

D₂ Dopamine Receptors Reduce N-Type Ca²⁺ Currents in Rat Neostriatal Cholinergic Interneurons Through a Membrane-Delimited, Protein-Kinase-C-Insensitive Pathway

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Yan, Zhen, Wen-Jie Song, and D. James Surmeier. D₂ dopamine receptors reduce N-type Ca²⁺ currents in rat neostriatal cholinergic interneurons through a membrane-delimited, protein-kinase-C-insensitive pathway. *J. Neurophysiol.* 77: 1003–1015, 1997. Dopamine has long been known to regulate the activity of striatal cholinergic interneurons and the release of acetylcholine. Yet, the cellular mechanisms by which this regulation occurs have not been elucidated. One way in which dopamine might act is by modulating voltage-dependent Ca²⁺ channels. To test this hypothesis, the impact of dopaminergic agonists on Ca²⁺ channels in neostriatal cholinergic interneurons was studied by combined whole cell voltage-clamp recording and single-cell reverse transcription–polymerase chain reactions. Cholinergic interneurons were identified by the presence of choline acetyltransferase mRNA. Nearly all interneurons tested (90%, *n* = 17) coexpressed D₂ (short and long isoforms) and D_{1b} (D₅) dopamine receptor mRNAs. D_{1a} receptor mRNA was found in only a small subset (20%) of the sample and D₃ and D₄ receptor mRNAs were undetectable. D₂ receptor agonists rapidly and reversibly reduced N-type Ca²⁺ currents. D_{1b}/D_{1a} receptor activation had little or no effect on Ca²⁺ currents. The D₂ receptor antagonist sulpiride blocked the effect of D₂ agonists. Dialysis with guanosine-5'-O-(2-thiodiphosphate) or brief exposure to the G protein (G_{i/o}) alkylating agent N-ethylmaleimide also blocked the D₂ modulation. The reduction in N-type currents was neither accompanied by kinetic slowing nor significantly reversed by depolarizing prepulses. The D₂ receptor effects were mediated by a membrane-delimited pathway, because the modulation was not seen in cell-attached patches when agonist was applied to the bath and was not disrupted by perturbations in cytosolic signaling pathways known to be linked to D₂ receptors. Activation of M2 muscarinic receptors occluded the D₂ modulation, suggesting a shared signaling element. However, activation of protein kinase C attenuated the M2 modulation without significantly affecting the D₂ modulation. Taken together, our results suggest that activation of D₂ dopamine receptors in cholinergic interneurons reduces N-type Ca²⁺ currents via a membrane-delimited, G_{i/o} class G protein pathway that is not regulated by protein kinase C. This signaling pathway may underlie the ability of D₂ receptors to reduce striatal acetylcholine release.

INTRODUCTION

Dysfunctions in striatal dopaminergic signaling underlie a variety of basal ganglia disorders, such as Parkinson's disease and schizophrenia (Davis et al. 1991; Wooten 1990). Neostriatal cholinergic interneurons have long been known to be a recipient of this dopaminergic input (Lehman and Langer 1983). Through the release of acetylcholine (ACh), these giant interneurons exert a powerful neuromodulatory effect on medium spiny projection neurons (Howe and Surmeier 1995; Izzo and Bolam 1988; Misgeld et al. 1986). Although the interneuronal release of ACh is regulated by

dopamine (DA) (DeBoer et al. 1993; Di Chiara et al. 1994; Lehman and Langer 1983; Stoof et al. 1982), it is unclear how this is accomplished.

In other cells, DA exerts its effects through activation of G protein-coupled receptors (Sibley 1995). These receptors can be grouped into two classes: a D₁ class (D_{1a}, D_{1b}) and a D₂ class (D₂, D₃, D₄). In situ hybridization studies have shown that many cholinergic interneurons express D₂ mRNA (Le Moine et al. 1991; Weiner et al. 1991) and a fraction expresses D_{1a} receptor mRNA (Le Moine et al. 1991). Immunocytochemical studies have corroborated the localization of D₂ receptors (Ariano et al. 1989; Levey et al. 1993) and recently have suggested that some interneurons may also express D_{1b} receptor protein (Bergson et al. 1995a,b).

One way in which DA receptors might regulate ACh release is by modulating voltage-dependent Ca²⁺ channels (Dunlap et al. 1995). In medium spiny neostriatal neurons, D₁-class DA receptors regulate Ca²⁺ channels through an adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase and protein phosphatase cascade (Surmeier et al. 1995a). Considerably less is known about the ion channels modulated by D₂-class receptors in neostriatal neurons. In other cell types, D₂-class receptors have been found to reduce Ca²⁺ currents through pertussis toxin (PTX)-sensitive G proteins (Brown and Seabrook 1995; Lledo et al. 1992; Seabrook et al. 1994a,b). In some situations this modulation may be mediated by inhibition of adenylyl cyclase (Vallar and Meldolesi 1989) but, at least in pituitary cells, a direct G protein interaction seems to be involved (Lledo et al. 1992). The ability of D₂-class agonists to decrease ACh release in the striatum (Bertorelli et al. 1992; Stoof and Keibian 1982) suggests that similar mechanisms may be involved in cholinergic interneurons.

In addition to being regulated by DA, the release of ACh is modulated by muscarinic autoreceptors (Dolezal and Wecker 1990; James and Cubeddu 1987). M2 receptors reduce N-type Ca²⁺ currents in striatal cholinergic interneurons through a pathway very similar to that used by D₂-class receptors in other cell types (Yan and Surmeier 1996). The inhibitory effects of D₂-class receptors and muscarinic receptors on striatal ACh release are nonadditive (Drukarch et al. 1990), suggesting that there may be a shared signaling mechanism. What this shared element might be has yet to be determined.

METHODS

Acute dissociation procedure

Neostriatal neurons from rats (>3 wk) were acutely dissociated with the use of procedures similar to those we have previously

described (Bargas et al. 1994; Surmeier et al. 1995a). In brief, rats were anesthetized with methoxyflurane and decapitated; brains were quickly removed, iced, and then blocked for slicing. The blocked tissue was cut in 400- μm slices with a Vibroslice (Campden Instruments, London, UK) while being bathed in a low- Ca^{2+} (100 μM), *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)-buffered salt solution (composition, in mM: 140 sodium isethionate, 2 KCl, 4 MgCl_2 , 0.1 CaCl_2 , 23 glucose, and 15 HEPES, pH 7.4, 300–305 mosM/l). Slices were then incubated for 1–6 h at room temperature (20–22°C) in NaHCO_3 -buffered saline bubbled with 95% O_2 -5% CO_2 (composition, in mM: 126 NaCl, 2.5 KCl, 2 CaCl_2 , 2 MgCl_2 , 26 NaHCO_3 , 1.25 NaH_2PO_4 , 1 pyruvic acid, 0.2 ascorbic acid, 0.1 N^G -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 were then removed from the low- Ca^{2+} buffer, and regions of the dorsal neostriatum were dissected and placed in an oxygenated Cell-Stir chamber (Wheaton, Millville, NJ) containing pronase (Sigma protease Type XIV, 1–3 mg/ml) in HEPES-buffered Hanks' balanced salt solution (Sigma) at 35°C. Dissections were limited to tissue rostral to the anterior commissure to reduce the possibility of contamination from the globus pallidus. After 20–40 min of enzyme digestion, tissue was rinsed three times in the low- Ca^{2+} , HEPES-buffered saline and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was then plated into a 35-mm Lux Petri dish mounted on the stage of an inverted microscope containing HEPES-buffered Hanks' balanced salt solution saline.

Whole cell recordings

Whole cell recordings of Ba^{2+} currents through Ca^{2+} channels employed standard techniques (Hamill et al. 1981; Surmeier et al. 1995a). Electrodes were pulled from Corning 7052 glass and fire polished before use. The internal solution consisted of (in mM): 180 *N*-methyl-D-glucamine, 40 HEPES, 4 MgCl_2 , 0.1 1,2 bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid, 12 phosphocreatine, 2 Na_2ATP , 0.2 Na_3GTP , and 0.1 leupeptin, pH adjusted to 7.2–7.3 with H_2SO_4 , 265–270 mosM/l. The pH of *N*-methyl-D-glucamine solutions was measured with a Corning Model 476570 probe. The external solution consisted of (in mM): 135 NaCl, 20 CsCl, 1 MgCl_2 , 10 HEPES, 0.001 tetrodotoxin, 2 BaCl_2 , and 10 glucose, pH adjusted to 7.3 with NaOH, 300–305 mosM/l. All reagents were obtained from Sigma except for sulfuric acid (Fluka, Ronkowa, NY).

