J Neurophysiol
87: 1046–1056, 2002; 10.1152/jn.00459.2001.

D2 Receptors Inhibit the Secretory Process Downstream From Calcium Influx in Dopaminergic Neurons: Implication of K^+ Channels

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Received 4 June 2001; accepted in final form 24 October 2001

Congar, Patrice, Annie Bergevin, and Louis-Eric Trudeau. D2 receptors inhibit the secretory process downstream from calcium influx in dopaminergic neurons: implication of K^+ channels. *J Neurophysiol* 87: 1046–1056, 2002; 10.1152/jn.00459.2001. Dopaminergic (DAergic) neurons possess D2-like somatodendritic and terminal autoreceptors that modulate cellular excitability and dopamine (DA) release. The cellular and molecular processes underlying the rapid presynaptic inhibition of DA release by D2 receptors remain unclear. Using a culture system in which isolated DAergic neurons establish self-innervating synapses ("autapses") that release both DA and glutamate, we studied the mechanism by which presynaptic D2 receptors inhibit glutamate-mediated excitatory postsynaptic currents (EPSCs). Action-potential evoked EPSCs were reversibly inhibited by quinpirole, a selective D2 receptor agonist. This inhibition was slightly reduced by the inward rectifier K^+ channel blocker barium, largely prevented by the voltage-dependent K^+ channel blocker 4-aminopyridine, and completely blocked by their combined application. The lack of a residual inhibition of EPSCs under these conditions argues against the implication of a direct inhibition of presynaptic Ca^{2+} channels. To evaluate the possibility of a direct inhibition of the secretory process, spontaneous miniature EPSCs were evoked by the Ca^{2+} ionophore ionomycin. Ionomycin-evoked release was insensitive to cadmium and dramatically reduced by quinpirole, providing evidence for a direct inhibition of quantal release at a step downstream to Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Surprisingly, this effect of quinpirole on ionomycin-evoked release was blocked by 4-aminopyridine. These results suggest that D2 receptor activation decreases neurotransmitter release from DAergic neurons through a presynaptic mechanism in which K^+ channels directly inhibit the secretory process.

INTRODUCTION

Dopaminergic neurons possess D2-type autoreceptors (Farnebo and Hamberger 1971; Missale et al. 1998), which serve at least three functions: *1*) somatodendritic autoreceptors decrease neuronal excitability (Chiodo and Kapatos 1992; Lacey et al. 1987, 1988); *2*) terminal (i.e., presynaptic) autoreceptors reduce dopamine (DA) synthesis and packaging (Onali et al. 1988; Pothos et al. 1998; Wolf and Roth 1990); *3*) terminal autoreceptors rapidly inhibit impulse-dependent DA release (Cass and Zahniser 1991; Kennedy et al. 1992; Stamford et al. 1991). The cellular and molecular processes underlying the rapid presynaptic inhibition of DA release by terminal D2 receptors remain unclear.

Three general classes of presynaptic mechanisms are thought to be responsible for the modulation of neurotransmitter release by G protein-coupled receptors (Bouron 2001; Meir et al. 1999; Miller 1998; Wu and Saggau 1997) and could be involved in D2 receptor-mediated presynaptic inhibition. A first mechanism is a direct inhibition of Ca^{2+} channels in synaptic terminals. It has been reported that through a D2-type receptor, DA can inhibit somatodendritic voltage-dependent Ca^{2+} channels (VDCCs) in dopaminergic (DAergic) neurons (Cardozo and Bean 1995). If a similar modulation occurs in nerve terminals, this could have a direct impact on DA release. This hypothesis is currently untested. A second potential mechanism is a K^+ conductance increase in the presynaptic terminal. This would lead indirectly to a decrease in Ca^{2+} influx through action potential shunting. Voltage-dependent K^+ channels are well-established effectors of somatodendritic D2 receptors (Lacey et al. 1987–1989). Several K^+ conductances can be modulated by somatodendritic D2 autoreceptors (Chiodo and Kapatos 1992; Freedman and Weight 1988; Kim et al. 1995; Lin et al. 1998; Liu et al. 1994; Roeper et al. 1990; Sun et al. 2000; Surmeier et al. 1993; Tanaka et al. 1996; Uchida et al. 2000). The contribution of such conductances to the action of terminal D2 autoreceptors has not been clearly established. The K^+ channel blockers 4-aminopyridine (4-AP) and tetraethylammonium (TEA) reduce quinpirole-induced inhibition of [^{3}H]DA release in the striatal slice preparation (Cass and Zahniser 1991). However, the significance of such results needs to be investigated in a preparation allowing a more direct evaluation of regulatory processes localized to nerve terminals. A third mechanism of presynaptic inhibition that could be involved is a direct inhibition of the secretory process in nerve terminals, downstream of Ca^{2+} influx (Bouron 2001; Meir et al. 1999; Miller 1998; Parnas et al. 2000; Trudeau et al. 1996; Wu and Saggau 1997). To our knowledge, the contribution of such a mechanism to D2 receptor-mediated presynaptic inhibition in DAergic neurons has never been directly tested.

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Because the release of DA from nerve terminals does not evoke detectable synaptic currents (Sulzer et al. 1998; present study), neither in vivo nor in vitro, its measurement poses a technical challenge. Here we have taken advantage of a culture system in which isolated DAergic neurons establish synapses that release glutamate in addition to DA. Such glutamatergic synaptic currents are inhibited by terminal D2 receptor activation through a presynaptic site of action (Joyce and Rayport 2000; Sulzer et al. 1998), thus providing an advantageous system to study terminal D2 receptor function. In the present study, we specifically evaluated the implication of presynaptic K^+ channels and the possibility of a direct modulation of the secretory process.

METHODS

Cell culture

Single neuron microcultures were prepared using dissociated ventral tegmental area (VTA) cells from neonatal rat pups, according to recently described protocols (Bourque and Trudeau 2000; Michel and Trudeau 2000) derived from Cardozo (1993) and Sulzer et al. (1998). Neurons were plated onto preestablished midbrain astrocytic monolayers on 15-mm-diameter round precoated glass coverslips.

Astrocytes were prepared from the mesencephalon of cryoanesthetized neonatal (postnatal day 1–3) Sprague-Dawley rat pups. Cells were enzymatically dissociated using papain (Worthington Biochemical Corp., Lakewood, NJ) and were grown in basal medium Eagle with Earl's Salts (Gibco, Burlington, Ontario) supplemented with penicillin/streptomycin, GlutaMAX-1 (Gibco), Mito + serum extender (VWR Canlab, Montreal, Quebec), and 10% heat-inactivated fetal calf serum (Gibco). Five days before plating neurons, astrocytes were trypsinized and plated at a concentration of 60,000 living astrocytes per milliliter on agarose-covered glass coverslips which had been previously sprayed with collagen/poly-D-lysine (each at 0.5 mg/ml) micro-droplets (50–150 μM in diameter). This permitted the establishment of small groups of isolated cells.

To prepare neurons, a 1- to 2-mm-thick coronal slice was cut at the level of the midbrain flexure. Two small blocks of tissue containing the left and right portion of the VTA were dissected out using a custom tissue micro-punch. After dissociation, neurons were diluted at a density titrated to optimize neuronal viability and the establishment of single neuron microcultures (80–100,000 living cells per milliliter) and plated onto astrocyte micro-islands. Cell cultures were incubated at 37°C in 5% CO_2 atmosphere and maintained in neurobasal A/B27 medium (Gibco) supplemented with penicillin/streptomycin, GlutaMAX-1 (Gibco), and 5% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT).

