Distinct Mechanisms of Presynaptic Inhibition at GABAergic Synapses of the Rat Substantia Nigra Pars Compacta

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Giustizieri, Michela, Giorgio Bernardi, Nicola B. Mercuri, and Nicola Berretta. Distinct mechanisms of presynaptic inhibition at GABAergic synapses of the rat substantia nigra pars compacta. J Neurophysiol 94: 1992–2003, 2005. First published June 8, 2005; doi:10.1152/jn.00171.2005. We investigated the mechanisms of presynaptic inhibition of GABAergic neurotransmission by group III metabotropic glutamate receptors (mGluRs) and GABAB receptors, in dopamine (DA) neurons of the substantia nigra pars compacta (SNc). Both the group III mGluRs agonist L-(+)-2-amino-4-phosphonobutyric acid (AP4, 100 μM) and the GABAB receptor agonist baclofen (10 μM) reversibly depressed the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) to 48.5 ± 2.7 and 79.3 ± 1.6% (means ± SE) of control, respectively. On the contrary, the frequency of action potential-independent miniature IPSCs (mIPSCs), recorded in tetrodotoxin (TTX, 1 μM) and cadmium (100 μM) were insensitive to AP4 but were reduced by baclofen to 49.7 ± 8.6% of control. When the contribution of voltage-dependent calcium channels (VDCCs) to synaptic transmission was boosted with external barium (1 mM), AP4 became effective in reducing TTX-resistant mIPSCs to 65.4 ± 7.6% of control, thus confirming a mechanism of presynaptic inhibition involving modulation of VDCCs. Differently from AP4, baclofen inhibited to 58.5 ± 6.7% of control the frequency mIPSCs recorded in TTX and the calcium ionophore ionomycin (2 μM), which promotes Ca2+-dependent, but VDCC-independent, transmitter release. Moreover, in the presence of α-latrotoxin (0.3 nM), to promote a Ca2+-independent vesicular release of GABA, baclofen reduced mIPSC frequency to 48.1 ± 3.2% of control, while AP4 was ineffective. These results indicate that group III mGluRs depress GABA release to DA neurons of the SNc through inhibition of presynaptic VDCCs, while presynaptic GABAB receptors directly impair transmitter exocytosis.

INTRODUCTION

Presynaptic inhibition is a mechanism of modulation commonly observed at most synapses of the central and peripheral nervous system. It is initiated in response to activation of a wide range of presynaptic receptors and leads to reduction in the probability of vesicle fusion onto the presynaptic membrane (Wu and Saggau 1997). Although reduction in the amount of released neurotransmitter in response to action potentials invading the presynaptic terminal is the final outcome of any form of presynaptic inhibition, the intracellular mechanisms responsible for such an effect may vary depending on the receptors involved or synaptic location. The most common mechanism of action consists in inhibition of voltage-dependent calcium channels (VDCCs) located on the presynaptic boutons. This leads to a reduced elevation in intracellular Ca2+ after action potential invasion of the terminal, thus reducing the efficacy of the Ca2+-dependent exocytotic machinery. This mechanism of action has been demonstrated in response to activation of a variety of presynaptic receptors, in particular mGluRs (Capogna 2004; Scanziani et al. 1995; Takahashi et al. 1996; Zhang and Schmidt 1999) and GABAB receptors (Chen and van den Pol 1998; Isaacson 1998; Takahashi et al. 1998; Wu and Saggau 1995). However, additional mechanisms of presynaptic inhibition have been proposed involving a direct impairment of transmitter exocytosis. Indeed, activation of presynaptic receptors can reduce the frequency of miniature postsynaptic currents even when extracellular Ca2+ is removed or Ca2+ channels are blocked by cadmium (Dittman and Regehr 1996; Doi et al. 2002; Kolaj et al. 2004; Rohrbacher et al. 1997; Scanziani et al. 1992). Furthermore, stimulation of presynaptic receptors may reduce the frequency of miniature postsynaptic currents triggered by toxins like the lanthamide gadolinium and α-latrotoxin (Capogna et al. 1996b), which induce neurotransmitter release independently of Ca2+ influx through VDCCs (Capogna et al. 1996a). Therefore presynaptic inhibition of evoked synaptic transmission may also directly target the recruitment of synaptic vesicle in the terminal, downstream of Ca2+ influx (Sakaba and Neher 2003).

