an outlier). Previous work has shown that these neurons express high levels of the $G_{q\alpha}$-linked M1 receptor. *C*, Dialysis with the calcineurin autoinhibitory peptide (25 μM) blocked the NPA modulation of the slow tail currents in enkephalin-expressing neurons but not that of the peak currents ($n = 4$). *Inset*, The gel shows *ENK* but not *SP* amplicons derived from the recorded neuron. Voltage protocol is shown at the *top*.

significantly ($n = 6$; $p < 0.05$, Kruskal–Wallis), even in the presence of BAYK 8644 (Fig. 7*A*).

D_2 receptor stimulation also shortened the duration of tetraethylammonium (TEA)-enhanced Ca^{2+} spikes in medium spiny neurons. When held at −60 mV in the presence of TEA (20 mM), a brief current stimulus evoked an all-or-none Ca^{2+} spike in medium spiny neurons (Kita et al., 1985; Bargas et al., 1989). In this recording situation, the duration of the Ca^{2+} spike was 210 ± 35 msec (mean ± SD; $n = 40$). If nicardipine (5 μM) or nitrendipine (5 μM) were added, the Ca^{2+} spike was reduced in duration to 150 ± 40 msec ($n = 15$). As with the L-type channel antagonists, quinpirole (10 μM) reduced the duration of the TEA-induced Ca^{2+} spike in all three cells tested (mean duration = 160 ± 30 msec) (Fig. 7*B*, *top*). In the presence of nicardipine (5 μM), quinpirole failed to exert any further reduction of the Ca^{2+} spike in three of five neurons (Fig. 7*B*, *bottom*).

By providing a sustained depolarizing influence, L-type Ca^{2+} currents enhance repetitive activity evoked from depolarized membrane potentials in medium spiny neurons (Hernandez-Lopez et al., 1997). D_2 receptor-mediated suppression of these currents should diminish evoked spiking. Intracellular recordings from medium spiny neurons in tissue slices confirmed this conjecture. Neurons were slightly depolarized (approximately −65 mV) by steady current injection, and then repetitive activity was evoked by current steps. From these "upstate" membrane potentials, quinpirole (10 μM) diminished evoked spiking in 6 of 10 neurons (Fig. 7*C*). The suppression of repetitive activity was particularly evident with small current injections that come close to mimicking *in vivo* conditions (Fig. 7*D*, *top*). But, the reduction in firing frequency induced by quinpirole was evident via the whole intensity–frequency plot (Fig. 7*D*). In quinpirole-responsive neurons, the half-maximum frequency was reduced from 45 ± 10 to 33 ± 12 Hz by quinpirole ($n = 6$; $p < 0.05$, Kruskal–Wallis). The ability of quinpirole to alter evoked activity was suppressed by blockade of L-type channels with nicardipine (5 μM; $n = 3$). Taken together, these results clearly argue that D_2 receptor modulation of L-type Ca^{2+} channels results in a suppression of repetitive spiking evoked from depolarized potentials in medium spiny neurons.

DISCUSSION

D_2 receptors in striatal medium spiny neurons activate a PLC–IP_3–calcineurin cascade

The results presented show that activation of D_2 receptors reduces currents through L-type Ca^{2+} channels, leading to a suppression of evoked spike activity in enkephalin-expressing striatal medium spiny neurons. Even though nearly all striatal effects of D_2 receptor activation are ascribed to the inhibition of adenylyl cyclase activity (Sibley, 1995), this signaling linkage was not responsible for the modulation of L-type Ca^{2+} channels. Manipulation of adenylyl cyclase activity, cAMP metabolism, and dialysis with a competitive inhibitor of cAMP had no effect on the modulation. Rather, the D_2 receptor modulation depended on $G_{\beta\gamma}$ protein activation of a PLCβ1-signaling cascade, mobilization of intracellular Ca^{2+}, and activation of calcineurin. This conclusion is based on five observations. First, the D_2 receptor suppression of L-type currents was blocked by inhibition of $G_{\beta\gamma}$ signaling. Second, medium spiny neurons expressed readily detectable levels of PLCβ1 mRNA (but not that of other PLCβ isoforms), and the modulation was blocked by inhibitors of PLCβ1. Third, D_2 receptor stimulation induced the release of Ca^{2+} from intracellular stores. Fourth, disruption of IP_3 signaling or chelation of intracellular Ca^{2+} blocked the modulation. Lastly, inhibition of the Ca^{2+}-dependent phosphatase calcineurin blocked the modulation. Dephosphorylation by calcineurin has been shown to mediate reductions in L-type Ca^{2+} currents in a variety of cell types (Chad and Eckert, 1986; Armstrong et al., 1991; Lukyanetz et al., 1998). In an intact preparation, the D_2 receptor-triggered activation of calcineurin may act cooperatively with a direct Ca^{2+}–calmodulin-mediated inactivation