Electrophysiology and dye labeling

Electrophysiological experiments were performed at room temperature on neurons maintained for 12–20 days in culture. Cultures were transferred to a custom-made small volume laminar perfusion flow recording chamber mounted on an inverted microscope and continuously superfused using a gravity flow system (2.5–3 ml/min) with a standard extracellular bathing solution containing the following (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 6 sucrose, 10 glucose, pH 7.4, 300 mOsm. Drugs were bath applied, with a delay between valve opening and onset of drug action of approximately 15 s. Autaptic neurons were recorded using the ruptured or perforated (amphotericin B 150–200 μg/ml) whole-cell patch-clamp technique. Patch pipettes (3–5 MΩ) contained the following (in mM): 140 $KMeSO_4$, 10 NaCl, 0.1 EGTA, 4 MgATP, 0.5 GTP (Tris salt), 10 HEPES, pH 7.4, 300 mOsm. Spontaneous miniature excitatory postsynaptic currents (EPSCs) were recorded in the presence of 0.5 μM tetrodotoxin (TTX; Alomone Labs, Jerusalem, Israel). Currents were recorded using a PC-501 patch-clamp amplifier (Warner Instruments Corp., Hamden, CT), filtered at 1 KHz and digitized at 2-5 KHz using a Digidata 1200 A/D converter (Axon Instruments, Foster City, CA) interfaced to a Pentium PC and Pclamp 8 software (Axon Instruments). Junction potentials were nulled before the establishment of the whole-cell configuration. The capacitative compensation circuit of the amplifier was used to reduce capacitative transients. Series resistance (typically 7–12 MΩ) was corrected by 70–80%. During whole-cell recording, autaptic responses were evoked by brief (1 ms) depolarizing voltage steps from a holding potential (V_H) of -50 mV, at a frequency of 0.05 Hz. In DAergic neurons, this usually elicited an "action current" followed by a 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)-sensitive glutamate-mediated inward autaptic EPSC. In non-DAergic neurons, autaptic currents displayed reversal potentials close to -50 mV and were sensitive to 5 μM SR-95531, a γ-aminobutyric acid-A ($GABA_A$) receptor antagonist (not shown; Michel and Trudeau 2000). Miniature synaptic currents were analyzed using MiniAnalysis 5.2 software (Synaptosoft, Leonia, NJ). Data in the text and figures are expressed as mean ± SE. Unless otherwise indicated, data were statistically analyzed using the paired Wilcoxon signed-rank test with a probability value of $P < 0.05$ used as an indication of significant differences ($^*P < 0.05$; $^{**}P < 0.005$).

Fluorescence imaging of intracellular Ca^{2+} transients

For experiments using dynamic fluorescence imaging of intracellular Ca^{2+} levels, cells were loaded for 30–45 min at room temperature with Fura-2 AM (5 μM) (Molecular Probes Inc., Eugene, OR). Calcium transients were elicited by extracellular stimulation delivered by a bipolar theta glass stimulating pipette filled with standard extracellular bathing solution. Fluorescence excitation at 340/380 nm was driven by a computer-controlled fast optical switch (DX-1000; Stanford Photonics, Palo Alto, CA). Fluorescence images were collected using a Gen-III+ intensified progressive line scan CCD camera (Stanford Photonics) at a frequency of 0.5 Hz, with minimal exposure times (264 ms) to minimize bleaching. Image acquisition and analysis was performed using Axon Imaging Workbench 4.0 (Axon Instruments).

Immunocytochemistry

In most of the patch-clamp and imaging experiments, the microdot location was marked after recording using blue fluorescent microspheres (Duke Scientific Corp., Palo Alto, CA) that were deposited using a patch pipette onto the agarose substrate, near the microdot. This facilitated the subsequent identification of the recorded DAergic neurons by post hoc immunofluorescent staining for tyrosine hydroxylase. Cells were fixed with 4% paraformaldehyde. Tyrosine hydroxylase was localized using a monoclonal antibody (Sigma Chemicals, St. Louis, MO) and Alexa-488 or Alexa-546-conjugated secondary antibodies (Molecular Probes). Images of immunofluorescent labeling were acquired using a Hamamatsu Orca-II digital-cooled CCD camera using Esee and Isee software (Inovision Corp., Raleigh, NC). When required, stacks of images at different focal depths were acquired using a z-motor and reconstructed after deconvolution to reject out-of-focus signal.

RESULTS

Modulation of glutamatergic EPSCs by D2 receptors in isolated DAergic neurons

Considering that dopamine release does not evoke synaptic currents, alternate approaches are required to study the activity and modulation of the nerve terminals established by DAergic neurons. Although techniques such as amperometry allow the detection of individual DA quanta at randomly selected release

sites (Pothos et al. 1998), it is difficult to confirm that such sites represent bona fide nerve terminals. In addition, such recordings fail to provide information on the overall efficacy of the release process in individual DAergic neurons. In the present study, we have thus taken advantage of the ability of DAergic neurons in primary culture to co-release glutamate (Joyce and Rayport 2000; Sulzer et al. 1998) to monitor the effects of terminal D2-type DA receptor activation on synaptic efficacy. Experiments were performed on single neuron VTA microcultures (Bourque and Trudeau 2000; Michel and Trudeau 2000; Sulzer et al. 1998) (Fig. 1*A*). After whole-cell recording, the position of the neuron was marked by depositing blue fluorescent microspheres and the identity of the neuron as DAergic confirmed by tyrosine hydroxylase (TH) immunofluorescence labeling (Fig. 1*A*). During whole-cell recording, brief (1 ms) depolarizing voltage steps from a holding potential of −50 to +20 mV evoked postsynaptic, CNQX-sensitive α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated autaptic EPSCs (Fig. 1*B*, see also Sulzer et al. 1998). It is noteworthy that, as previously reported by Sulzer et al. (1998), we did not observe any component in the evoked autaptic response that was sensitive to DA receptor antagonists (not shown), neither in ruptured nor in perforated whole-cell patch-clamp modes. Direct application of the selective D2 receptor agonist quinpirole (5 μM) also did not evoke detectable somatodendritic membrane currents (not shown). These observations suggest that under our experimental conditions, somatodendritic D2-type DA receptors were either not present or not effectively coupled to potassium channels.

However, bath application of quinpirole (5 μM) robustly and reversibly decreased the peak amplitude of autaptic EPSCs in isolated DAergic neurons to 48.2 ± 2.4% of control ($n = 64$, $P < 0.001$, Fig. 1, *C–E*). Multiple applications of quinpirole separated by a 10-min washout period reproducibly inhibited EPSC amplitude (not shown). Although all neurons that were sensitive to quinpirole were subsequently confirmed to be TH positive, our cultures also contained a significant proportion of GABAergic neurons and a variable proportion of TH-negative glutamatergic neurons. Those TH-negative neurons were in all cases insensitive to quinpirole [see GABAergic inhibitory postsynaptic currents (IPSCs), $n = 5$, Fig. 1*E*]. In all further experiments, the sensitivity of a neuron to quinpirole thus served as a valid marker of DAergic phenotype, as initially demonstrated by Rayport et al. (1992). We nonetheless performed postrecording immunofluorescent identification of neurons in most experiments.

In a subset of experiments, quinpirole was applied in the presence of the selective D2 receptor antagonist sulpiride (5