Cell-attached patch recording

Recordings were obtained with an internal solution consisting of (in mM) 110 BaCl_2 and 10 HEPES, pH adjusted to 7.4 with tetraethylammonium hydroxide. Electrodes were made as for whole cell recordings except that they were coated with Sylgard. After seal formation, the transmembrane potential was nominally zeroed by bathing the cell in an isotonic K^+ solution containing (in mM) 140 potassium gluconate, 1 MgCl_2 , 5 ethylene glycol-bis(b-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, 10 HEPES, and 5 glucose, pH adjusted to 7.4 with KOH, 300–305 mosM/l). Transmembrane currents were evoked by stepping the electrode to –20 mV from a holding potential of +80 mV. The protocol is shown with the conventional polarity in the figures for clarity.

Receptor ligands and second-messenger reagents were made up as concentrated stocks in water or dimethyl sulfoxide and stored at –70°C. The DA receptor ligands DA, (–)-quinpirole, *R*(–)-propylnorapomorphine (NPA), *R*(+)-SCH-23390, (–) sulpiride, (+)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide and the muscarinic receptor agonist oxotremorine methiodide (oxo-M) were obtained from RBI, (Natick, MA). 8-(4-chlorophenolthio)-adenosine 3':5'-cyclic mono-

phosphate (cpt-cAMP), forskolin, 1,9-dideoxy forskolin, phorbol-12-myristate-13-acetate (PMA), and 4 α -phorbol were obtained from Sigma. The protein kinase C (PKC) inhibitors PKC_{19-31} and chelerythrine chloride were obtained from LC Laboratories (Woburn, MA). The calcium channel blocker ω -conotoxin GVIA (ω -CgTx) was obtained from Peninsula Labs (Belmont, CA) or Calbiochem, (San Diego, CA). Nifedipine was obtained from RBI and ω -agatoxin IVA (ω -AgTx) was a gift from Pfizer (Groton, CT). Aliquots were thawed and diluted the day of use. Final dilutions were made in external media containing 0.01–0.1% cytochrome C when ω -CgTx and/or ω -AgTx were used. The involvement of $\text{G}_{i/o}$ proteins was studied with the use of *N*-ethylmaleimide (NEM) (Sigma).

Recordings were obtained with an Axon Instruments 200A patch-clamp amplifier, controlled and monitored with a Quadra 900 Macintosh computer running AxoData (version 1.1) with a 125-kHz interface (Instrutech, Greatneck, NY). Electrode resistances were typically 2–4 $\text{M}\Omega$ in the bath. After seal rupture, series resistance (4–10 $\text{M}\Omega$) was compensated (70–90%) and periodically monitored. Recordings were made only from large neurons (>10 pF) that had short (<75 μm) proximal dendrites. The adequacy of voltage control was assessed after compensation by examining the tail currents generated by strong depolarizations. Drugs were applied with a gravity-fed “sewer pipe” manifold system. The application capillary (~150 μm ID) was positioned a few hundred micrometers from the cell under study. Solution changes were effected by electronic valves controlling the inflow to a manifold feeding a single outlet capillary. Solution changes typically were complete within <1 s; the time constant of Cd^{2+} block of Ca^{2+} current was 300 ms.

Statistical methods

Data analyses were performed with AxoGraph (Axon Instruments, version 2.0) and Kaleidagraph (Albeck Software, Reading, PA, version 3.0.4). 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); outliers are shown as asterisks or circles. Changes in current amplitude produced by receptor agonists were measured 20–30 s after beginning ligand exposure; at this point, the agonist-induced modulation had typically reached a quasisteady state.

The onset of the D_2 modulation was fit with a single- or double-exponential curve of the form $I = I_0 + I_1 \exp[-(t - t_0)/\tau_1] + I_2 \exp[-(t - t_0)/\tau_2]$, where I was peak current, t was time and t_0 was the time at which the drug was applied.

Single-neuron RNA harvest and reverse transcription–polymerase chain reaction analysis

After recording, cells 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 above). 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 the contents were ejected into a 0.5-ml Eppendorf tube containing (in μl) 5 diethyl pyrocarbonate-treated water, 1 RNAsin (28,000 U/ml), 1 dithiothreitol (0.1 M) and 1 oligo(dT) (0.5 $\mu\text{g}/\mu\text{l}$). The mixture was heated to 70°C for 10 min and incubated on ice for 1 min. Single-strand cDNA was synthesized from the cellular mRNA by addition of SuperScript II RT (1 μl , 200 U/ μl) and buffer [4 μl , 5X First

Strand Buffer: 250 mM tris(hydroxymethyl)aminomethane-HCl, 375 mM KCl, 15 mM MgCl₂, dithiothreitol (1 μl, 0.1 M), and mixed deoxynucleotide triphosphates (dNTPs) (1 μl, 10 mM). The reaction mixture (20 μl) was transferred to a 42°C water bath and incubated for 50 min. The reaction was terminated by heating the mixture to 70°C for 15 min and then icing. The RNA strand in the RNA-DNA hybrid was then removed by addition of 1 μl RNase H (2 U/μl) and incubation for 20 min at 37°C. All reagents except for RNasin (Promega, Madison, WI) were obtained from GIBCO BRL (Grand Island, NY). The cDNA from the reverse transcription (RT) of RNA in single neostriatal neurons was subjected to polymerase chain reaction (PCR) to detect the expression of various mRNAs.

The choline acetyltransferase (ChAT) mRNA (Brice et al. 1989) was identified with the use of a pair of primers flanking a splicing site near to the 3' terminus of the coding region. The upper primer was 5'-ATG GCC ATT GAC AAC CAT CTT CTG (nucleotides 1729–1752) and was located on exon 14. The lower primer was 5'-CCT TGA ACT GCA GAG GTC TCT CAT (nucleotides 2029–2052) and was located on exon 15. The size of the amplified ChAT cDNA was 324 base pairs (bp).

DA receptor mRNA specific primers were designed against the region coding for the third cytoplasmic loop or carboxyl tail. The PCR strategy employed was similar to that previously described by Vrana et al. (1995). Primers used for D_{1a} receptor mRNA (Genebank accession #M35077) detection were 5'-CAG TCC ATG CCA AGA ATT GCC AGA-3' (nucleotides 1119–1142) and 5'-AAT CGA TGC AGA ATG GCT GGG TCT-3' (nucleotides 1320–1343). The size of the amplified D_{1a} cDNA was 225 bp. Primers used for D₂ receptor mRNA (Genebank accession #X17458) detection were 5'-GCA GTC GAG CTT TCA GAG CC-3' (nucleotides 829–848) and 5'-TCT GCG GCT CAT CGT CTT AAG-3' (nucleotides 1212–1232). The size of the amplified D₂ cDNA was 404 (long isoform) and 317 (short isoform) bp. Primers used for D₃ receptor mRNA (Genebank accession #X53944) detection were 5'-CAT CCC ATT CGG CAG TTT TCA A-3' (nucleotides 823–844) and 5'-TGG GTG TCT CAA GGC AGT GTC T-3' (nucleotides 1002–1023). The size of the amplified D₃ cDNA was 201 bp. Primers used for D₄ receptor mRNA (Genebank accession #M84009) detection were 5'-TCA TGC TAC TGC TTT ACT GGG CCA-3' (nucleotides 729–752) and 5'-TCT GAG AGA GGT CTG ACT CTG GTC-3' (nucleotides 928–951). The size of the amplified D₄ cDNA was 223 bp. Primers used for D_{1b} receptor mRNA (Genebank accession #M69118) detection were 5'-AGT CGT GGA GCC TAT GAA CCT GAC-3' (nucleotides 1495–1518) and 5'-GCG TCG TTG GAG AGA TTT GAG ACA-3' (nucleotides 1988–2011). The size of the amplified D_{1b} cDNA was 517 bp.

PCR amplification was carried out with a thermal cycler (MJ Research, Watertown, MA) with thin-walled plastic tubes. Reaction mixtures contained 2–2.5 mM MgCl₂, 0.5 mM of each of the dNTPs, 0.8–1 μM primers, 2.5 U Taq DNA polymerase (Promega), 5 μl 10X Buffer (Promega), and 1–10 μl of the cDNA template made from the single-cell RT reaction. The thermal cycling program for these PCR amplifications was as follows: 94°C for 1 min, 58°C for 1 min, and 74°C for 1.5 min. This was performed for 45 cycles to obtain the signal for ChAT mRNA.