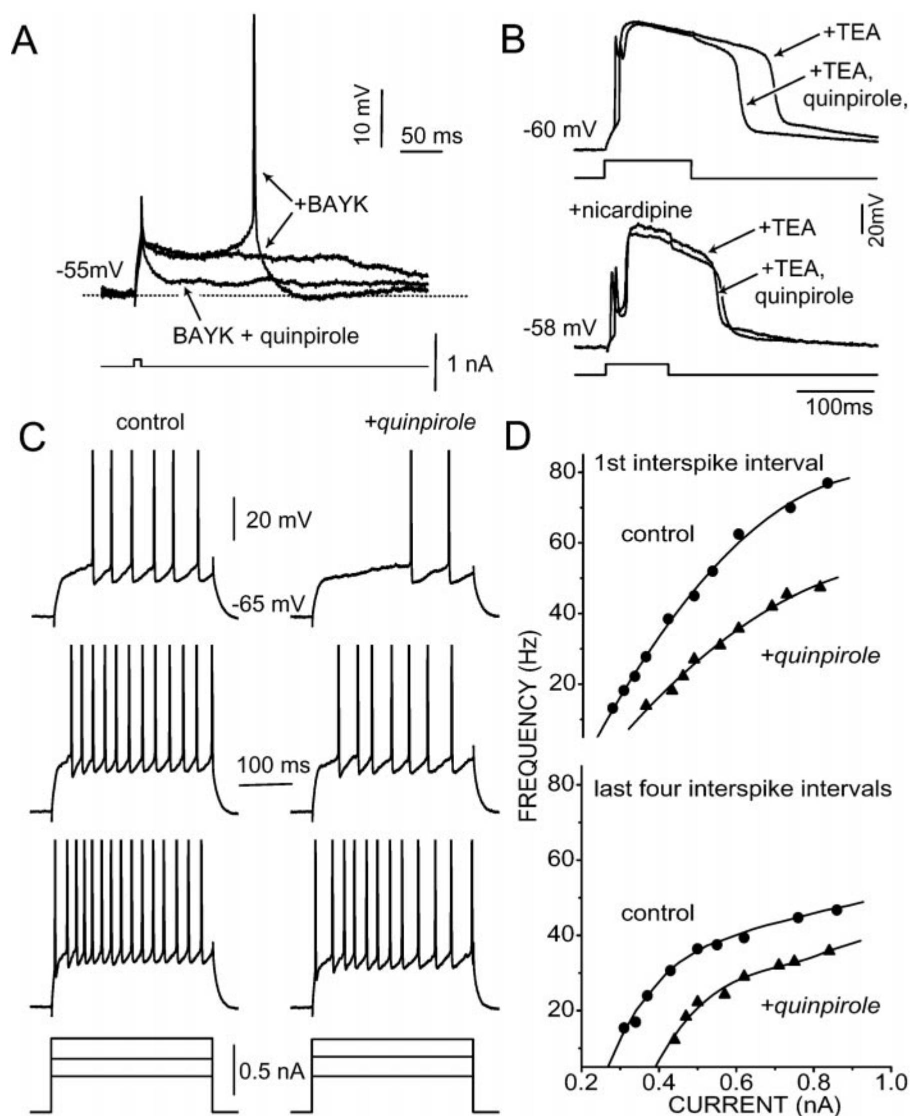

Figure 7. D_2 receptor stimulation suppresses evoked activity in medium spiny neurons recorded in brain slices. *A*, At depolarized membrane potentials mimicking the upstate in medium spiny neurons, a brief current pulse (see protocol *below* the *traces*) evokes a sustained depolarization in the presence of BAYK 8644 (2.5 μM). The depolarization was significantly shortened by the addition of quinpirole (10 μM). Similar results were seen in all six cells tested. *B*, Intracellular recordings from medium spiny neurons in the presence of TEA (20 mM) are shown. Cells were maintained near −60 mV. *Top*, Quinpirole (10 μM) shortened the duration of the TEA spike (median reduction = 25%; n = 3). *Bottom*, In the presence of nicardipine (5 μM), the TEA spike was of shorter duration. The addition of quinpirole had little or no effect in the presence of nicardipine (median reduction = 2%; n = 3). *C*, Activity evoked by intracellular current injection from a depolarized (approximately −65 mV) membrane potential was suppressed by quinpirole. *Left*, Records evoked by increasing current steps (300 msec in duration) in control conditions are shown. *Right*, Records taken from the same cell after the addition of quinpirole (10 μM) are shown. Note that in the presence of quinpirole, the discharge frequency decreased for similar current steps. *D*, Plots of discharge frequency as a function of injected current for the neuron in *C* are shown. *Top*, The plot is the frequency (reciprocal of first interspike interval) in the presence and absence of quinpirole (10 μM). *Bottom*, The average of the last four interspike intervals in the evoked train in the presence and absence of quinpirole is shown. Similar results were obtained in five other responsive neurons.

process (Imredy and Yue, 1994; Peterson et al., 1999) to suppress currents through L-type Ca^{2+} channels further.