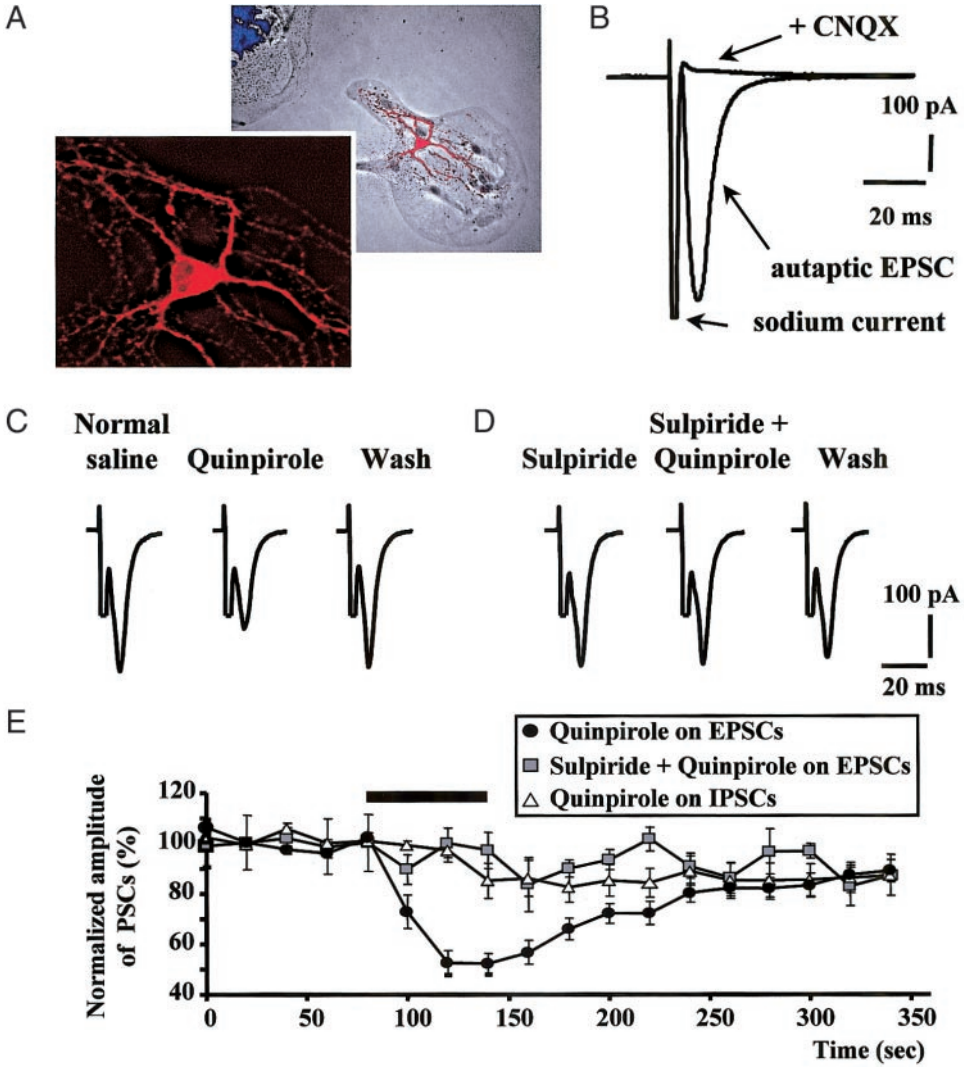

FIG. 1. Inhibition of autaptic excitatory postsynaptic current (EPSC) amplitude by quinpirole in isolated dopaminergic (DAergic) neurons. *A*: image of an isolated midbrain neuron identified as DAergic by tyrosine hydroxylase (TH) immunofluorescence. Blue fluorescent microspheres (*top left*) facilitated the identification of the field following immunocytochemistry. Note in the inset the presence of multiple TH-positive varicosities. *B*: autaptic EPSC recorded in an isolated DAergic neuron ($V_H = -50$ mV). The EPSC is preceded by a sodium "action current." The EPSC is blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM). *C*: autaptic EPSC amplitude was reversibly decreased by the D2 receptor agonist quinpirole (5 μM) ($V_H = -50$ mV). Note that in these and subsequent figures, each trace is an average of 3–5 sweeps and action current waveforms have been clipped for clarity. *D*: the effect of quinpirole on EPSC amplitude was blocked by the D2 antagonist sulpiride (5 μM). *E*: time course of the average effect of quinpirole (alone or together with sulpiride) on autaptic EPSC and inhibitory postsynaptic current (IPSC) amplitude in DAergic and GABAergic neurons.

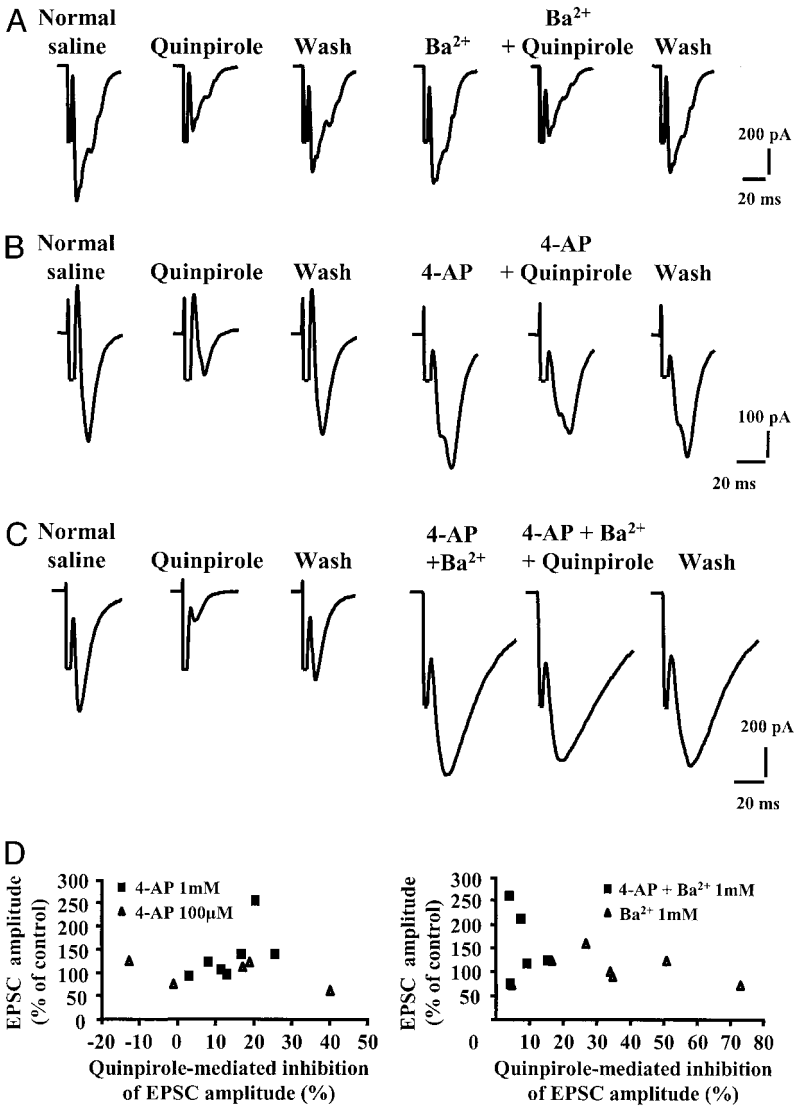

FIG. 2. Implication of K^+ channels in D2 receptor-mediated presynaptic inhibition. *A*: whole-cell recordings from isolated DAergic neurons ($V_H = -50$ mV). A first application of quinpirole (5 μM) inhibited EPSC amplitude. In the presence of barium (1 mM) the effect of quinpirole was slightly reduced. *B*: whole-cell recordings from a different DAergic neuron. A first application of quinpirole inhibited EPSC amplitude. In the presence of 4-aminopyridine (4-AP; 100 μM) the effect of quinpirole was considerably reduced. *C*: whole-cell recordings from a different DAergic neuron. A first application of quinpirole inhibited EPSC amplitude. In the presence of 4-AP (1 mM) and Ba^{2+} (1 mM) the effect of quinpirole was almost completely blocked. *D*: lack of correlation between the effect of 4-AP or Ba^{2+} on EPSC amplitude and the ability of these blockers to reduce quinpirole-mediated inhibition of EPSC amplitude.

μM). Although by itself sulpiride did not significantly change EPSC amplitude (90.3 ± 12.9% of control, $n = 4$, $P > 0.05$), it completely antagonized the inhibitory effect of quinpirole on EPSC amplitude (93.3 ± 2.1% of control, $n = 4$, $P > 0.05$, Fig. 1, *D* and *E*). Therefore the modulation of synaptic efficacy by quinpirole in this model is selectively mediated by D2-type DA receptors on DAergic neurons.

Implication of potassium conductances

We first investigated the possible implication of G protein-activated inward rectifier K^+ (GIRK)-type conductances in the effect of quinpirole by using barium, a blocker of GIRK-type channels. Although barium at a maximally effective concentration (1 mM) did not significantly modify the amplitude of EPSCs (106.2 ± 11.8% of control amplitude, $n = 7$, $P > 0.05$), it slightly but significantly reduced the inhibitory effect of quinpirole (from 46.3 ± 9.8 to 31.0 ± 8.7% inhibition, $n = 7$, paired data, $P < 0.01$) (Fig. 2*A*–Fig. 3). Thus a significant fraction of the effect of quinpirole, about 30%, seems to be due to a modulation of presynaptic GIRK-type K^+ channels.