To maximize the probability of detecting DA receptor mRNAs, a two-stage multiplex amplification was performed. In the first step, the DA receptor cDNAs were selectively amplified with the use of one half of the single-cell cDNA (10 μl) as a template in a multiplex PCR reaction. All five DA receptor primers were added to a reaction mixture containing the same reagents as with conventional PCR except for slightly elevated MgCl₂ (4.0 mM) and dNTPs (1.0 mM) (Chamberlain and Chamberlain 1994). Twenty cycles were performed with the use of the following parameters: 94°C for 1 min, 58°C for 1 min, 72°C for 3 min. An aliquot of this PCR product (2 μl) was then used as a template for a second

round (40 cycles) of "touchdown" PCR amplification with each pair of specific primers. In this round, after the first 20 cycles the annealing temperature was decreased by 1° every other cycle, resulting in a final annealing temperature of 48°C.

PCR products (amplicons) were visualized by staining with ethidium bromide and separated by electrophoresis in 1.5–2% agarose gels. Representative products were sequenced with the use of a dye termination procedure by either the University of Tennessee Molecular Resource Center or St. Jude Children's Research Hospital sequencing facility and were found to match published sequences.

Care was taken to ensure that the PCR signal arose from cellular mRNA. 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.

RESULTS

Striatal cholinergic interneurons express primarily D₂ and D_{1b} DA receptor mRNAs

Giant aspiny, cholinergic interneurons make up 1–3% of the total neuronal population in the dorsal striatum (Bolam and Bennett 1995). In the acutely dissociated preparation, large neurons can readily be distinguished from medium spiny projection neurons (Fig. 1A). RT-PCR analysis of neurons with whole cell capacitances >10 pF revealed that ~90% expressed detectable levels of ChAT mRNA ($n = 48$) (Fig. 1B).

RT-PCR analysis was also used to determine how the expression of DA receptor subtypes was coordinated in identified cholinergic interneurons. These experiments used a two-step amplification procedure that was designed to maximize the probability of detecting low-abundance transcripts (Surmeier et al. 1996). These studies revealed that all cholinergic interneurons express D₂ mRNA (17/17) (Fig. 1, B and C). Long and short isoforms of the D₂ receptor mRNA were consistently coexpressed. D_{1b} mRNA was also detected in nearly 90% (26/29) of the interneurons tested, whereas D_{1a} mRNA was found in only ~20% (6/29). D₃ and D₄ mRNAs were not found in any of the neurons examined ($n = 17$). The plot in Fig. 1C summarizes how DA receptor mRNA expression was coordinated in a set of interneurons in which each cell was probed for all five receptor mRNAs ($n = 17$).

Activation of D₂ receptors reduces neostriatal Ca²⁺ currents

The application of the D₂ agonists (–)-quinpirole (10 μM) or NPA (10 μM) rapidly and reversibly decreased Ba²⁺ currents through Ca²⁺ channels, as did the application of DA (10 μM) (Fig. 2, A and B). The median reduction in peak Ba²⁺ current produced by NPA or DA was 20% ($n = 29$), whereas quinpirole was less potent (14%, $n = 30$). The application of D₁-class agonists, on the other hand, produced little or no reduction of Ba²⁺ currents ($n = 18$, data not shown). Because D_{1b} receptor mRNA was found in nearly 90% of the cholinergic neurons sampled (Fig. 1C), these results suggest that D_{1b} receptors were not coupled to somatodendritic Ca²⁺ channels in our recording condition

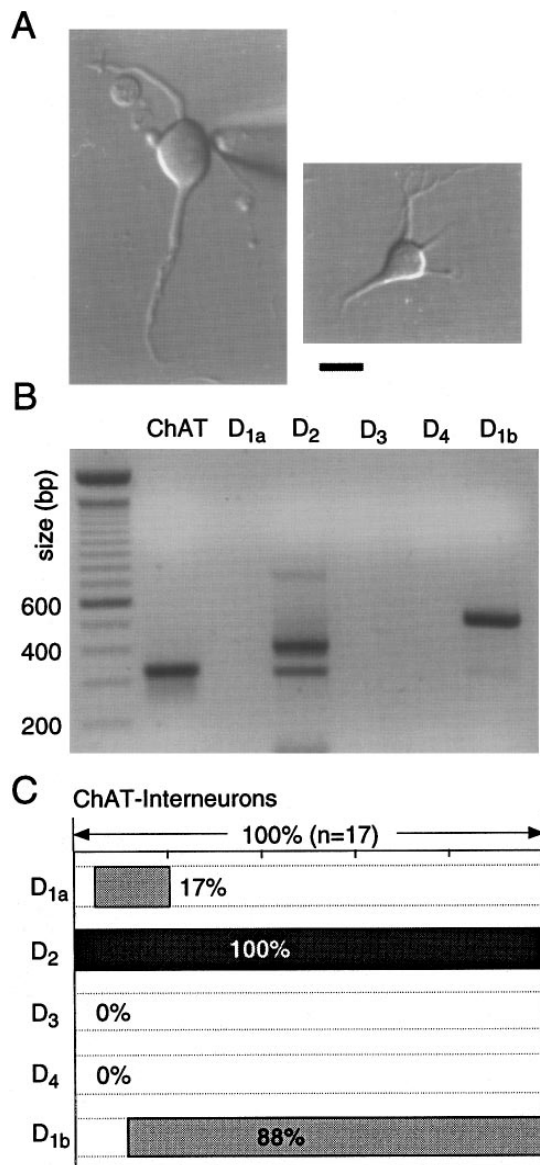


FIG. 1. Cholinergic interneurons express primarily D₂ and D_{1b} dopamine receptor mRNAs. *A*: photomicrograph of an acutely isolated neostriatal large interneuron and a medium-sized neuron. Scale bar: 10 μ m. *B*: polymerase chain reaction (PCR) profile of a single choline acetyltransferase (ChAT)-positive neuron having detectable levels of D₂ and D_{1b} mRNAs. *C*: bar plot showing the coordinated expression of dopamine receptor subtypes D_{1a}-D₄ in ChAT-positive neurons detected by the multiplex PCR method ($n = 17$).

(although D_{1b} functional assays were not performed in cells subjected to RT-PCR analysis).

To verify that D₂ receptors were mediating the modulation seen with quinpirole or NPA, the ability of D₂-class antagonist (-) sulpiride to prevent their action was examined. As shown in Fig. 2C, sulpiride (1 μ M) almost completely eliminated the effects of quinpirole (10 μ M). Removing the antagonist restored the ability of quinpirole to modulate currents. Similar results were seen in every cell tested ($n = 3$, see Fig. 2C, inset).

D₂ receptors target N-type Ca²⁺ currents

Our previous studies have shown that neostriatal cholinergic interneurons express five distinct types of Ca²⁺ channel

α 1 subunit (Yan and Surmeier 1996). To determine which of these channels was affected by activation of D₂ receptors, specific channel antagonists were used. Block of L-type channels with nifedipine (5 μ M) did not affect the D₂ modulation (data not shown). On the other hand, the application of the N channel antagonist ω -CgTx (1 μ M) almost com-