We have focused our attention on two previously described forms of presynaptic inhibition at GABAergic synapses of dopamine (DA) neurons of the substantia nigra pars compacta (SNc), mediated by glutamate and GABA, through activation of mGluRs (Bonci et al. 1997) and GABAB receptors (Haussner and Yung 1994), respectively. Midbrain DA neurons are part of the basal ganglia circuitry and play an essential role in motor control as well as in cognitive functions and affective behaviors (Groenewegen 2003; Montague et al. 1996; Pillon et al. 2003). The great majority of synapses to these neurons are GABAergic (Smith and Bolam 1990), arising from local GABAergic networks, the pallidum, the striatum, and the nucleus accumbens (Grosova et al. 1982; Haber et al. 1985; Smith and Bolam 1990; Tepper et al. 1995; Walaas and Fonnum 1980). These synaptic inputs provide a tonic inhibition to DA neurons and control their firing activity (Celada et al. 1999; Collingridge and Davies 1981; Engberg et al. 1993; Paldini and Tepper 1999; Paladini et al. 1999). Glutamatergic axons are also present in this area, originating from the cortex, the subthalamus, and pedunculopontine nuclei (Hammond et al. 1978; Scarnati et al. 1986; Sesack et al. 1989) and contribute...
with their pre- and postsynaptic actions to the modulation of DA neurons excitability, such that alteration in the glutamatergic neurotransmission in this area is thought to be associated to basal ganglia disorders, like Parkinson’s disease (Greenamyre 2001; Rouse et al. 2000). The discovery that both mGluRs and GABA<sub>B</sub> receptors depress VDCCs in GABAergic neurons projecting to the ventral midbrain (Stefani et al. 1994, 1996, 1999) has prompted the hypothesis that both receptors exert their functional role of presynaptic inhibition at GABAergic synapses of the SNc through a converging mechanism of inhibition of presynaptic VDCCs, but evidence exists in midbrain embryonic cultures of a VDCC-independent mechanism of action by GABA<sub>B</sub> receptors (Jarolimek and Misgeld 1992; Rohrbacher et al. 1997). We now present an in-depth investigation of the mechanism of presynaptic inhibition of GABAergic synapses to DA neurons of the SNc in response to group III mGluRs and GABA<sub>B</sub> receptor stimulation. This characterization provides new insights into the role of these presynaptic receptors in the processing of information in the SNc and their potential target in the treatment of Parkinson’s disease.

METHODS

Wistar rats (18–25 days old) were anesthetized by inhalation of 2-bromo-2-chloro-1,1,1-trifluoroethane and killed by decapitation. All experiments follow international guidelines on the ethical use of animals from the European Communities Council Directive of 24 November 1986 (86/609/EEC). The brain was rapidly removed, and a tissue block containing the midbrain was immersed in artificial cerebrospinal fluid solution (ACSF) composed of (in mM) 126 NaCl, 2.5 KCl, 1.2 MgCl<sub>2</sub>, 2.4 CaCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 24 NaHCO<sub>3</sub>, and 11 glucose; saturated with 95% O<sub>2</sub>-5% CO<sub>2</sub> (pH 7.4, 303 mosM). Horizontal slices (250 μm) of the ventral midbrain, containing the substantia nigra, were cut at 8–10° C using a vibratome (Leica VT1000S, Leica Microsystems, Wetzlar, Germany). After an incubation period of 1 h in ACSF at 33.5°C, a single slice was transferred to a submerged recording chamber (2.5–3 ml/min, 33.5°C), on the stage of an upright microscope (Axioskop FS, Zeiss, Göttinngen, Germany), equipped for infrared video microscopy (Hamamatsu, Tokyo), allowing a direct visualization of the recorded neurons.

Electrophysiology

Presumed dopamine neurons were recorded in whole cell patch-clamp configuration using 1.5-mm borosilicate glass electrodes (3–4 MΩ), pulled with a vertical puller (PP-83 Narishige) and filled with (in mM) 133 CsCl, 2 MgCl<sub>2</sub>, 0.1 EGTA, 10 HEPES, and 10 QX-314; pH adjusted to 7.3 with CsOH (280 mosM). Membrane currents were recorded in voltage-clamp mode at a holding potential of −60 mV, using a differential amplifier (Multiclamp 700A, Axon Instruments, Union City, CA). Signals were filtered at 1 kHz, digitized at 10 kHz with Digidata 1320 (Axon Instruments), and acquired with the pClamp9 software (Axon Instruments). No series resistance compensation was implemented, to keep a low signal-to-noise ratio, however, series resistance and whole cell capacitance were monitored continuously during the experiment, and recordings were discarded if series resistance changed by >15% from control conditions. Evoked synaptic responses were elicited at 0.033 Hz, using a bipolar stainless steel stimulating electrode (FHC, Bowdoinham, ME), close to the recorded cell.