We next used 4-AP to investigate the implication of voltage-dependent K^+ (K_v) conductances in the presynaptic effect of quinpirole. Bath application of 100 μM 4-AP, similar to barium, caused no reliable change in EPSC amplitude by itself (99.7 ± 10.6% of control, $n = 6$, $P > 0.05$). However, 4-AP

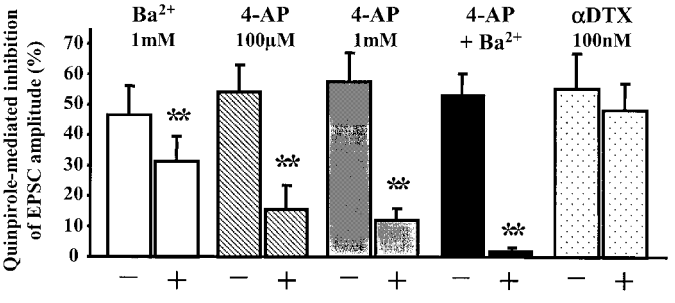

FIG. 3. Effect of K^+ channels blockers on quinpirole-mediated inhibition. Summary diagram illustrating the average inhibition of autaptic EPSC amplitude by quinpirole in experiments performed with K^+ channel blockers. The left column in each pair illustrates the effect of quinpirole under control conditions (−). The right column in each pair illustrates the effect of quinpirole in the presence of the K^+ channel blocker (+). A combination of barium and 4-AP completely blocked the presynaptic effect of quinpirole (black columns) (**$P < 0.01$).

significantly and reversibly reduced the effect of quinpirole (from 56.0 ± 9.6 to 16.2 ± 8.3% inhibition, $n = 6$, paired data, $P < 0.001$) (Fig. 2*B*–Fig. 3). This effect of 4-AP was only slightly increased at 1 mM (from 57.2 ± 9.4 to 12.0 ± 3.9% inhibition, $n = 7$, paired data, $P < 0.001$) (Fig. 3), a concentration which also failed to significantly increase EPSC amplitude (135.2 ± 23% of control, $n = 7$, $P > 0.05$). It is important to note that the block of quinpirole's ability to inhibit EPSCs was not caused by some kind of "saturation" of evoked release due to the enhancement of EPSC amplitude by 4-AP. This is illustrated by the complete lack of correlation between the ability of 4-AP to increase EPSC amplitude by itself and its ability to reduce quinpirole-mediated presynaptic inhibition, both at 100 μM ($r = 0.48$, Pearson, $P = 0.41$) and at 1 mM ($r = -0.58$, Pearson, $P = 0.17$) (Fig. 2*D*). Thus a major part of the effect of quinpirole (about 70%) implicates presynaptic 4-AP-sensitive K^+ channels.

In a final attempt to completely reverse the effect of quinpirole, we applied barium (1 mM) and 4-AP (1 mM) together. In the presence of these two blockers, some neurons showed a notable increase in EPSC amplitude (Fig. 2*C*), but on average, the amplitude of EPSCs was again not reliably increased (156.9 ± 33.8% of control, $n = 5$, $P > 0.05$). However, the inhibitory effect of quinpirole was completely and reversibly prevented (from 53.0 ± 6.9 to 1.8 ± 0.6% inhibition, $n = 7$, paired data, $P < 0.001$) (Fig. 2*C*–Fig. 3). Again, the block of quinpirole's ability to inhibit EPSCs was not caused by some kind of saturation of evoked release due to the enhancement of EPSC amplitude by 4-AP and Ba^{2+}. This is illustrated by the complete lack of correlation between the ability of the combination of 4-AP and Ba^{2+} to increase EPSC amplitude by itself and its ability to reduce quinpirole-mediated presynaptic inhibition ($r = 0.31$, Pearson, $P = 0.60$) (Fig. 2*D*).

Because 4-AP blocks a wide variety of K^+ channels containing K_v subunits, it was of interest to determine whether an antagonist with a narrower selectivity would also block quinpirole-induced presynaptic inhibition in DAergic neurons. We thus examined the effect of α-dendrotoxin (α-DTX), a more selective blocker of K_v channels (Coetzee et al. 1999; Meir et al. 1999). Using 100 nM α-DTX, we failed to observe any significant increase in EPSC amplitude (102.5 ± 6.6% of control, $n = 5$, $P > 0.05$) nor any decrease in the presynaptic effect of quinpirole (from 55.9 ± 11.4 to 48.5 ± 8.4% inhibition, $n = 5$, paired data, $P > 0.05$) (Fig. 3). In two of the five neurons tested, the quinpirole-induced inhibition of EPSCs was first measured in control saline, then in the presence of α-DTX, and finally again in the presence of 4-AP. In those two neurons quinpirole induced a similar inhibition of EPSC amplitude in the absence and in the presence of α-DTX, but its effect was significantly blocked in the presence of 4-AP (from 48.5 ± 8.4 to 7.2 ± 4.2% inhibition, not shown). These results suggest that quinpirole produces its presynaptic effect through modulation of 4-AP-sensitive but α-DTX-insensitive K^+ channels.

Quinpirole does not affect Ca^{2+} influx into DAergic neurons

If one of the mechanisms through which quinpirole inhibits autaptic EPSC amplitude in DAergic neurons is a direct inhibition of presynaptic Ca^{2+} channels, then it would be expected that at least a component of quinpirole's effect should remain in the presence of the K^+ channel blockers. This was not observed in our experiments. Nevertheless, it has been reported that DA can reduce somatodendritic voltage-dependent Ca^{2+}

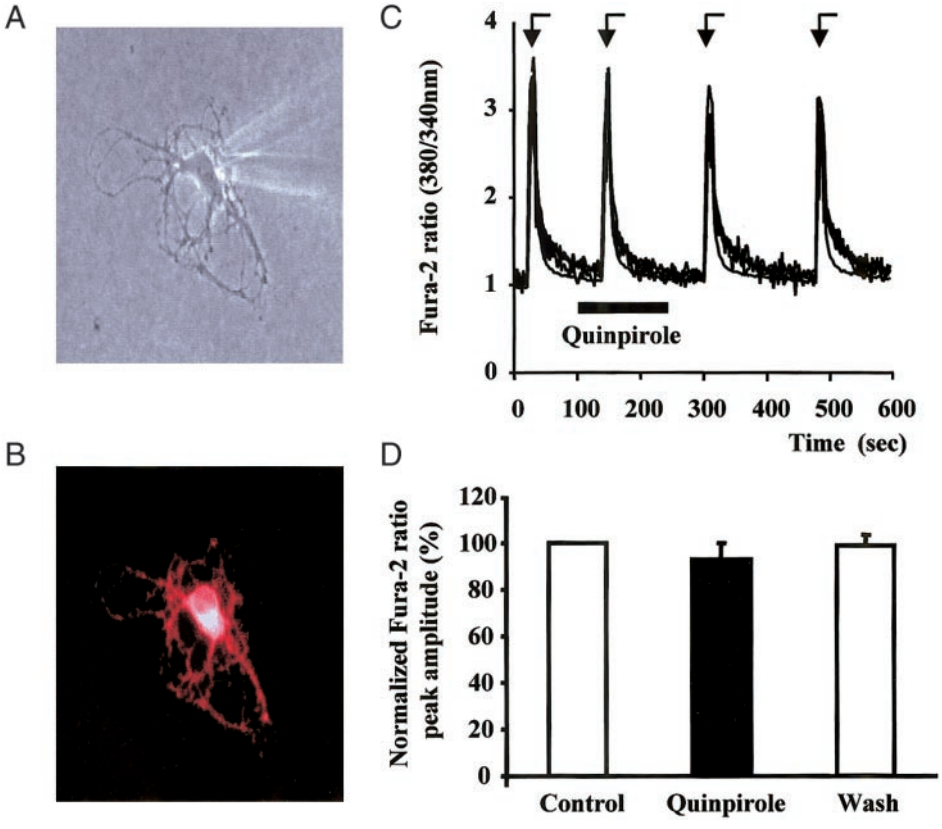

FIG. 4. Lack of effect of quinpirole on Ca^{2+} influx in DAergic neurons. *A*: phase contrast image of an isolated neuron. Note the presence of the extracellular stimulating pipette to the right of the image. *B*: TH immunofluorescent labeling confirming the DAergic phenotype of the neuron used for Fura-2 imaging. *C*: time course of Fura-2 ratio intensity measurements from three areas on the neuron shown in *A* and *B*. Extracellular stimulation trains (arrows) induced reproducible rises in intracellular Ca^{2+} as reflected by an increase in the 380/340 nm Fura-2 ratio. Quinpirole (5 μM) failed to cause any detectable change in Ca^{2+} influx. *D*: summary diagram illustrating the normalized average amplitude of electrically evoked rises in Fura-2 ratios during the control period, in the presence of quinpirole and during washout of quinpirole.