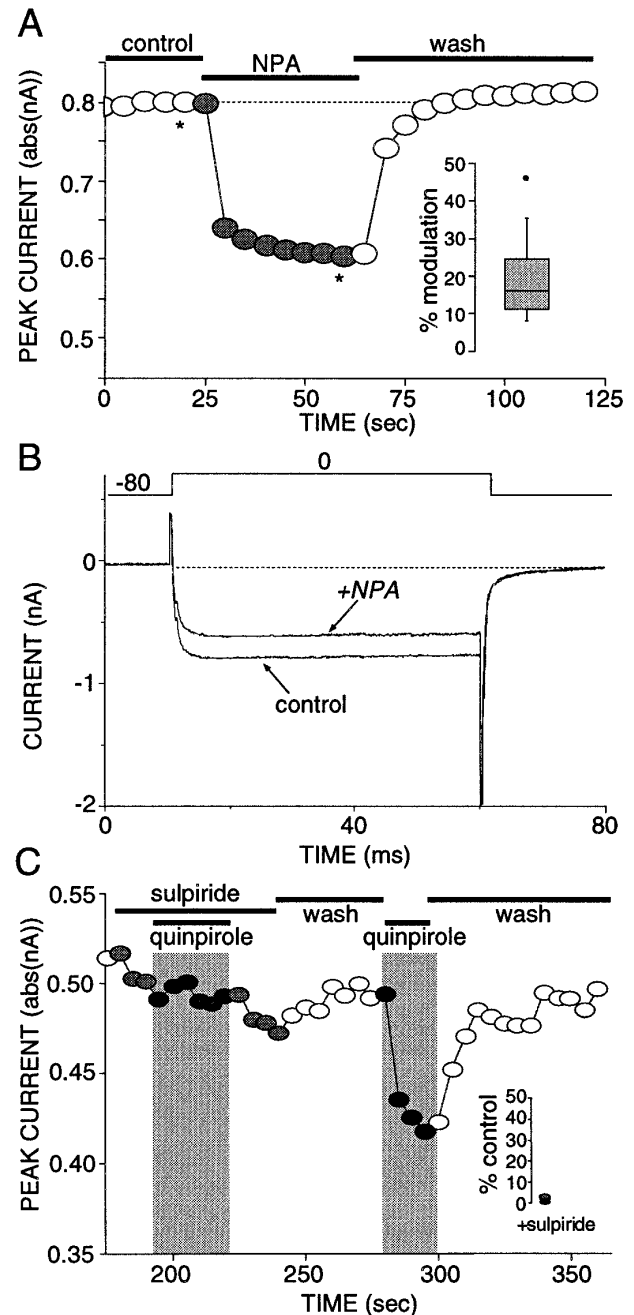


FIG. 2. D₂-class agonists produce a reversible decrease in Ba²⁺ currents that was blocked by D₂ receptor antagonists. *A*: plot of peak current evoked by a voltage step to 0 mV as a function of time and agonist application. The D₂-class agonist R(-)-propylnorapomorphine (NPA) (10 μ M) rapidly and reversibly reduced peak currents. *B*: current traces from the data used to construct A. Asterisks: points in the record used. *C*: plot of peak current evoked by a voltage ramp protocol as a function of time and ligand application. In the presence of (-) sulpiride (1 μ M), quinpirole had little effect; washing led to recovery of the quinpirole effect.

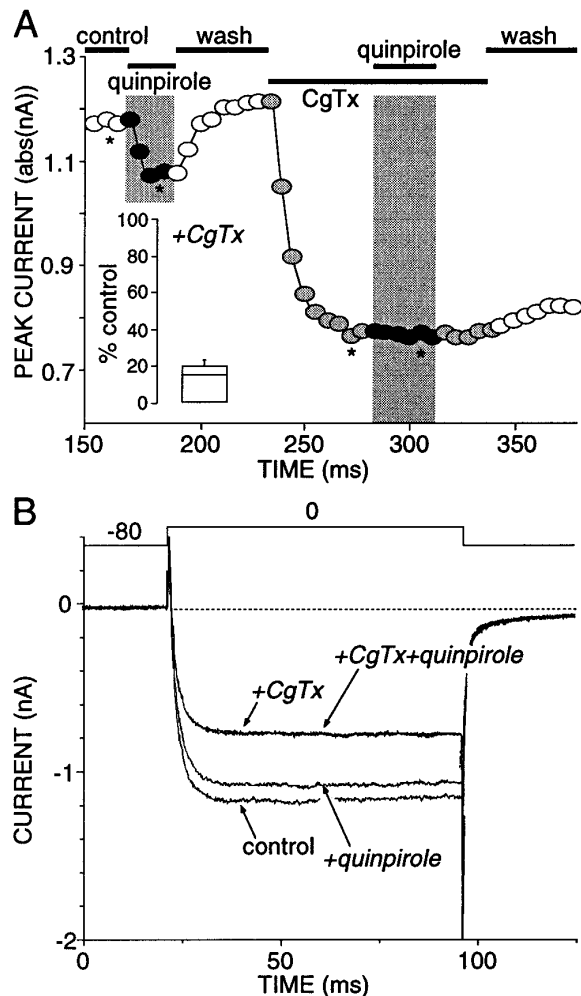


FIG. 3. Activation of D_2 receptors reduces N-type Ca^{2+} currents. *A*: plot of peak current evoked by a step to 0 mV as a function of time and drug application. The modulation by quinpirole was eliminated by block of N-type channels with ω -conotoxin GVIA (ω -CgTx) (1 μ M). *Inset*: box plot summary of the percent control modulation by quinpirole in the presence of ω -CgTx ($n = 7$). *B*: representative current traces from the records used to generate the plot in *A*, showing the modulation effect of quinpirole before and after application of ω -CgTx. Asterisks in *A*: times the current traces shown in *B* were collected.

pletely eliminated the effects of D_2 receptor agonists. In Fig. 3*A*, a time course from one of these experiments is shown. Before block of N-type channels, quinpirole reduced peak currents (Fig. 3, *A* and *B*). Subsequent to the application of ω -CgTx, quinpirole had no detectable effect (Fig. 3, *A* and *B*). Figure 3*A*, *inset*, is a box plot summarizing the modulation by quinpirole after the block of N-type channels ($n = 7$). On average, >80% of the D_2 modulation involved regulation of N-type channels.

D_2 receptors act through a NEM-sensitive G protein

Previous studies in cell lines have shown that D_2 receptors are capable of acting through PTX-sensitive G proteins ($G_{i/o}$ class) to reduce Ca^{2+} currents (Brown and Seabrook 1995; Lledo et al. 1992). If the D_2 effects in cholinergic interneurons also involved PTX-sensitive G proteins, then a brief application of the sulfhydryl alkylating agent NEM should block the modulation, because NEM has been shown to

disrupt coupling of PTX-sensitive G proteins to Ca^{2+} channels (Shapiro et al. 1994). We have previously shown that NEM treatment of cholinergic interneurons uncouples M_2 muscarinic receptors from N-type channels as effectively as PTX treatment (Yan and Surmeier 1996).

The results from one of these experiments are shown in Fig. 4. In this neuron, quinpirole (10 μ M) and the muscarinic receptor agonist oxo-M (10 μ M) both reduced peak currents (Fig. 4, *A* and *B*). A brief (2 min) application of NEM (50 μ M) significantly reduced the response both to oxo-M and to quinpirole (Fig. 4, *A* and *C*). Figure 4*A*, *inset*, is a box plot of the percent modulation (relative to control) after the application of NEM in four experiments. Although it was slightly more effective in blocking the M_2 modulation, NEM reduced the D_2 modulation by >60% on average, suggesting that a $G_{i/o}$ -class G protein mediated a significant component of the modulation.

To provide independent evidence for the involvement of G proteins in the modulation, the impact of guanosine-5'-O-(2-thiodiphosphate) (GDP β S) dialysis was examined. GDP β S competes with endogenous GTP for the nucleotide binding site on $G\alpha$ proteins, blocking receptor-mediated activation (Eckstein et al. 1979). As summarized in Fig. 4*D*, dialysis with GDP β S (2 mM) significantly reduced the modulation produced by D_2 agonists and muscarinic agonists, arguing that the D_2 modulation was dependent on G proteins.

Modulation by D_2 agonists is not reversed by strong depolarization

A common characteristic of agonist-induced reductions in Ca^{2+} current is that strong depolarization partially reverses the modulation (Bean 1989; Hille 1994). The muscarinic modulation of Ca^{2+} currents in the cholinergic interneuron is nearly halved by a prepulse to +100 mV (Yan and Surmeier 1996). To determine whether this feature was shared by the D_2 modulation, D_2 agonists were applied with and without a depolarizing prepulse and the modulations compared. As shown in Fig. 5, the reduction in currents produced by D_2 agonists was almost unaffected by depolarizing the membrane to +100 mV. In a sample of 11 cells, the median modulation produced by D_2 agonists after a prepulse was >80% of the control value (Fig. 5*B*, *inset*).

D_2 dopaminergic modulation is not affected by intracellular cAMP levels or inhibition of PKC

D_2 receptors can inhibit adenylyl cyclase, reducing cytosolic cAMP levels and protein kinase A activity (Stoof and Keibian 1984; Vallar and Meldolesi 1989). Protein kinase A is known to regulate the activity of several types of Ca^{2+} channels found in the brain (Fournier et al. 1993; Mogul et al. 1993; Surmeier et al. 1995a). If this were the mechanism by which D_2 receptors reduced Ca^{2+} currents in cholinergic interneurons, then bath application of the membrane permeant cAMP analogue cpt-cAMP should reverse the modulation by countering the reduction in cytosolic cAMP levels. However, in all interneurons tested, the D_2 modulation of Ca^{2+} channels was unaffected by bath application of cpt-cAMP (500 μ M) ($n = 4$, data not shown). As an alternative strategy, cells were exposed to the adenylyl cyclase activator forskolin (10 μ M) to increase the intracellular cAMP levels