Although previous biochemical studies of striatal slices have not reported D_2 receptor stimulation of PLC (Gupta and Mishra, 1990; Rubinstein and Hitzemann, 1990), striatal cellular heterogeneity complicates the interpretation of these studies. Indiscriminate activation of striatal D_2 receptors can be expected to have two opposing effects. One is activation of PLC in enkephalinergic medium spiny neurons. The other is diminished acetylcholine release (Drukarch et al., 1990) and a reduction in M1 muscarinic receptor stimulation of PLC in medium spiny neurons (Akins et al., 1990; Bernard et al., 1992). Hence, there may be no net change in striatal PLC activity after global D_2 receptor activation. Although they have not provided a clear picture of the signaling mechanism, studies using heterologous expression systems have shown that D_2 receptors are capable of stimulating PLC and mobilizing intracellular Ca^{2+} pools (Vallar et al., 1990; MacKenzie et al., 1994; Yang et al., 1995).

The ability of striatal D_2 receptors to mobilize intracellular Ca^{2+} stores, reduce L-type Ca^{2+} channel currents, and suppress evoked activity effectively reconciles an apparently divergent set of observations. On one hand, D_2 receptor activation is known to increase striatal calcineurin and MAP kinase activity via Ca^{2+}-dependent mechanisms (Nishi et al., 1997; Yan et al., 1999). On the other hand, blockade of D_2 receptors or diminished D_2 receptor tone is known to increase striatal immediate early gene (IEG) and peptide expression (Chesselet et al., 1998). D_2 receptor activation also is necessary for certain forms of striatal use-dependent synaptic plasticity (Calabresi et al., 1992). Our results directly demonstrate calcineurin activation by D_2 receptors and provide a mechanism for Ca^{2+}-dependent MAP kinase activation. Calcineurin-mediated suppression of L-type Ca^{2+} currents will reduce glutamate-induced CRE-binding protein phosphorylation and IEG induction (Rajadhyaksha et al., 1999). By the same token, this D_2 receptor-signaling pathway provides a ready alternative to disinhibition of adenylyl cyclase (Ward and Dorsa, 1999) in explaining the ability of D_2 receptor antagonists to increase sharply striatal IEG induction after cortical stimulation (Berretta et al., 1999).

D_2 receptor activation selectively suppresses activity in enkephalin-expressing medium spiny neurons

In addition to reconciling these more recent observations, our results provide the first direct evidence for one of the oldest conjectures about dopaminergic regulation of striatal activity, namely, that D_2 receptor activation selectively suppresses the activity of enkephalin-expressing medium spiny neurons (Albin et al., 1989). This conjecture has served as a cornerstone of basal ganglia models and treatment strategies for Parkinson's disease for over a decade. Yet, the evidence for this conjecture has been indirect or inconclusive (Nicola et al., 2000).

Our results show that D_2 receptor stimulation inhibits activity evoked from relatively depolarized membrane potentials mimicking the upstate produced by excitatory cortical or thalamic inputs

(Wilson and Kawaguchi, 1996). *In vivo*, medium spiny neurons move between this depolarized upstate in which they generate spikes and a hyperpolarized "downstate" in which they are quiescent. Although other voltage-dependent channel types are modulated in concert (Surmeier et al., 1992; Surmeier and Kitai, 1993; Waszczak et al., 1998), the D_2 receptor suppression of L-type Ca^{2+} currents is critical to this inhibition of activity. Why? Unlike N- and P/Q-type voltage-dependent Ca^{2+} channels in medium spiny neurons, L-type channels are active in the subthreshold potential range of the upstate (Bargas et al., 1994; Song and Surmeier, 1996). This property allows them to exert an important influence on the membrane potential near spike threshold, pushing the membrane potential closer or pulling it farther away from spike generation. Medium spiny neurons expressing D_1 receptors use this property of L-type Ca^{2+} currents to enhance evoked activity in the presence of dopamine (Surmeier et al., 1995; Hernandez-Lopez et al., 1997). In contrast, activation of D_2 receptors in enkephalin-expressing neurons should reduce both the magnitude and duration of the response to cortical or thalamic excitatory synaptic input, as predicted over a decade ago by Albin et al. (1989). Moreover, by targeting a Ca^{2+} channel with privileged access to transcriptional regulation (Bading et al., 1993; Graef et al., 1999; Mermelstein et al., 2000), D_2 receptors exert a proximal control over gene expression tied to extrinsically driven activity. This proximal coupling may prove to be very important to long-term striatal adaptations triggered by alterations in dopaminergic signaling in Parkinson's disease, prolonged neuroleptic treatment, and drug abuse (Hornykiewcz, 1973; Meltzer and Stahl, 1976; Nestler and Aghajanian, 1997).

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