currents in DAergic neurons (Cardozo and Bean 1995). We thus monitored electrically evoked Ca^{2+} transients in the cell body and proximal dendrites of isolated DAergic neurons loaded with Fura-2 AM to evaluate whether quinpirole caused a generalized inhibition of voltage-gated Ca^{2+} channels. Intracellular Ca^{2+} transients were evoked by direct electrical stimulation of neurons using a theta glass pipette (2-s trains at 10 Hz, 0.5-ms pulse duration) (Fig. 4*C*). Calcium transients of constant amplitude could be evoked by successive stimulation trains using this technique. Quinpirole did not significantly affect the peak amplitude of these Ca^{2+} transients (93.3 ± 7.0% of control; $n = 5$; $P > 0.05$) (Fig. 4, *C* and *D*). A similar small, nonsignificant decrement was measured in TH-negative neurons (93.20 ± 1.5% of control) ($n = 7$; not shown). Although intracellular Ca^{2+} was not quantified directly in nerve terminals, an approach which would be difficult with microcultures in which axons tend to bundle and run alongside dendrites, this set of data supports the idea that quinpirole-induced inhibition is largely independent of global modifications of Ca^{2+} handling.

Absence of effect of quinpirole on spontaneous miniature synaptic events in DAergic neurons

To determine whether D2 receptors can directly inhibit the secretory process in DAergic neurons, we next investigated the effect of quinpirole on miniature EPSCs (mEPSCs), recorded in the presence of TTX (0.5 μM), in isolated DAergic neurons. In all experiments, the sensitivity to quinpirole of the evoked autaptic EPSC was first confirmed (in the absence of TTX), prior to the beginning of mEPSC recordings (Fig. 5*A*). In a preliminary set of experiments, we studied mEPSCs using 1- to 2-min sampling episodes; in these experiments, DAergic neurons were found to display highly variable but usually low levels of spontaneous synaptic activity [from 0 to 10 Hz; on average 1.5 ± 0.7 Hz (median 0.8 Hz, $n = 13$) (Fig. 5*B*)]. Due to this low and variable frequency, the analysis of the effect of quinpirole on the frequency and amplitude of spontaneous mEPSCs was performed using prolonged recordings (10 min control, 10 min quinpirole, 10 min wash). In these experiments, DAergic autaptic neurons still displayed variable and low levels of spontaneous synaptic activity [from 0.6 to 5 Hz; on average 2.6 ± 0.9 Hz; median value 1.6 Hz, $n = 5$ (Fig. 5*B*)]. Bath application of quinpirole did not decrease the basal frequency of mEPSCs. To the contrary, the frequency of spontaneous mEPSCs had a tendency to increase, although this did not reach statistical significance [153.9 ± 33.0% of control, $n = 5$, paired data, Wilcoxon signed-rank test, $P = 0.715$ (Fig. 5, *B* and *C*)]. Cumulative probability distributions of mEPSC inter-event intervals were analyzed for each neurons using the Kolmogorov-Smirnov test: three of the five neurons displayed a slight but significant ($P < 0.01$) shift of the distribution to the left (i.e., a small increase in frequency) while the two others displayed a slight but significant shift of the distribution to the right (i.e., a small decrease in frequency). Although in each tested neuron autaptic EPSC amplitudes were found to be robustly inhibited by quinpirole (not shown), the frequency of spontaneous mEPSCs in these same cells thus showed no systematic and quantitatively important chances. The mean amplitude of the mEPSCs was also not significantly modified by quinpirole (97.8 ± 2.2% of control, $n = 5$, paired data, Wilcoxon signed-rank test, $P = 0.34$). Again, the cumulative probability distributions of mEPSC amplitudes were analyzed for each neuron: two of the five neurons displayed a slight but significant shift of the distribution to the left (i.e., a small increase in amplitude), while two others displayed a slight but significant shift of the distribution to the right (i.e., a small decrease in frequency) and the last one was not modified. Thus no systematic and quantitatively important change in mEPSC amplitude accompanied the inhibition of autaptic EPSC amplitude.

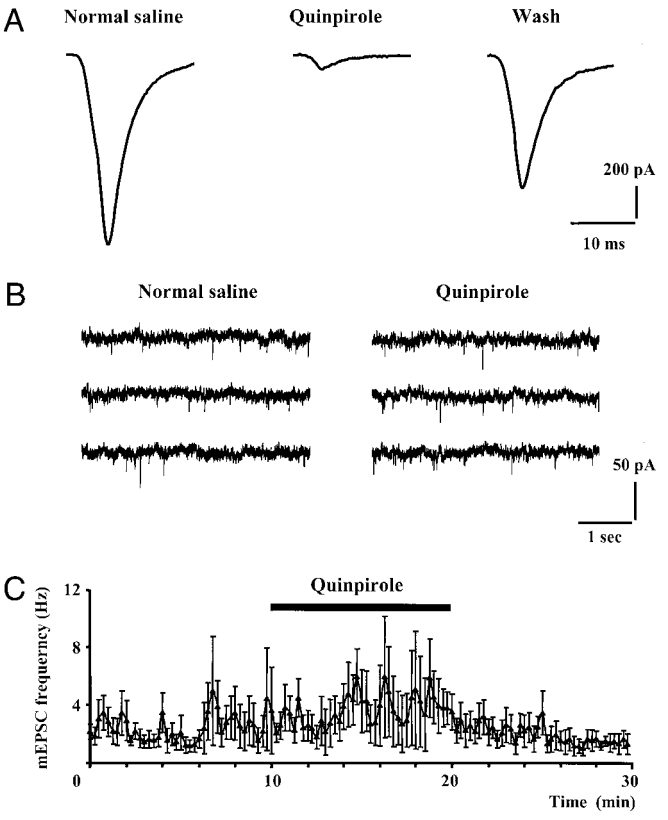

FIG. 5. Lack of effect of quinpirole on spontaneous autaptic miniature EPSCs. *A*: whole-cell recordings from an isolated DAergic neuron ($V_H = -50$ mV). Quinpirole (5 μM) reversibly inhibited the evoked EPSC amplitude. The action current has been subtracted for clarity. *B*: whole-cell recordings of spontaneous miniature EPSCs (mEPSCs) in the same isolated DAergic neuron in the presence of 0.5 μM tetrodotoxin (TTX), in control conditions (normal saline), and in the presence of 5 μM quinpirole (quinpirole) ($V_H = -50$ mV). *C*: average time course of the effect of quinpirole on autaptic spontaneous mEPSC frequency. Note that although in each cell tested, quinpirole significantly and reversibly decreased the amplitude of evoked EPSC, it did not significantly modify the frequency of spontaneous mEPSCs recorded in the same neurons.