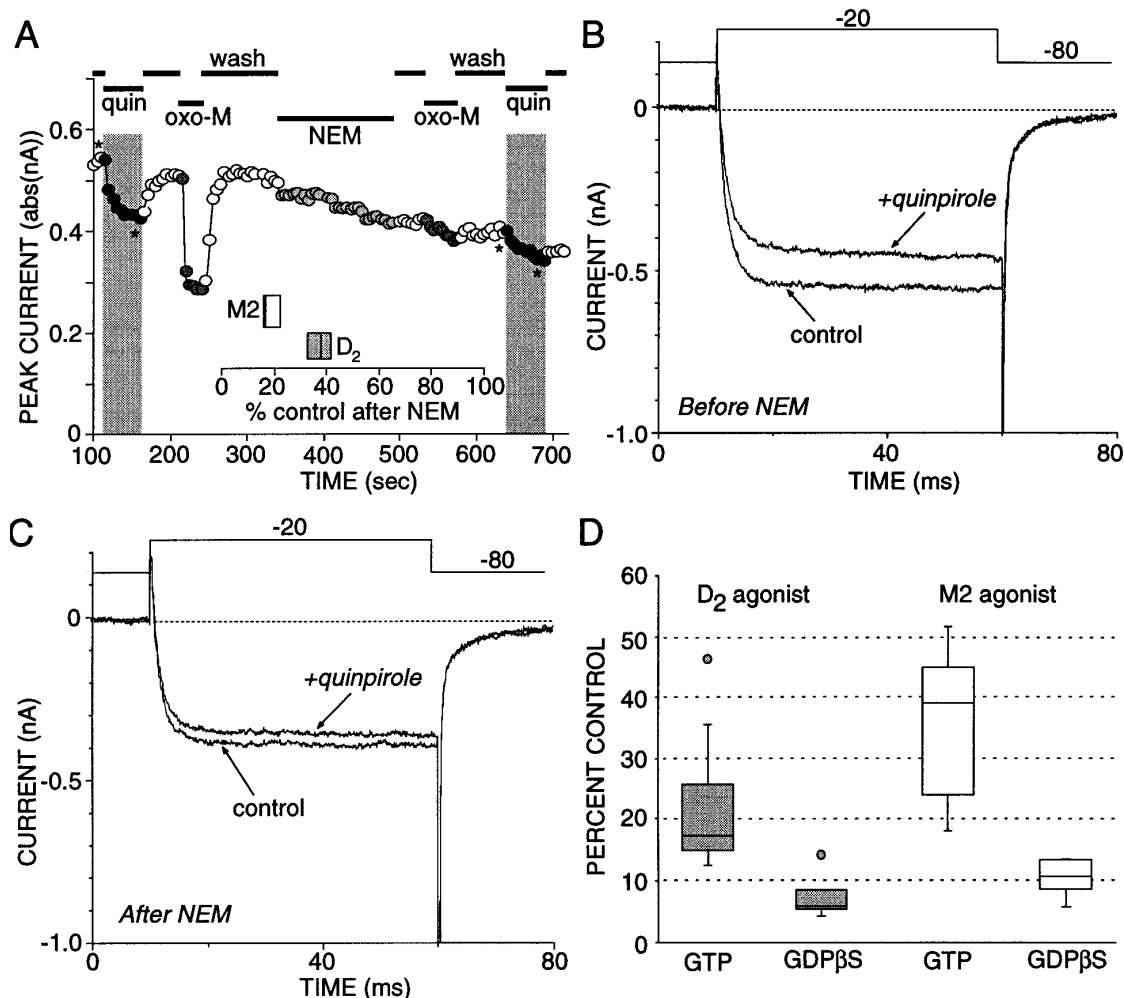


FIG. 4. Modulation by D_2 receptors depends on a $G_{i/o}$ -class G protein. *A*: plot of peak current evoked by a step to -20 mV as a function of time and drug application. Both quinpirole ($10 \mu\text{M}$) and oxotremorine methiodide (oxo-M) ($10 \mu\text{M}$) reduced peak currents; these effects were almost completely eliminated by a 2-min application of *N*-ethylmaleimide (NEM) ($50 \mu\text{M}$). *Inset*: box plot showing the D_2 and M2 modulations relative to control values after a 2-min application of NEM. *B*: representative current traces (at time points denoted in *A* by *) showing the modulation by quinpirole before application of NEM. *C*: representative current traces (at time points denoted in *A* by *) showing the effect of quinpirole after application of NEM. *D*: box plot of the percent reduction in peak current produced by the D_2 -class agonist NPA ($10 \mu\text{M}$) and the muscarinic agonist oxo-M ($10 \mu\text{M}$) in cells dialyzed with a normal internal containing GTP (0.2 mM) and in cells dialyzed with guanosine-5'-O-(2-thiodiphosphate) (GDP β S) (2 mM). Both receptor-mediated effects on Ca^{2+} currents were greatly reduced by GDP β S dialysis.

and then D_2 agonists were applied. In agreement with the cAMP analogue experiments, the response to D_2 agonists was not noticeably altered by forskolin ($n = 5$) (data not shown).

In chick dorsal root ganglion neurons, noradrenaline reduces N-type Ca^{2+} currents by activating receptors coupled to G_i -class G proteins. The signaling pathway leading to reduction in N-type current in these cells depends on PKC (Diversé-Pierluissi and Dunlap 1993; Rane et al. 1989). Because D_2 receptors also couple to G_i -class proteins, it is possible that a similar mechanism mediated our response. To test this hypothesis, neurons were dialyzed with the PKC inhibitor chelerythrine chloride ($20 \mu\text{M}$) or PKC $_{19-31}$ ($50 \mu\text{M}$) and D_2 -class agonists were applied. Although both effectively blocked the effects of phorbol esters (see below), they had no detectable effect on the modulation produced by D_2 -class agonists ($n = 8$, data not shown). The D_2 modulation was also not noticeably affected by chelation of intra-

cellular Ca^{2+} to low-nanomolar levels by dialysis with solutions containing 10 mM ethylene glycol-bis(b-aminoethyl ether) *N,N,N',N'*-tetraacetic acid ($n = 6$, median reduction = 17%, data not shown).

D₂ modulation has fast onset kinetics and is membrane delimited

A number of studies have suggested that neuronal G protein-coupled receptors can influence ion channels through a membrane-delimited pathway (Brown 1993; Hescheler and Schultz 1993; Hille 1994). One of the characteristics of this type of coupling is rapid onset kinetics. To see whether the D_2 modulation of Ca^{2+} channels was fast enough to be consistent with this sort of mechanism, onset kinetics was measured at low and high agonist concentrations. In these protocols, a short (30 ms) depolarizing step was repeated once a second (faster rates led to N current inactivation). When a high concentration ($200 \mu\text{M}$)

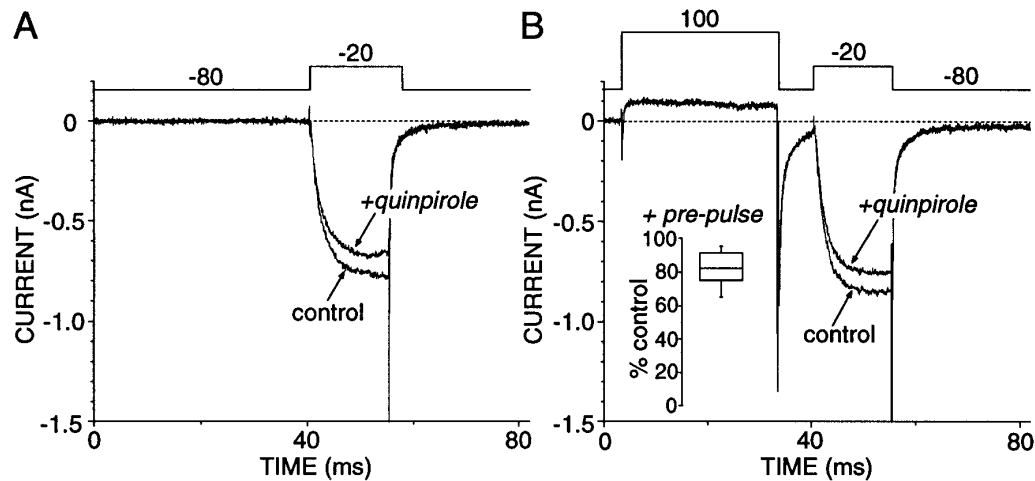


FIG. 5. Effects of D_2 receptor activation on N-type currents were not attenuated by depolarizing prepulses. *A*: currents evoked by a step to -20 mV before and after quinpirole ($10 \mu\text{M}$) application. *B*: currents in the same neuron evoked by a brief step to $+100$ mV, a return to -80 mV, and then a test step to -20 mV in the presence and absence of quinpirole. Note that the % reduction was not altered by the prepulse. *Inset*: box plot of the modulation produced by D_2 -class agonists after a depolarizing prepulse (as a percentage of the modulation in the absence of a depolarizing prepulse) in a sample of 11 cells.

of quinpirole was applied, currents were rapidly reduced (Fig. 6, *A* and *B*). The onset of the modulation was typically biexponential, with a principal, fast time constant of <1 s (Fig. 6*B*). At lower agonist concentrations ($10 \mu\text{M}$), the onset kinetics were slower (~ 2 s). Figure 6*B*, *inset*, shows a box plot summary of the onset time constants at high ($200 \mu\text{M}$) and low ($10 \mu\text{M}$) agonist concentrations in four experiments. These onset kinetics are well within the range of those reported for membrane-delimited signaling pathways in other systems (Hille 1994).