Data analysis

Evoked and spontaneous inhibitory postsynaptic currents (sIPSCs) were analyzed off-line using the pClamp9 software package (Axon Instruments). Spontaneous and miniature events were detected through an algorithm based on the minimization of the sum of squared errors data and a template function. Single-template waveforms were created by averaging a number of spontaneous events, following visual inspection of a representative trace in each cell. The template matching threshold was set between 5 and 5.5, providing a good balance to avoid detection of false events. Data are expressed as means ± SE. To statistically evaluate the effects on evoked responses, a two-tailed Student t-test was used to compare the mean amplitude of evoked synaptic currents in control conditions and during the last 2 min of experimental challenge, using P < 0.05 as threshold for statistical significance. Changes in spontaneous events amplitude or interevent interval was determined in each single neuron by comparison of their cumulative distributions, with the Kolmogorov-Smirnov (K-S) test, using P < 0.02 as threshold for statistical significance. Group analysis of variations in amplitude and interevent interval of spontaneous events in all neurons exposed to a specific experimental challenge were evaluated using the two-tailed Student t-test, using P < 0.05 as threshold for statistical significance.

Drugs

Drugs used were 6-cyano-7-nitroquinolinicline-2,3-dione (CNQX), d(-)-2-amino-5-phosphonoepoatoic acid (AP5), baclofen, l(+)-2-amino-4-phosphonobutyric acid (AP4), (RS)-a-methyl-4-sulfonofenylglycine (MSG), and (2S,3R)-3-[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxipropyl]phenylmethyl phosphinic acid (CGP 55845) from Tocris Cookson (Bristol, UK); tetrodotoxin (TTX) and α-latrotoxin (α-LTX) from Alomone Labs (Jerusalem, Israel); ionomycin (free acid, streptomycyes conglobatus), from Calbiochem (San Diego, CA). Drugs were prepared in stock solutions (×1,000) of distilled water, except for α-LTX dissolved in 50% glycerol, and bath applied.

RESULTS

Whole cell patch-clamp recordings were obtained from a total of 136 neurons located within the SNc. The Cs<sup>+</sup>- and QX-314-containing filling solution in the patch electrodes precluded any meaningful electrophysiological identification of the recorded neurons on the basis of an I<sub>h</sub> current or postsynaptic response to dopamine (Mercuri et al. 1992; Perkins and Wong 1995); however, when spontaneous current spikes were observed in cell-attached mode, they never exceed the basal frequency of 1–3 Hz, typical of the DA neurons (Grace and Onn 1989). Moreover, cells were visually identified as presumed DAergic neurons on the basis of their location closed to the medial terminal nucleus of the accessory optic tract and their typical fusiform cell body and long unbranched proximal dendrites, extending in the plane of the slice (Grace and Onn 1989). All experiments were performed in the continuous presence of the glutamate ionotropic receptor antagonists CNQX (10 μM) and AP5 (50 μM). Moreover, any postsynaptic response due to opening of K<sup>+</sup> conductances was prevented by the internal dialysis with CsCl and QX-314 through the patch pipette.

Effect of AP4 and baclofen on evoked IPSCs

Synaptic responses were evoked by means of a stimulating electrode placed within the substantia nigra. They consisted of a fast IPSC, mediated by GABA<sub>B</sub> receptors, as it was completely blocked by picrotoxin (100 μM; not shown). Bath perfusion of the selective group III mGluRs agonist AP4...
Schoepp et al. 1999) resulted in a reversible IPSC depression, that attained a maximal effect of 53.0 ± 7.5% of control, at a concentration of 100 μM (P < 0.01, paired Student’s t-test, n = 10; Fig. 1B). The group II/III mGluRs antagonist MSPG (Jane et al. 1995) prevented AP4 effect (Fig. 1A). Thus IPSC amplitude in MSPG (100 μM) and AP4 (100 μM) was 95.7 ± 7.7% of control in MSPG alone (P > 0.9, paired Student’s t-test, n = 4). The effect of AP4 was consistent with previous observations showing a similar degree of AP4-induced depression, reaching a plateau at concentrations ≥30 μM (Bonci et al. 1997). IPSC amplitude was also inhibited (15.3 ± 3.2% of control; P < 0.01, paired Student’s t-test, n = 9) by the GABA<sub>B</sub> receptor agonist baclofen (10 μM; Fig. 1D), and this effect was prevented by the selective GABA<sub>B</sub> receptor antagonist CGP 55845 (Fig. 1C). IPSC amplitude in CGP 55845 (1 μM) and baclofen (10 μM) was 95.5 ± 11.0% of control in CGP 55845 alone (P > 0.5, paired Student’s t-test, n = 4).