The failure of the marked inhibition of evoked EPSC amplitude to be accompanied by a decrease in mEPSC frequency suggests that either the basal state of the secretory process is not directly inhibited by D2 receptor activation or the inhibition only acts on Ca^{2+}-evoked exocytosis. To distinguish between these possibilities, we enhanced the frequency of CNQX-sensitive mEPSCs using the Ca^{2+} ionophore ionomycin (2.5 μM). Ionomycin is known to elicit neurotransmitter release by directly producing an elevation of intraterminal Ca^{2+}, effectively bypassing the requirement for presynaptic VDCC activation (Angleson and Betz 2001; Capogna et al. 1996). Its effect is completely insensitive to the Ca^{2+} channel

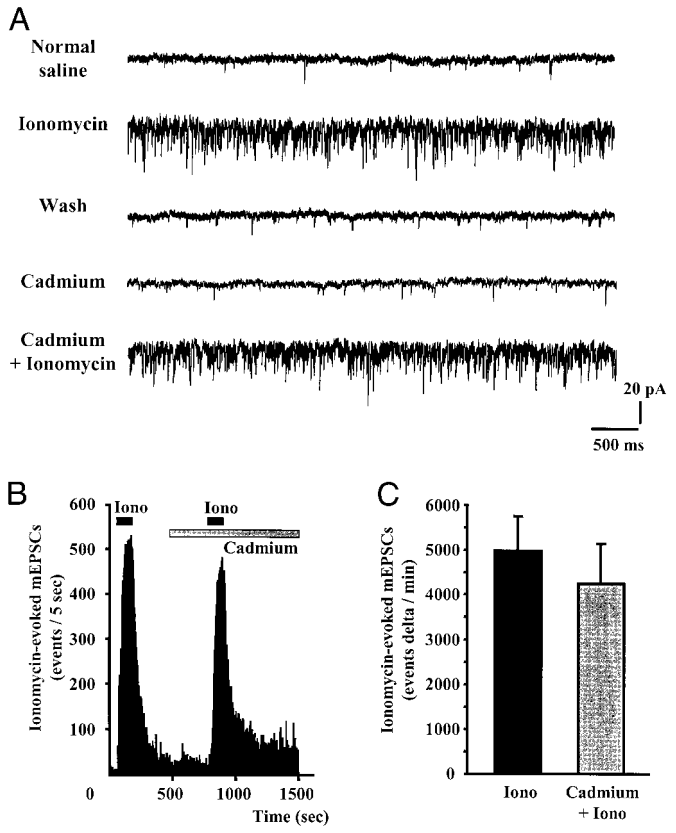

FIG. 6. The Ca^{2+} ionophore ionomycin enhances miniature EPSC frequency independently from voltage-dependent calcium channels. *A*: whole-cell recordings of mEPSCs in isolated DAergic neurons in the presence of 0.5 μM TTX ($V_H = -50$ mV). Ionomycin (2.5 μM) reversibly enhanced mEPSC frequency. The effect of ionomycin was not significantly modified by the Ca^{2+} channel blocker cadmium (100 μM). *B*: histogram illustrating the time course of the two ionomycin-induced increases in mEPSC frequency in the same neuron, successively in the absence and presence of cadmium. *C*: summary histograms illustrating the maximum effect of ionomycin on mEPSC frequency under control conditions (*left*) and in the presence of cadmium (*right*). Note that cadmium did not significantly modify the ionomycin-induced increase in mEPSC frequency.

blocker cadmium and thus independent of the activation of Ca^{2+} channels in the presynaptic terminal (Capogna et al. 1996). In a first set of experiments, we first verified that, under our experimental conditions, the ability of ionomycin to increase mEPSC frequency was indeed not significantly affected by blocking voltage-dependent Ca^{2+} channels with cadmium. For each stimulation, the ability of ionomycin to increase mEPSC frequency was expressed as a difference score (delta) obtained by subtracting the number of mEPSCs within a 60-s period of time before from that during the application of ionomycin, at the peak of the effect. A marked and reversible increase of mEPSC frequency was observed following a brief (2 min) application of ionomycin (from 3.0 ± 1.4 to 92.1 ± 12.6 Hz, $n = 6$, Fig. 6*A*). After a 10-min washout and a complete recovery of mEPSC frequency, a second stimulation with ionomycin was then performed in the presence of cadmium ($CdCl_2$, 100 μM, preapplied for 5 min). Cadmium did not significantly change the ability of ionomycin to enhance mEPSC frequency [from 5.0 ± 1.3 to 84.4 ± 14.2 Hz, not significantly different from the first stimulation; $n = 6$, paired data, $P > 0.05$ (Fig. 6, *A–C*)]. These results thus confirm that ionomycin-induced neurotransmitter release in isolated DAergic neurons is independent of presynaptic VDCC activation. Under such conditions, the ability of an agent to modulate ionomycin-evoked release should thus reflect an action on a process downstream of Ca^{2+} influx through VDCCs.

We next investigated the effect of quinpirole on ionomycin-stimulated mEPSCs. The neurons were first confirmed to show a robust and reversible quinpirole-induced decrease in autaptic EPSC amplitude (not shown). Then, after a washout period of ≥10 min, mEPSCs were recorded in the presence of TTX. As described above, a marked and reversible increase of mEPSC frequency was observed following a brief (2 min) application of ionomycin [from 0.2 ± 0.1 to 23.4 ± 7.2 Hz, $n = 7$ (Fig. 7*A*)]. After a 10-min washout and a complete recovery of mEPSC frequency, a second stimulation with ionomycin was then performed in the presence of quinpirole (5 μM, preapplied for 1 min). Finally, after an additional 10-min washout of quinpirole and ionomycin, a third application of ionomycin

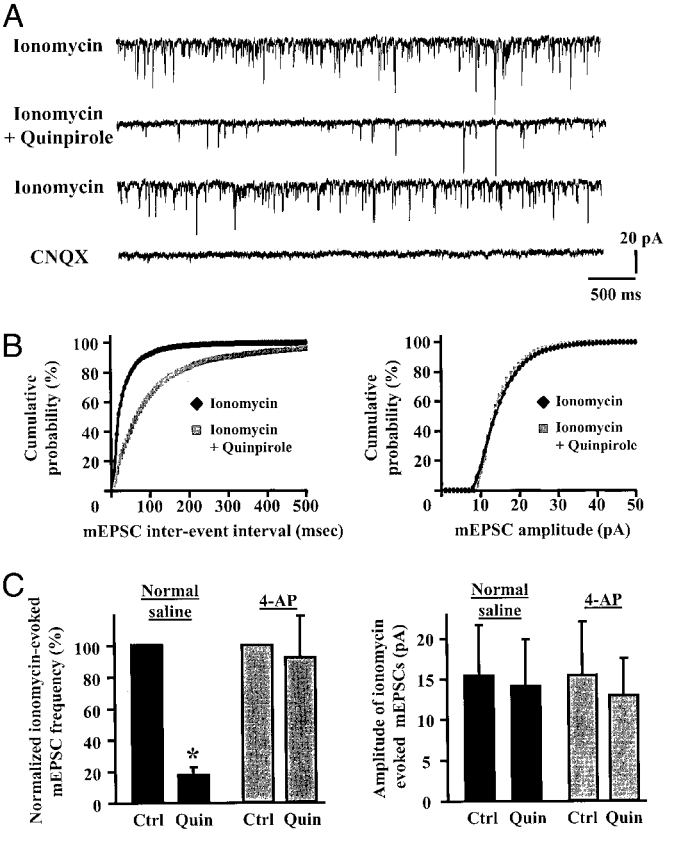

FIG. 7. Inhibition of ionomycin-evoked mEPSCs by quinpirole. *A*: whole-cell recordings of mEPSCs in isolated quinpirole-sensitive DAergic neurons in the presence of 0.5 μM TTX ($V_H = -50$ mV). Ionomycin (2.5 μM) reversibly enhanced mEPSC frequency. The effect of ionomycin was reversibly reduced by quinpirole. All synaptic events were blocked by the AMPA/kainate receptor antagonist CNQX (5 μM). *B*: cumulative probability distribution of ionomycin-evoked mEPSC frequency (*left*) and amplitude (*right)* from 7 experiments (pooled data). Note that quinpirole strongly inhibited ionomycin-evoked mEPSC frequency (Kolmogorov-Smirnov, $P < 0.001$) and caused only a very small decrease in their amplitude (K-S, $P < 0.001$). *C*: summary histograms illustrating the effect of ionomycin on mEPSC frequency (*left*) and mean amplitude (*right*) under control conditions (Ctrl) and in the presence of quinpirole (Quin). Quinpirole strongly inhibited ionomycin-evoked release in normal saline (black columns), but this effect was greatly reduced in the presence of the K^+ channel blocker 4-AP (gray columns). Quinpirole did not cause a significant decrease in mEPSC amplitude when comparing the average mEPSC amplitude of individual experiments.