Although suggestive, rapid onset kinetics are not unequivocal evidence for membrane-delimited signaling. A more direct test is to record Ba^{2+} currents in the cell-attached recording configuration and apply agonists to the bath. If the modulation by D_2 -class agonists was mediated by a soluble second messenger, then their effects should be similar to those seen in whole cell recordings. However, in all the neurons studied ($n = 4$), bath application of NPA failed to alter the Ba^{2+} currents in cell-attached macropatches (Fig. 6*C*), despite the fact that application of same agonist to adjacent interneurons recorded in the whole cell configuration produced a robust modulation of currents. These results are consistent with the hypothesis that D_2 receptors modulate N-type Ca^{2+} channels through a membrane-delimited G protein pathway.

Dopaminergic and muscarinic modulations are subadditive

Activation of M_2 -class muscarinic receptors in cholinergic interneurons also reduces Ba^{2+} current through N-type Ca^{2+} channels (Yan and Surmeier 1996). As shown here for D_2 receptors, the M_2 -class modulation was fast, membrane delimited, and mediated by $G_{i/o}$ -class G proteins. As a consequence, it is possible that these two signaling pathways share common elements. One sign of this would be a subadditivity in the modulation of currents. To test for this eventuality, D_2 - and M_2 -class agonists were applied alone and together. Data from one of these experiments is shown in Fig. 7*A*, where peak current (evoked by a test step) is plotted as a function of time and drug application. Application of NPA

($10 \mu\text{M}$) (Fig. 7*B*) or the muscarinic agonist oxo-M ($10 \mu\text{M}$) alone reduced Ba^{2+} currents by 15 and 23%, respectively. However, in the presence of the muscarinic agonist, the application of NPA did not reduce currents further (Fig. 7*C*). A similar pattern of results was observed in all 13 cells tested. In the presence of oxo-M, the median D_2 modulation was only 10–20% of that seen in the absence of oxo-M (Fig. 7*A*, *inset*).

Activation of PKC disrupts the muscarinic but not the dopaminergic modulation

In several cell types, G protein-mediated inhibition of N-type Ca^{2+} channels can be disrupted by activation of PKC (Swartz 1993). To test whether interneurons possessed a similar sensitivity, PKC was activated by external application of PMA and its effect on the D_2 and M_2 receptor evoked modulations was studied. As shown in Fig. 8*A*, before PMA treatment, both oxo-M and NPA reduced currents (Fig. 8, *A* and *B*). PMA (500 nM) treatment led to a runup in peak currents (Swartz 1993; Yang and Tsien 1993). After PMA exposure, oxo-M had substantially less of an impact on currents, whereas NPA continued to reduce currents to a similar extent (Fig. 8, *A* and *C*). Figure 8*A*, *inset*, is a box plot summarizing the results from this and other experiments. After PMA treatment, oxo-M produced (on average) only $\sim 30\%$ of the modulation seen before treatment ($n = 17$). On the other hand, D_2 agonists reduced currents nearly as well, the median modulation being $\sim 80\%$ of the pretreatment value ($n = 14$). Application of the inactive phorbol analogue 4α -phorbol (500 nM) was without effect ($n = 5$, data not shown). Furthermore, dialysis with the PKC inhibitors PKC_{19-31} ($20 \mu\text{M}$) ($n = 2$) or chelerythrine chloride ($5 \mu\text{M}$) ($n = 3$) blocked the effects of bath application of PMA (data not shown), arguing that its effects were mediated by PKC activation.

DISCUSSION

Cholinergic interneurons express primarily D_2 and D_{1b} mRNAs

Although they make up only a few percent of all the neurons in the dorsal neostriatum, giant aspiny interneurons

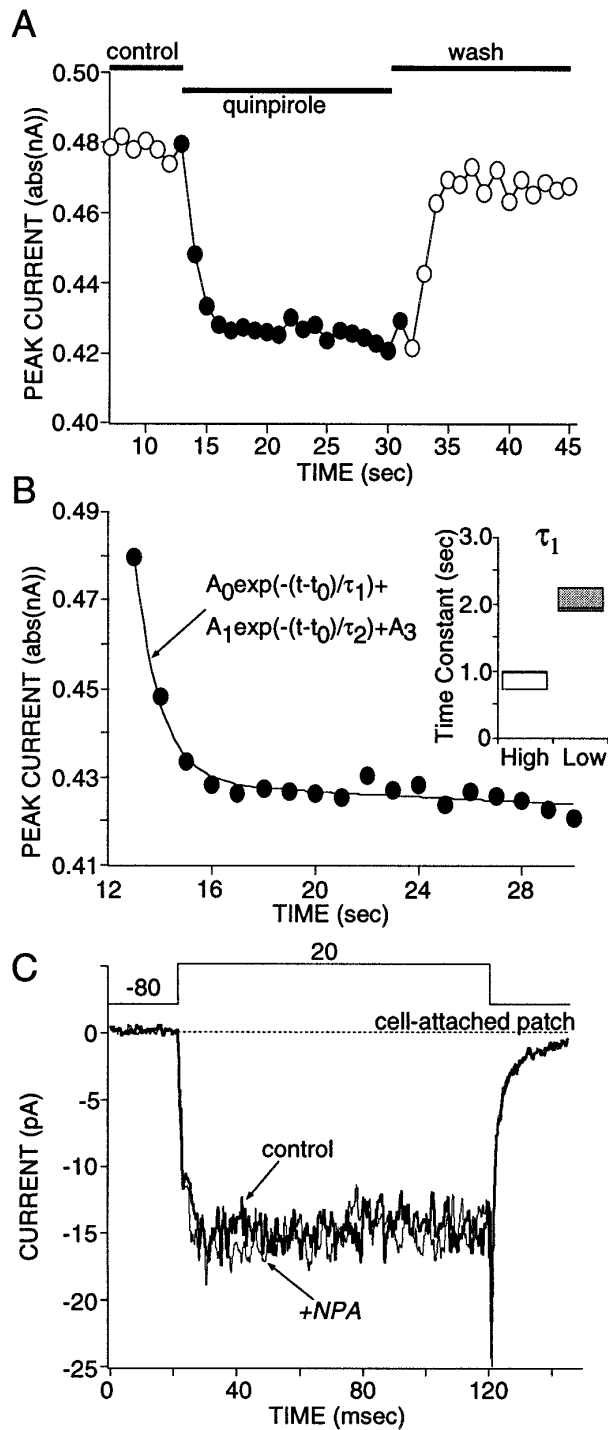


FIG. 6. D_2 receptor modulation was rapid and membrane delimited. **A**: plot of peak current evoked by a step to 0 mV repeated at 1 Hz as a function of time and quinpirole ($200 \mu\text{M}$) application. **B**: exponential fit of the onset of the modulation. Fitted line was determined by a least-squares algorithm; the fitted time constant is shown. *Inset*: box plot summary of onset time constants at high concentration ($200 \mu\text{M}$) of quinpirole ($n = 4$) and low concentration ($10 \mu\text{M}$) of quinpirole ($n = 4$). **C**: cell-attached patch recording of Ba^{2+} currents evoked by a step depolarization to +20 mV from a holding potential of -80 mV in the presence and absence of bath-applied NPA ($10 \mu\text{M}$). Each trace is an average of 3 successive sweeps. NPA did not reduce current amplitudes in any of the cells tested ($n = 4$). Shock artifact has been suppressed.

could readily be visualized in the dissociated preparation and subsequently identified by RT-PCR detection of ChAT mRNA (cf. Kawaguchi 1993). These neurons invariably had detectable levels of mRNA for both short and long isoforms of the D_2 DA receptor. The majority of interneurons (90%) also coexpressed mRNA for the D_{1b} DA receptor. This finding is in agreement with a recent immunocytochemical work showing that many large, presumed cholinergic interneurons expressed D_{1b} -like receptor protein (Bergson et al. 1995a,b). In contrast, D_{1a} receptor mRNA was found in only ~20% of our sample. This estimate is in good agreement with previous ones based on in situ hybridization (Le Moine et al. 1991; Weiner et al. 1991).

D_3 and D_4 mRNAs were not detected in any cholinergic interneuron. The apparent absence of these mRNAs was not a consequence of an inability to detect these mRNAs per se, because they have been detected in many medium spiny projection neurons with the use of identical techniques ($n > 50$) (Surmeier et al. 1996).