**Effect of AP4 and baclofen on sIPSCs**

To explore in greater detail the mechanism of presynaptic inhibition by group III mGluRs and GABA<sub>B</sub> receptors in this area, we examined the effects of AP4 and baclofen on sIPSCs. In each tested neuron (n = 10) AP4 (100 μM) caused a reversible rightward shift of the interevent cumulative distribution (P < 0.01, K-S test), that was associated, in 4 of 10 neurons, to reduction of sIPSC amplitude (P < 0.01, K-S test; Fig. 2, A and B). Overall, AP4 (100 μM) caused a significant increase in sIPSC interevent interval (P < 0.001, paired Student’s t-test, n = 10), associated to a small but non significant reduction of their amplitude (P = 0.07 paired Student’s t-test, n = 10; Fig. 2B, Table 1).

Perfusion of baclofen (10 μM) caused in each cell (n = 8) a reversible rightward shift of the interevent cumulative distribution (P < 0.001, K-S test), that was associated, in three of eight neurons, to reduction of sIPSC amplitude (P < 0.001, K-S test; Fig. 2, C and D). Overall, both the reduction of sIPSC amplitude and the increase in their interevent interval was statistically significant (P < 0.02 and P < 0.001, respectively, paired Student’s t-test, n = 10; Fig. 2D, Table 1).

**Effect of AP4 and baclofen on mIPSCs**

The effects of AP4 and baclofen on GABAergic transmission were further explored by looking at the effects of these agonists on action potential-independent miniature IPSCs (mIPSCs). In the presence of TTX (1 μM), in two only, of nine
neurons, 100 μM AP4 significantly increased (P < 0.0001, K-S test) mIPSC interevent interval, without changing mIPSC amplitude (P > 0.1, K-S test). Thus no significant change resulted from group comparison of both mIPSC amplitude (P > 0.3, paired Student’s t-test, n = 9) and interevent interval (P > 0.3, paired Student’s t-test, n = 9; Table 1).

By contrast, baclofen (10 μM) was still effective in reducing GABAergic transmission in the presence of TTX (1 μM). In each neuron (n = 6), baclofen increased mIPSC interevent interval (P < 0.0005, K-S test) without reducing their amplitude (P > 0.03, K-S test). Therefore considering all tested neurons, a significant increase of mIPSC interevent interval was observed (P < 0.05, paired Student’s t-test, n = 6) with no change in their amplitude (P > 0.5, paired Student’s t-test, n = 6; Table 1).

Effect of AP4 and baclofen on mIPSCs in cadmium

The difference in AP4 and baclofen action was further confirmed in experiments performed in the continuous presence of TTX (1 μM) and CdCl2 (100 μM), to abolish the contribution of VDCCs to the spontaneous release of GABA. In these conditions, in no neuron AP4 (100 μM) caused changes in mIPSC interevent interval cumulative distribution (P > 0.1, K-S test, n = 5) or in their amplitude distribution (P > 0.1, K-S test, n = 5; Fig. 3, A and B). Thus no significant change resulted from group comparison of both mIPSC amplitude (P > 0.4, paired Student’s t-test, n = 5) and interevent interval (P > 0.5, paired Student’s t-test, n = 5; Fig. 3B, Table 1).
TABLE 1. Summary data of spontaneous and miniature inhibitory postsynaptic currents amplitude and interevent interval in different experimental conditions, before and after AP4 (100 μM) or baclofen (10 μM) perfusion