was performed. The delta value of the stimulation with ionomycin in the presence of quinpirole was compared with the mean delta value of the first and third stimulations to account for any gradual rundown in the effectiveness of ionomycin to stimulate mEPSC frequency. We found that quinpirole produced a profound inhibition of ionomycin-induced mEPSCs [by 82.8 ± 5.2%; $n = 7$, paired data, $P < 0.05$ (Fig. 7, *A–C*)]. Although the overall average amplitude of ionomycin-evoked mEPSCs was not significantly decreased by quinpirole (Fig. 5*C*), an analysis of the cumulative probability distribution of all events using the Kolmogorov-Smirnov test nonetheless showed that the large decrease in mEPSC frequency was accompanied by a very small decrease in mEPSC amplitude in the presence of quinpirole [$P < 0.001$, $n = 7$ (Fig. 7*B*)]. This was likely due to a reduction in the summation of mEPSCs that sometimes occurred at the peak of ionomycin's effect.

These results indicate that D2-type autoreceptors located on presynaptic terminals can inhibit quantal glutamate release from VTA DAergic neurons by a mechanism that occurs at a step beyond Ca^{2+} influx through presynaptic VDCCs. These results are somewhat surprising considering that barium and 4-AP completely blocked quinpirole's ability to inhibit evoked EPSC amplitude (Figs. 2 and 3), leaving little place for an additional mechanism. One possibility is that both mechanisms could be somehow interdependent. To test this hypothesis, we determined the effect of 4-AP (1 mM) on the modulation of ionomycin-evoked mEPSCs by quinpirole. We found that 4-AP by itself significantly affected neither basal mEPSC frequency (0.8 ± 0.3 Hz, $n = 5$, $P > 0.05$) nor the effectiveness of ionomycin to trigger mEPSCs (to 20.8 ± 7.4 Hz, $n = 5$, $P > 0.05$). However, 4-AP completely blocked quinpirole's ability to inhibit ionomycin-evoked mEPSCs [8.0 ± 26.2% inhibition, $n = 5$, paired data, $P > 0.05$ (Fig. 7*C*)]. These results show that the inhibition of the secretory process by quinpirole in DAergic neurons is somehow dependent on 4-AP-sensitive K^+ channels.

DISCUSSION

To investigate the mechanism of D2 receptor-mediated presynaptic inhibition, we have taken advantage of a VTA primary culture system in which postnatally derived DAergic neurons establish functional synapses that release both DA and glutamate (Bourque and Trudeau 2000; Congar and Trudeau 1999; Sulzer et al. 1998). One major advantage of this system is that the release of glutamate at synapses generates prominent glutamate-mediated fast EPSCs that can be readily measured by electrophysiological techniques and that can serve as an index of the activity of the synaptic terminals established by individual DAergic neurons.

In a first set of experiments, we have confirmed that, as previously reported by Sulzer et al. (1998), autaptic EPSCs recorded from isolated DAergic neurons are reversibly and reproducibly inhibited by the selective D2 agonist quinpirole, an effect that is antagonized by the D2 antagonist sulpiride. Moreover, this effect was specific to DAergic neurons, since autaptic responses evoked in isolated TH-negative neurons were insensitive to quinpirole.

Implication of presynaptic K^+ channels

Although the precise identification of the subtypes of K^+ channels implicated in D2-mediated presynaptic inhibition was beyond the scope of the present study, our results strongly suggest that two different types of potassium conductances play a key role in this mechanism. A combined application of 4-AP and barium, blockers of K_v-type and GIRK-type K^+ channels, respectively, completely blocked quinpirole-mediated presynaptic inhibition. While 4-AP blocked approximately 70% of the effect of quinpirole, barium blocked approximately 30%. Moreover, we failed to observe any effect of α-DTX, a voltage-gated K^+ channel blocker with a narrower selectivity. This pharmacological profile is very similar to that recently reported by Manzoni and Williams (1999), who characterized a 4-AP-sensitive, α-DTX-insensitive mechanism responsible for presynaptic inhibition of glutamate release by μ-opioid receptors at excitatory afferents to DAergic neurons in VTA slices. The sensitivity of presynaptic voltage-gated potassium channels to α-DTX is variable and depends on the precise subunit composition of the channels (for review see Coetzee et al. 1999; Meir et al. 1999). The absence of effect of α-DTX seems to exclude the implication of a large subset of the K_v1 family.

Our finding of a prominent implication of 4-AP-sensitive K^+ conductances in the presynaptic effect of quinpirole provides direct evidence supporting the hypothesis proposed by Cass and Zahniser (1991) that a major component of the mechanism by which D2 autoreceptors decrease DA release involves presynaptic K^+ channels (see also Cass and Zahniser 1990). Our results also suggest that in addition to 4-AP-sensitive K^+ channels, presynaptic GIRK-type channels could also play a role. Although these channels are mostly somatodendritic in neurons, their localization on nerve terminals in some preparations has been recently suggested (Morishige et al. 1996; Ponce et al. 1996). To our knowledge, the present study is the first to suggest the implication of barium-sensitive GIRK-type channels in D2 receptor-mediated presynaptic inhibition in DAergic neurons.

Although our results strongly suggest that presynaptic D2 receptors inhibit evoked glutamate release in VTA DAergic neurons mainly through the modulation of barium- and 4-AP-sensitive K^+ channels located on nerve terminals, one cannot exclude off-hand the possibility that quinpirole inhibits EPSC amplitude at least in part by impairing action potential propagation at axonal branch points. 4-AP may thus block quinpirole-induced presynaptic inhibition by facilitating action potential propagation. Recent work has indeed showed that 4-AP-sensitive K^+ channels localized in axons are able to modulate action potential propagation (Debanne et al. 1997, 1999; Tan and Llano 1999). Nevertheless, in our experiments, neither barium nor 4-AP, even applied together at a maximally effective concentration, reliably and significantly enhanced autaptic EPSC amplitude by themselves. This suggests that the control of action potential propagation by barium and 4-AP-sensitive K^+ conductances is probably quite weak in DAergic neurons under our experimental conditions. In addition, this observation argues against the possibility of some kind of "occlusion" of quinpirole's effect because of a saturation of the neurotransmitter release process. Finally, even if present, a mechanism implicating a change in action potential propaga-

tion would not readily account for our data on the modulation of mEPSCs.

Implication of presynaptic Ca^{2+} channels

Because DA receptors can directly inhibit Ca^{2+} channels in a number of preparations (Missale et al. 1998), including acutely dissociated midbrain neurons (Cardozo and Bean 1995), the possibility that a similar effect occurs in the nerve terminals of DAergic neurons has to be considered. However, to our knowledge, this question has not been directly addressed in previous studies. Our results are incompatible with the hypothesis that a direct modulation of presynaptic voltage-dependent Ca^{2+} channels plays a significant role. First, we found that in the presence of 4-AP and barium, conditions that should not prevent direct Ca^{2+} channel modulation, quinpirole fails to cause any significant decrease in autaptic EPSC amplitude. Second, although our Ca^{2+}-imaging experiments do not allow us to make strong conclusions about nerve terminals, they nonetheless show that D2 autoreceptor stimulation fails to cause any generalized inhibition of electrically evoked Ca^{2+} influx in the cell body and proximal processes of DAergic neurons. Finally, in experiments where ionomycin was used to trigger cadmium-insensitive Ca^{2+}-dependent exocytosis, under conditions where voltage-dependent Ca^{2+} channel activation was bypassed, the D2 receptor agonist quinpirole still caused a large decrease in mEPSC frequency. Thus although we cannot completely exclude the possibility of an indirect modulation of terminal Ca^{2+} influx as an additional mechanism (for example through the activation of terminal K^+ conductances by D2 autoreceptors, leading to an indirect decrease in action potential-evoked elevations of local Ca^{2+} concentration in synaptic terminals), a major component of presynaptic inhibition of transmitter release by D2 receptors seems to occur independently of a direct inhibition of Ca^{2+} channels, downstream of Ca^{2+} influx (see following text). Interestingly, a very similar mechanism of presynaptic inhibition depending on barium- and 4-AP-sensitive K^+ conductances and independent of the modulation of presynaptic Ca^{2+} channels, has also been identified in the nucleus accumbens (inhibition of glutamate release by cannabinoids) (Robbe et al. 2001) and in the periaqueductal gray (inhibition of GABA release by opioids) (Vaughan et al. 1997).