D_2 receptors reduce N-type Ca^{2+} channel currents through a membrane-delimited pathway

D_2 receptor agonists, but not D_1 -class agonists, rapidly and reversibly reduced Ba^{2+} current through Ca^{2+} channels. In light of the fact that D_2 receptor isoforms were the only D_2 -class receptor mRNAs found in our sample of interneurons, it seems highly likely that these effects were mediated by D_2 receptors, not D_3 or D_4 receptors.

In other cell types, D_2 receptors have been linked to inhibition of voltage-dependent Ca^{2+} channels (Brown and Seabrook 1995; Lledo et al. 1992; Seabrook et al. 1994a,b). As expected from biochemical studies (Stoof and Keibadian 1984), the signaling pathways employed in these cells involved a PTX-sensitive G protein of the G_i or G_o class ($G_{i/o}$). In peripheral and central neurons, brief exposure to NEM has been shown to selectively disrupt PTX-sensitive G proteins (Shapiro et al. 1994; Yan and Surmeier 1996). In neostriatal cholinergic interneurons, the D_2 dopaminergic modulation of N-type Ca^{2+} channels was significantly reduced by a brief exposure to NEM, arguing that PTX-sensitive G proteins were responsible for the D_2 modulation.

The best described of the cytosolic consequences of D_2 receptor activation in the dorsal striatum is the reduction in cAMP levels following the inhibition of adenylyl cyclase (Stoof and Keibadian 1984). Both short and long forms of the D_2 receptor are known to activate G_i proteins that inhibit adenylyl cyclase (Senogles 1994). However, cholinergic interneurons appear to preferentially express a type of adenylyl cyclase (type II) that is not inhibited by G_i proteins (Furuyama et al. 1993; Mons and Cooper 1994; Taussig et al. 1994). This fact and the failure of forskolin or superfusion with cAMP analogues to alter the D_2 response is consistent with the proposition that D_2 receptors exert their effects on N-type channels through some mechanism other than inhibition of adenylyl cyclase.

Two other cytosolic signaling pathways are known to be linked to D_2 receptors. In transfected cell lines, D_2 receptors can potentiate Ca^{2+} -mediated stimulation of phospholipase A2 and the production of arachidonic acid (Piomelli et al. 1991). Arachidonic acid reduces voltage-dependent Ca^{2+} currents in neostriatal neurons (unpublished observations).

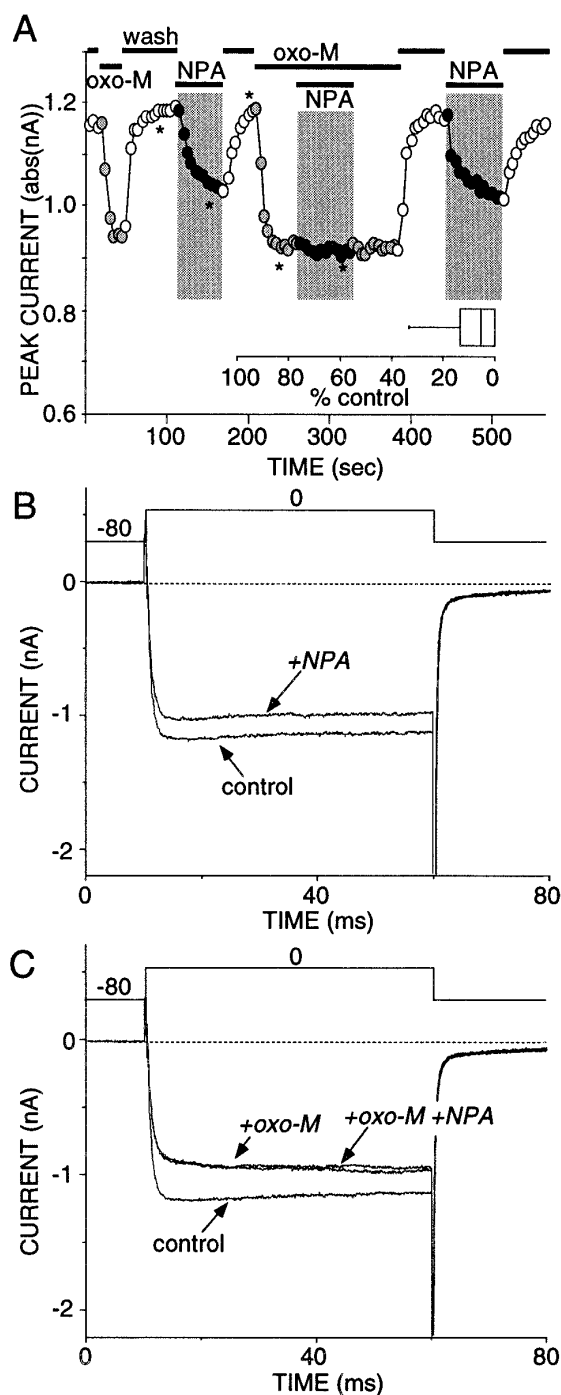


FIG. 7. D_2 and M2 muscarinic modulations of Ca^{2+} channels are subadditive. *A*: plot of peak current evoked by a step to 0 mV as a function of time and ligand application. The muscarinic agonist oxo-M (10 μM) alone and the D_2 -class agonist NPA (10 μM) alone reduced Ba^{2+} currents. Application of NPA in the presence of oxo-M did not produce any additional inhibition. After oxo-M was washed off, NPA again produced a robust modulation. *Inset*: box plot summarizing the effects of D_2 -class agonists in the presence of oxo-M (as a percentage of the modulation in the absence of oxo-M) in a sample of 13 cells. *B*: representative current traces showing the modulation by NPA alone (at time points denoted in *A* by *). *C*: representative current traces showing the modulation by oxo-M alone and by the coapplication of NPA and oxo-M (at time points denoted in *A* by *).

However, chelation of intracellular Ca^{2+} to low-nanomolar levels and the use of Ba^{2+} as the charge carrier failed to block the effects of D_2 agonists, arguing that this pathway was not crucial to the modulation. Furthermore, PKC potentiates this D_2 linkage (Di Marzo et al. 1992), yet the D_2 modulation was unaffected by PKC activation or inhibition (see below). This observation also argues against a direct PKC mediation of the D_2 effects. In chick neurons, stimulation of G_i proteins (as might be expected following D_2 receptor activation) leads to activation of PKC and reduction of N-type Ca^{2+} currents (Diversé-Pierluissi and Dunlap 1993; Rane et al. 1989). Clearly, this mechanism does not seem to be involved here.

In the absence of compelling evidence for a cytosolic messenger, the most likely signaling pathway is one that is restricted to the membrane. So-called membrane-delimited signaling pathways have been described in several peripheral and central neurons (Brown 1993; Hescheler and Schultz 1993; Hille 1994). The arguments in favor of a membrane-delimited model here are similar to those presented for other cell types. First, as mentioned above, known cytosolic pathways do not seem to mediate the modulation. Second, the modulation was rapid, taking ~ 1 s to stabilize at high agonist concentrations (this time constant is an upper bound placed on our recordings by the drug delivery system). And third, the modulation was not seen in cell-attached patch recordings when agonist was applied outside the patch to the bath (cytosolic 2nd messengers should not be impeded by this recording configuration).

Membrane-delimited modulation of N-type Ca^{2+} channels is often mediated by PTX-sensitive G proteins, as in our case. The alterations in channel behavior produced by this type of pathway are typically voltage dependent (Hille 1994). For example, activation of M2 muscarinic receptors in cholinergic interneurons slows the kinetics of N-type currents activated by moderate depolarization and reduces peak currents through a mechanism that is largely reversed by strong depolarization (Yan and Surmeier 1996). This type of modulation has been hypothesized to be the result of a shift in channel gating from a "willing" to a "reluctant" mode (Bean 1989) subsequent to G protein binding to the channel (Boland and Beam 1993).

The D_2 modulation of N-type currents, in contrast, did not produce a noticeable slowing of current kinetics and was not reversed significantly by strong depolarization. Membrane-delimited modulations of N-type Ca^{2+} channels that do not produce a shift in gating have been described in a number of cell types (Hille 1994). Typically, however, these signaling pathways rely on non-PTX-sensitive G proteins in contrast to the D_2 modulation.