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AP4</th>
<th>t</th>
<th>n</th>
<th>Control</th>
<th>Baclofen</th>
<th>t</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude, pA</td>
<td>34.7 ± 1.8</td>
<td>32.8 ± 1.6</td>
<td>2.086</td>
<td>10</td>
<td>36.5 ± 2.5</td>
<td>33.7 ± 2.3***</td>
<td>3.382</td>
<td>8</td>
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<tr>
<td>Amplitude, pA</td>
<td>73.6 ± 6.9</td>
<td>93.7 ± 9.7****</td>
<td>6.216</td>
<td>9</td>
<td>77.7 ± 8.4</td>
<td>164.0 ± 19.7****</td>
<td>6.741</td>
<td>6</td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>26.1 ± 3.5</td>
<td>25.4 ± 3.9</td>
<td>1.082</td>
<td>5</td>
<td>26.5 ± 1.7</td>
<td>26.1 ± 1.5</td>
<td>0.609</td>
<td>11</td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>118.3 ± 34.6</td>
<td>122.1 ± 32.3</td>
<td>1.047</td>
<td>15</td>
<td>90.4 ± 4.1</td>
<td>192.8 ± 36.3*</td>
<td>2.971</td>
<td>12</td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>25.9 ± 1.6</td>
<td>25.7 ± 1.7</td>
<td>0.767</td>
<td>11</td>
<td>27.7 ± 2.0</td>
<td>27.0 ± 2.5</td>
<td>1.165</td>
<td>9</td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>112.5 ± 13.0</td>
<td>114.2 ± 14.7</td>
<td>0.591</td>
<td>10</td>
<td>108.6 ± 9.7</td>
<td>233.0 ± 25.8**</td>
<td>4.130</td>
<td>5</td>
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<tr>
<td>Amplitude, pA</td>
<td>32.8 ± 2.7</td>
<td>29.5 ± 2.2***</td>
<td>3.689</td>
<td>36</td>
<td>30.1 ± 1.6</td>
<td>24.8 ± 1.9****</td>
<td>5.854</td>
<td>12</td>
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<tr>
<td>Amplitude, pA</td>
<td>67.5 ± 7.7</td>
<td>102.9 ± 15.4****</td>
<td>4.637</td>
<td>11</td>
<td>61.2 ± 3.7</td>
<td>121.1 ± 11.4****</td>
<td>7.263</td>
<td>10</td>
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<td>Amplitude, pA</td>
<td>43.3 ± 4.4</td>
<td>43.9 ± 4.6</td>
<td>0.738</td>
<td>15</td>
<td>42.7 ± 4.9</td>
<td>33.1 ± 3.1***</td>
<td>3.964</td>
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<td>Amplitude, pA</td>
<td>46.4 ± 2.7</td>
<td>46.0 ± 2.7</td>
<td>1.290</td>
<td>10</td>
<td>47.8 ± 3.7</td>
<td>114.3 ± 28.0*</td>
<td>2.628</td>
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<td>Amplitude, pA</td>
<td>41.6 ± 4.4</td>
<td>41.9 ± 4.6</td>
<td>0.206</td>
<td>15</td>
<td>39.1 ± 2.7</td>
<td>31.6 ± 1.4**</td>
<td>3.165</td>
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<tr>
<td>Amplitude, pA</td>
<td>52.3 ± 6.7</td>
<td>52.7 ± 7.3</td>
<td>0.341</td>
<td>10</td>
<td>47.8 ± 3.7</td>
<td>103.2 ± 11.2****</td>
<td>6.607</td>
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</table>

Values are means ± SE. Data are compared using the 2-tailed paired Student’s t-test. The relative t values are also shown for each pair of data. *P < 0.05, **P < 0.02, ***P < 0.005, ****P < 0.001. Data are taken before and after L-(+)-2-amino-4-phosphonobutyric acid (AP4). sIPSC, spontaneous inhibitory postsynaptic current.

Effect of AP4 and baclofen on mIPSCs in barium

Conversely, baclofen (10 μM), in the presence of TTX (1 μM) and CdCl₂ (100 μM), still produced in each neuron (n = 5) a significant rightward shift of the interevent cumulative distribution (P < 0.005, K-S test) with no associated reduction of mIPSC amplitude (P > 0.5, K-S test; Fig. 3, C and D). Thus considering all tested neurons, a significant increase of mIPSC interevent interval was observed (P < 0.02, paired Student’s t-test, n = 5) with no change in their amplitude (P > 0.3, paired Student’s t-test, n = 5; Fig. 3D, Table 1).

Effect of AP4 and baclofen on mIPSCs in barium

The lack of effect of AP4 on mIPSCs suggests that group III mGlURs decrease GABAergic transmission by acting on the VDCCs opened after action potential invasion of the presynaptic terminal. Alternatively though, group III mGlURs may act on TTX-sensitive Na⁺ conductances, hence reducing action-dependent synaptic events only. To discriminate between these two possibilities, we repeated the same experiments in the presence of TTX and BaCl₂. We envisaged that, by blocking K⁺ conductances with Ba²⁺, presynaptic calcium channels may still contribute to the spontaneous release of GABA as a result of presynaptic terminal depolarization, although in the absence of action potentials. Indeed, in control experiments we found that mIPSC interevent interval, recorded in TTX (1 μM), decreased from 99.7 ± 12.6 to 65.7 ± 7.4 ms (P < 0.02, paired Student’s t-test, n = 9) after perfusion with BaCl₂ (1 mM). A subsequent addition of CdCl₂ (100 μM), in the continuous presence of TTX and BaCl₂ increased mIPSC interevent interval to 138.9 ± 19.6 ms (P < 0.005, paired Student’s t-test, n = 9; data not shown).