Evidence for a direct inhibition of the secretory process

In contrast to its ability to reliably inhibit the amplitude of the evoked EPSCs, quinpirole did not inhibit the frequency of spontaneous mEPSCs. In the absence of additional data, this finding could be interpreted as evidence for a lack of direct inhibition of the secretory process in nerve terminals. However, two considerations warrant a reconsideration of such a conclusion. First, considering the relatively low basal spontaneous mEPSC frequency (median 1.6 Hz), it may be considered that an inhibition of such a low frequency may be hard to detect. A second consideration is that although basal release may not be directly inhibited, this does not exclude that Ca^{2+}-evoked exocytosis may be inhibited at a late step. This could happen for example if some aspect of the coupling of exocytosis to Ca^{2+} sensing was inhibited by D2 receptor activation. Such a mechanism has recently been suggested to explain the modulation of neurotransmitter release by the cAMP system in nerve terminals (Sakaba and Neher 2001; Trudeau et al. 1998). Compatible with this last hypothesis, we found that quinpirole strongly inhibited the enhancement of mEPSC frequency evoked by the Ca^{2+} ionophore ionomycin. Such a method triggers Ca^{2+}-dependent exocytosis independently of the activity of presynaptic VDCCs (Capogna et al. 1996), as confirmed in the present study. Taken together, our findings thus suggest that presynaptic D2-type DA receptors inhibit a late step of glutamate exocytosis through a mechanism closely related to the Ca^{2+}-dependent activation of the release machinery. In light of our observations, it is interesting to note that in a recent study, Koga and Momiyama (2000) reported an inhibitory effect of D2 receptor activation on mEPSC frequency in VTA slices. They found that this inhibition was dependent on the external Ca^{2+} concentration. If such manipulations also modified basal intraterminal Ca^{2+} concentrations, the mechanisms identified in the present report and in the work of Koga and Momiyama may be similar.

In light of our finding of a complete block of quinpirole-mediated inhibition of autaptic EPSCs by 4-AP and barium, our results with ionomycin may be considered surprising: if the direct inhibition of exocytosis was a separate mechanism, independent of the K^+ channel-dependent mechanism, one would expect that in the presence of 4-AP and barium at least a component of quinpirole's effect should remain. Our finding that 4-AP also blocked the inhibition of ionomycin-evoked mEPSCs caused by quinpirole clearly suggests that these two mechanisms are at least partially interdependent and both implicate K_v conductances. But how could a K^+ channel-dependent modulation of the secretory process be mediated? Considering the now well-documented interaction between voltage-dependent Ca^{2+} channels and syntaxin, a core protein of the secretory machinery (Catterall 1999; Kim and Catterall 1997), one hypothesis, albeit highly speculative, is that some 4-AP-sensitive K^+ channels also directly interact with the secretory machinery and can thus be involved in regulating the efficacy of exocytosis. Much additional work will be required to test this possibility, but the recent finding that some K_v channel subunits indeed directly interact with syntaxin, a core protein of the secretory machinery (Fili et al. 2001), provides a molecular basis for such a hypothesis.

The mechanism identified in the present study could potentially be widely expressed. For example, activation of μ-opioid receptors in periaqueductal gray slices inhibits the frequency of miniature IPSCs (mIPSCs) through a mechanism that is sensitive to 4-AP but independent of changes in Ca^{2+} influx (Vaughan et al. 1997). Similarly, we have recently shown that in cultured mesencephalic GABAergic neurons, μ-opioid receptor activation inhibits the frequency of spontaneous mIPSCs as well as mIPSCs evoked by ionomycin through a mechanism sensitive to 4-AP (A. Bergevin, D. Girardot, M. J. Bourque, and L.-E. Trudeau, unpublished observations). Both of these observations could be explained by a K^+ channel-dependent inhibition of the secretory process.

In conclusion, the present set of results provide evidence that K^+ channels present on DAergic nerve terminals play a fundamental role in presynaptic inhibition of neurotransmitter release by D2-type receptors. In addition, our experiments suggest for the first time that D2 receptors can also directly inhibit the secretory process in nerve terminals at a step down-

stream from Ca^{2+} influx. The finding that these two mechanisms are apparently interrelated suggests a novel mechanism implicating a K^+ channel-dependent modulation of the secretory process.

Relevance of glutamate release by DAergic neurons to DA release

D2-mediated presynaptic inhibition of glutamate release has been previously documented in several structures including the nucleus accumbens (O'Donnell and Grace 1994), the striatum (Hsu et al. 1995, 1996; Kalivas and Duffy 1997; see also review of Nicola et al. 2000), the olfactory bulb (Berkowicz and Trombley 2000; Hsia et al. 1999), the supraoptic nucleus (Price and Pittman 2001), the subthalamic nucleus (Shen and Johnson 2000), the hippocampus (Hsu 1996), and the VTA (Koga and Momiyama 2000). Although this modulation has been defined as presynaptic in all studies, its molecular mechanisms were never clarified. The mechanism identified here could potentially shed light on the observations reported in the preceding reports.

Although we cannot reject the possibility that the mechanism that we have characterized here by measuring glutamate release from DAergic neurons is at least partly different from the mechanism regulating actual DA release, a number of arguments suggest that our experimental strategy provides information that is pertinent to both glutamate and DA release. First, glutamate release in cultured DAergic neurons is regulated by the glial cell line-derived neurotrophic factor receptor (Bourque and Trudeau 2000) and by the D2 receptor (Joyce and Rayport 2000; Sulzer et al. 1998; present study), two receptors that also regulate DA release in vivo. Second, we have recently shown that the vast majority (>85%) of synaptophysin-positive nerve terminals established by cultured DAergic neurons express VMAT-2, the vesicular DA transporter (Bourque and Trudeau 2000). In addition, Sulzer et al. (1998) have reported that the vast majority of synaptic varicosities established by cultured DAergic neurons contain both glutamate and DA. Thus although it is not known whether DA and glutamate are released from the same synaptic vesicles, these results suggest that most terminals established by cultured DAergic neurons have the capacity to store and release both glutamate and DA in a manner that is regulated through some of the same presynaptic mechanisms. Although the extrapolation of the present results to the release of DA may need additional experiments using different approaches, our work provides valuable information on the cellular mechanism of presynaptic inhibition mediated by D2-type receptors.

In conclusion, the present results provide new information on D2 receptor signaling in nerve terminals and suggest the possible existence of a novel type of presynaptic mechanism. The elucidation of this presynaptic mechanism may contribute to a more complete understanding of the physiology and pathology of DAergic systems in the brain.

We thank F. Michel and C. Jomphe for valuable help with some of the electrophysiological and immunohistochemical experiments. We are grateful to I. Jutras and M.-J. Bourque for technical assistance. Helpful comments on this manuscript were provided by Drs. A. Bouron, J.-C. Lacaille, G. Massicotte, and D. Lévesque.

This work was funded by the Canadian Institutes of Health Research (CIHR) and the EJLB Foundation. L.-E. Trudeau is a Michael Smith scholar of the CIHR. P. Congar was supported by the Neuroscience Canada Foundation and by the Groupe de Recherche sur le Système Nerveux Central of the Université de Montréal.

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