D_2 dopaminergic and M2 muscarinic signaling pathways share a common target but have differentiable components

M2 and D_2 signaling pathways differed in several other respects (see Table 1). One was that NEM was more effective in disrupting the M2 pathway than the D_2 pathway. Given the uncertainties in determining the susceptibility to NEM alkylation, it is difficult to infer much from this other than that the G proteins in these two pathways differ in structure or accessibility. This feature may be related to the impact of PKC on the two signaling pathways. PKC clearly

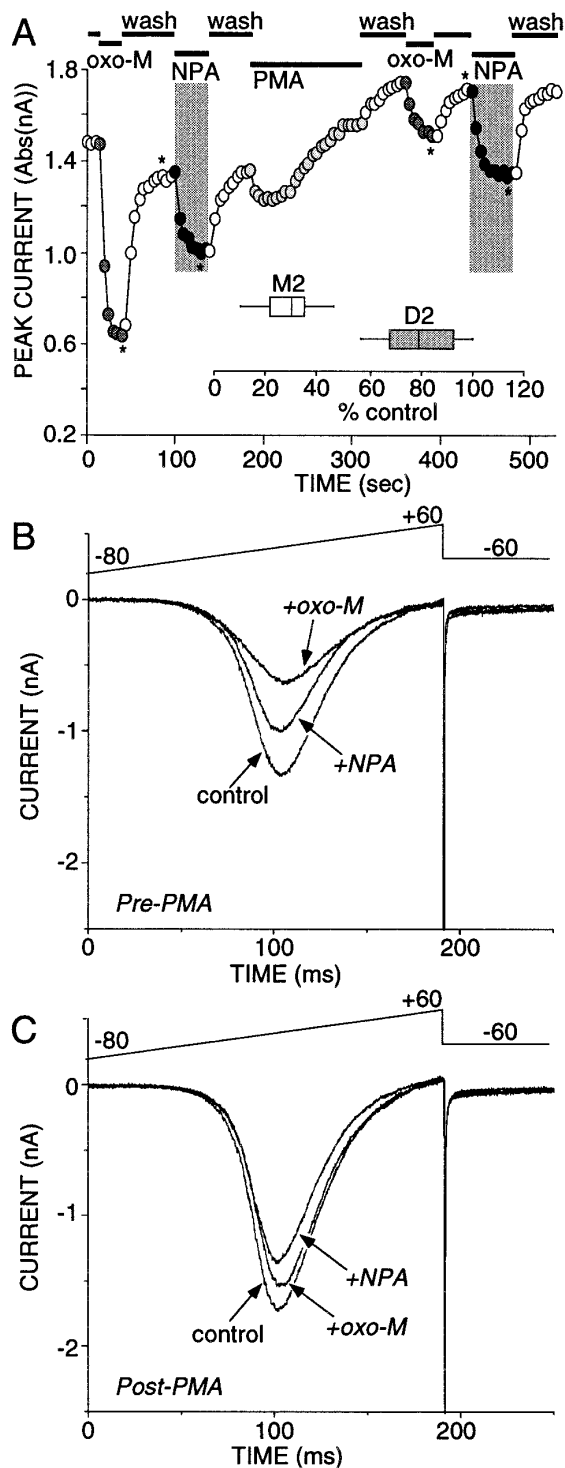


FIG. 8. Activation of protein kinase C (PKC) disrupted the M2 muscarinic modulation of Ca^{2+} currents, but not the D_2 dopaminergic modulation. *A*: plot of peak current evoked by a voltage ramp protocol as a function of time and drug application. Oxo-M ($10 \mu\text{M}$) and NPA ($10 \mu\text{M}$) reduced Ba^{2+} currents. After 2 min of treatment with phorbol-12-myristate-13-acetate (PMA, 500 nM), the oxo-M effect was substantially reduced, but the NPA effect was not affected. *Inset*: box plot summary of the modulation by M2 ($n = 17$) and D_2 ($n = 14$) receptors after treatment of PMA (relative to the pre-PMA control). *B*: representative current traces showing the modulation by oxo-M and NPA before the application of PMA (at time points denoted in *A* by *). *C*: representative current traces showing the modulation by oxo-M and NPA after the application of PMA (at time points denoted in *A* by *).

disrupted the muscarinic modulation of N-type channels but did not affect the D_2 modulation. This finding is consistent with previous studies showing that PKC disrupts voltage-dependent, G protein-mediated inhibition of N-type channels that is accompanied by kinetic slowing as with the M2 modulation (Swartz 1993; Swartz et al. 1993; Yan and Surmeier 1996; Zhu and Ikeda 1994a). However, it is unclear whether the key site of PKC phosphorylation is a G protein subunit or the ion channel.

If channel phosphorylation were the determinant of PKC's effect and both M2 and D_2 pathways used similar mechanisms, then they should target different subsets of N-type channels. There is ample evidence for alternative splicing and proteolytic processing of class B $\alpha 1$ subunits that could lead to functional heterogeneity (Hell et al. 1994; Williams et al. 1992). But, if this were the case, then M2 and D_2 modulations should be additive. In fact, they were distinctly subadditive, arguing that both pathways target the same channel population.

Assuming that D_2 and M2 pathways converge on the same N-type Ca^{2+} channels, how are they different? Recent work strongly suggests that G protein $\beta\gamma$ -subunits mediate voltage-dependent inhibition of N- and P/Q-type Ca^{2+} channels (Herlitze et al. 1996; Ikeda 1996; cf. Diversé-Pierluissi et al. 1995; Taussig et al. 1994; Zhu and Ikeda 1994b). Thus one would predict that the M2 signaling pathway employs $\beta\gamma$ -subunits to inhibit N-type channels. It is unclear at present whether voltage-independent modulation of N-type channels utilizes similar mechanisms. There is compelling evidence that G protein α_o -subunits are involved in the receptor-mediated, voltage-independent modulation of Ca^{2+} channels (Kleuss et al. 1991; Taussig et al. 1994), in particular that mediated by D_2 receptors (Baertschi et al. 1992; Lledo et al. 1992). But the ability of G α antibodies or antisense to disrupt receptor-mediated processes may reflect requirements of the receptor/G protein coupling, not G protein/ Ca^{2+} channel coupling (Hille 1994).

In this light, the most parsimonious hypothesis is that both voltage-dependent (M2) and voltage-independent (D_2) modulation of N-type channels are mediated by $\beta\gamma$ -subunits. The phenomenological differences between these two pathways suggest that they rely on different $\beta\Delta$ -subunits and/or have distinct channel binding sites (Kleuss et al. 1992, 1993). There are five cloned β -subunits and seven cloned γ -subunits (Clapham 1994; Watson et al. 1994), providing ample combinatorial diversity for such a scheme. It remains to be determined whether these putative differences in G protein linkage to N-type channels can account for the selective action of PKC on the M2 pathway.

Functional consequences

The D_2 -receptor-mediated reduction of Ca^{2+} current through N-type channels should attenuate the dendritic invasion of initial segment spikes (Sprutson et al. 1995) and the active augmentation of excitatory synaptic events arising from cortical or thalamic sources (Bernander et al. 1994; Kim and Connors 1993; Wilson 1993). Because membrane-delimited signaling is spatially and temporally focused (Brown 1993), this type of control could be of importance in regulating the computational functions of dendrites.

Ca^{2+} flux through N-type channels is also an important

TABLE 1.

| | Receptor Subtype | |
|--------------|--|--|
| | D ₂ | M2 |
| Similarities | Fast Membrane delimited NEM sensitive Targets N-type Ca ²⁺ channels | Fast Membrane delimited NEM sensitive Targets N-type Ca ²⁺ channels |
| Differences | Voltage independent PKC insensitive | Voltage dependent PKC sensitive |

NEM, *N*-ethylmaleimide; PKC, protein kinase C.

determinant of transmitter release (Dunlap et al. 1995). The inhibition of N-type Ca²⁺ channels provides a cellular mechanism for the well-established reduction of striatal ACh release by D₂ agonists (Di Chiara et al. 1994). The convergent regulation of N-type channels by M2 muscarinic and D₂ receptors also explains why the suppression of evoked ACh release by these receptors is subadditive (Drukarch et al. 1990; Stoof et al. 1992). However, these pathways are not functionally equivalent. For example, the voltage dependence of the M2 modulation suggests that repetitive terminal spiking should reverse autoreceptor inhibition of ACh release, whereas the D₂ inhibition of release should be insensitive to this type of activity. More importantly, perhaps, activation of PKC should effectively disconnect autoreceptors from the release machinery, leaving the D₂ control of ACh release intact. Understanding how PKC is regulated in cholinergic interneurons should be an important step toward clarifying the significance of this difference in cholinergic and dopaminergic signaling.

We thank Pfizer for the gift of ω -AgTx IVA, Dr. J. Flores-Hernandez for assisting in some of the experiments, and Dr. L. Dudkin for technical help.

This work was supported by the National Institute of Neurological Disorders and Stroke Grants NS-26473 to D. J. Surmeier and S. T. Kitai and NS-34696 to D. J. Surmeier, and a Parkinson's Disease Foundation fellowship to W. J. Song.

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Received 12 August 1996; accepted in final form 10 October 1996.

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