In each neuron (n = 11) recorded in the continuous presence of TTX (1 μM) and BaCl₂ (1 mM), AP4 (100 μM) caused a significant rightward shift of the interevent cumulative distribution (P < 0.005, K-S test; Fig. 3, E and F), associated, in 2 of 11 neurons, to reduction of mIPSC amplitude (P < 0.02, K-S test). Overall, both the reduction of mIPSC amplitude and the increase in their interevent interval was statistically significant (P < 0.005 and P < 0.001, respectively, paired Student’s t-test, n = 11; Fig. 3F, Table 1).

Baclofen (10 μM), in the presence of TTX and BaCl₂, was still effective in reducing GABAergic transmission. In each neuron (n = 11) baclofen increased mIPSC interevent interval (P < 0.0001, K-S test; Fig. 3, G and H), associated, in 8 of 11 cells, to reduction of their amplitude (P < 0.02, K-S test). Accordingly, from group analysis of all tested neurons, both the reduction of mIPSC amplitude and their increase in interevent interval resulted statistically significant (P < 0.001 both, paired Student’s t-test, n = 11; Fig. 3H, Table 1).

Effect of AP4 and baclofen on ionomycin-induced mIPSCs

The previous experiments indicate that group III mGlURs inhibit the release of GABA by acting on VDCCs, while the mechanism of action of GABA_B receptors is downstream Ca²⁺ influx through VDCCs. We further explored whether baclofen could also inhibit VDCC-independent, but Ca²⁺-dependent, miniature events. To this aim, we bypassed Ca²⁺ entry through VDCCs with the Ca²⁺-ionophore ionomycin.

As expected, perfusion of ionomycin (2 μM), in the presence of TTX (1 μM), resulted in a significant decrease of mIPSC interevent interval, from 92.0 ± 8.6 to 48.7 ± 3.3 ms (P < 0.001, paired Student t-test, n = 14), associated to increase of mIPSC amplitude, from 29.6 ± 2.2 to 42.4 ± 3.7 pA (P < 0.005, paired Student t-test, n = 14), that reached a plateau within 10–15 min of drug perfusion (Fig. 4, A–C).
Therefore in each one of the recorded cells a rightward shift of the interevent cumulative distribution was observed ($P < 0.0001$, K-S test), associated to increase of mIPSC amplitude ($P < 0.0001$, K-S test; Fig. 4C).

After perfusion in TTX (1 $\mu$M) and ionomycin (2 $\mu$M) for $\geq 15$ min, baclofen (10 $\mu$M) was added to the medium and a significant reduction of GABAergic transmission was observed. In each neuron ($n = 12$) baclofen increased mIPSC

![Diagram](http://jn.physiology.org/)

**FIG. 3.** Group III mGluRs inhibit the voltage-dependent calcium channel (VDCC)-dependent GABAergic transmission. Successive sample traces of miniature IPSCs (mIPSCs) before (top), during (middle), and after (bottom) perfusion of 100 $\mu$M AP4 (A and E) or 10 $\mu$M baclofen (C and G). Amplitude (left) and interevent interval (right) distributions of the mIPSCs recorded from the corresponding cells are shown in B, F, D, and H, below the traces. In the continuous presence of TTX (1 $\mu$M) and cadmium (100 $\mu$M), baclofen (D) significantly increased mIPSCs interevent intervals ($P < 0.0001$, K-S test) without changing their amplitude distribution ($P > 0.29$, K-S test), whereas AP4 (B) was ineffective ($P > 0.53$ for amplitude and $P > 0.23$ for interevent interval distribution, K-S test). Insets: group analysis histograms (means $\pm$ SE) of mIPSCs amplitude (left) and interevent interval (right) from 5 neurons in both B and D ($** P < 0.02$, 2-tailed paired Student’s t-test). F: in the continuous presence of TTX (1 $\mu$M) and barium (1 mM), AP4 increased mIPSCs interevent intervals ($P < 0.0001$, K-S test) without changing their amplitude distribution ($P > 0.08$, K-S test) and baclofen (H) increased significantly mIPSCs interevent intervals ($P < 0.0001$, K-S test) and decreased their amplitude distribution ($P < 0.001$, K-S test). Insets: group analysis histograms (means $\pm$ SE) of mIPSCs amplitude (left) and interevent interval (right) from 11 neurons in both F and H (*** $P < 0.005$, **** $P < 0.001$, 2-tailed paired Student’s t-test).
FIG. 4. GABA<sub>B</sub> receptors inhibit ionomycin-induced mIPSCs. A: successive sample traces of mIPSCs recorded in the continuous presence of TTX (1 µM, left) and TTX + ionomycin (2 µM, right). B: running average histogram (mean ± SE, bin size: 30 s) of mIPSCs amplitude (top) and interevent interval (bottom) from the cell in A, showing the time course of ionomycin effect. C: amplitude (left) and interevent interval (right) cumulative distributions of the mIPSCs recorded from the same cell shown in A, in TTX (control) and TTX + ionomycin (ionomycin). In this cell, ionomycin reduced mIPSCs interevent interval distribution ($P < 0.0001$, K-S test) and increased their amplitude ($P < 0.0001$, K-S test). Insets: group analysis histograms (means ± SE) of mIPSCs amplitude (left) and interevent interval (right) from a total of 14 neurons (**$P < 0.005$, ***$P < 0.001$, 2-tailed paired Student’s t-test). D: successive sample traces of mIPSCs recorded in the continuous presence of TTX (1 µM) and ionomycin (2 µM) before (top), during (middle), and after (bottom) perfusion of 100 µM AP4. E: in the cell shown in D, no significant changes were observed after perfusion of AP4 in the interevent interval ($P > 0.78$, K-S test) or in the amplitude distribution ($P > 0.18$, K-S test). F: successive sample traces of mIPSCs recorded in the continuous presence of TTX (1 µM) and ionomycin (2 µM) before (top), during (middle), and after (bottom) perfusion of 10 µM baclofen. G: baclofen increased mIPSCs interevent interval distribution ($P < 0.0001$, K-S test), and reduced their amplitude ($P < 0.0005$, K-S test) in the cell shown in F. Insets in E and G: group analysis histograms (means ± SE) of mIPSCs amplitude (left) and interevent interval (right) from 15 (E) and 12 (G) neurons (*$P < 0.05$, ***$P < 0.005$, 2-tailed paired Student’s t-test).
interevent interval (P < 0.0001, K-S test) and reduced their amplitude in 11 of 12 cells (P < 0.005, K-S test; Fig. 4, F and G). Overall, both the reduction of mIPSC amplitude and the increase in their interevent interval were statistically significant (P < 0.005 and P < 0.05, respectively, paired Student’s t-test, n = 12; Fig. 4G, Table 1). Conversely, mIPSCs in TTX (1 μM) and ionomycin (2 μM) were insensitive to AP4 (100 μM). In all tested cells (n = 15), AP4 (100 μM) did not produce significant changes in the cumulative distribution of mIPSC interevent interval (P > 0.1, K-S test) or amplitude (P > 0.1, K-S test; Fig. 4, D and E). Accordingly, no significant change resulted from group comparison of both mIPSC amplitude (P > 0.4, paired Student’s t-test, n = 15) and interevent interval (P > 0.2, paired Student’s t-test, n = 15; Fig. 4E, Table 1).

Effect of AP4 and baclofen on α-LTX-induced mIPSCs

We further explored the mechanism of GABA<sub>B</sub>-mediated presynaptic inhibition of GABAergic transmission by testing if baclofen could directly inhibit the GABA release machinery. To this aim, we used the active component of the black widow spider peptide α-latrotoxin (α-LTX). This peptide has been shown to promote Ca<sup>2+</sup>-independent vesicular release of neurotransmitters (Capogna et al. 1996a).

Bath perfusion of α-LTX (0.3 nM), in the presence of TTX (1 μM), resulted in a significant decrease of mIPSC interevent interval, from 76.1 ± 7.3 to 42.4 ± 2.7 ms (P < 0.005, paired Student t-test, n = 7), associated to increase of amplitude, from 29.4 ± 3.4 to 44.4 ± 3.9 pA (P < 0.001, paired Student t-test, n = 7), that reached a plateau within 10–15 min of drug perfusion (Fig. 5, A–C). Thus in each one of the recorded cells, a rightward shift of the interevent cumulative distribution was observed (P < 0.0001, K-S test), associated to increase of mIPSC amplitude (P < 0.0001, K-S test; Fig. 5C).

After perfusion in TTX (1 μM) and α-LTX (0.3 nM) for ≥15 min, baclofen (10 μM) was added to the medium, and a significant reduction of GABAergic transmission was observed. In each neuron (n = 9), baclofen reversibly induced an increase of mIPSC interevent interval (P < 0.0001, K-S test) and reduced their amplitude in five of nine cells (P < 0.001, K-S test; Fig. 5, F and G). Overall, both the reduction of mIPSC amplitude and the increase in their interevent interval were statistically significant (P < 0.02 and P < 0.001, respectively, paired Student’s t-test, n = 9; Fig. 5G, Table 1). Conversely, mIPSCs in TTX (1 μM) and α-LTX (0.3 nM) were insensitive to AP4 (100 μM). In all tested cells (n = 10), AP4 (100 μM) did not produce significant changes in the cumulative distribution of mIPSC interevent interval (P > 0.1, K-S test) or amplitude (P > 0.1, K-S test; Fig. 5, D and E). Accordingly, no significant change resulted from group comparison of both mIPSC amplitude (P > 0.8, paired Student’s t-test, n = 10) and interevent interval (P > 0.7, paired Student’s t-test, n = 10; Fig. 5E, Table 1).

DISCUSSION

The present results give evidence of two different mechanisms of presynaptic inhibition on GABAergic synapses of the SNC. One mechanism, activated in response to group III mGluRs stimulation, consists of inhibition of presynaptic VDCCs. The second is initiated by GABA<sub>B</sub> receptors stimulation and causes a direct impairment of the vesicle release machinery.

Our experimental protocol required intracellular dialysis with Cs<sup>+</sup> and QX-314 to abolish any postsynaptic change in K<sup>+</sup> conductance, and in so doing, any reliable electrophysiological identification of the recorded neurons was precluded (Mercuri et al. 1992; Perkins and Wong 1995). In spite of this experimental limitation, we can reasonably affirm that the data presented were obtained from DA neurons because their shape and location was typical of the DA SNC neurons (Grace and Onn 1989); their tonic 1- to 3-Hz firing activity, detected in cell attached mode, has previously been reported to be a hallmark of DA-sensitive neurons in this area (Berretta et al. 2000); and finally, neurons of the SNC with such a low-frequency tonic firing activity, detected in cell attached mode, have been immunohistochemically identified as tyrosine hydroxylase-positive in a previous report (Guatteo et al. 2000).

The inhibitory action of AP4 or baclofen could also be associated to inhibition of spontaneous events amplitude. This occurred with baclofen in barium, ionomycin, or α-LTX or with AP4 when mIPSCs were recorded in barium (Table 1). We can rule out that these effects are due to some form of postsynaptic action of baclofen or AP4 because neither of the two agonists affected mIPSC amplitude in TTX and cadmium (Fig. 3, A–D; Table 1). More likely, the release of GABA occurs from multiple release sites at the same synapse; therefore, in conditions of higher release probability, multiple events may overlap and generate larger events. Indeed, mIPSC mean amplitude in TTX alone or in TTX + Cd<sup>2+</sup> was smaller than that of sIPSCs (see Table 1), thus indicating that higher-amplitude events were due to action potential-dependent synaptic events. According to this hypothesis, the reduced occurrence of these higher amplitude events was proportional to the degree of reduction in probability of release by group III mGluRs or GABA<sub>B</sub> receptors.

Group III mGluRs-mediated presynaptic inhibition was absent when VDCCs were blocked by external cadmium, although supramaximal concentrations of AP4 had been used (Bonci et al. 1997). Moreover, when barium was added to the external medium, to boost the contribution of VDCCs, the inhibitory effect of AP4 on mIPSC frequency was even stronger than that observed on sIPSCs (P < 0.02, 2-tailed unpaired Student t-test; Fig. 6). Furthermore, the Ca<sup>2+</sup>-dependent, but VDCC-independent, release of GABA induced by ionomycin (Capogna et al. 1996a,b) was insensitive to AP4. These results indicate that group III mGluRs inhibit the Ca<sup>2+</sup>-dependent release of GABA that follows action potential invasion of the terminal exclusively by reducing the conductance of presynaptic VDCCs. It should also be noted that the effects on sIPSCs in basal conditions were relatively small; indeed, for sIPSC amplitude, they did not even reach the level for statistical significance (Table 1). This probably occurs because in our in vitro conditions most of the sIPSCs were in actual facts miniature events, as indicated by the high proportion of sIPSCs that were insensitive to TTX or TTX + Cd<sup>2+</sup> (Table 1). Notably, the degree of inhibition of sIPSCs by AP4 was smaller than that observed for evoked IPSCs (~20 vs. ~50%; see Figs. 1 and 6). The reason for this discrepancy may reside in the VDCC dependence of evoked synaptic responses as
FIG. 5. GABA<sub>B</sub> receptors inhibit mIPSCs induced by α-LTX. A: successive sample traces of mIPSCs recorded in the continuous presence of TTX (1 μM, left) and TTX + α-LTX (0.3 nM, right). B: running average histogram (mean ± SE; bin size: 30 s) of mIPSCs amplitude (top) and interevent interval (bottom) from the cell in A, showing the time course of α-LTX effect. C: amplitude (left) and interevent interval (right) distributions of the mIPSCs recorded from the same cells are shown in A in TTX (control) and TTX + α-LTX (α-LTX). In this cell, α-LTX reduced mIPSCs interevent interval distribution (P < 0.0001, K-S test) and increased their amplitude (P < 0.0001, K-S test). Insets: group analysis histograms (means ± SE) of mIPSCs amplitude (left) and interevent interval (right) from a total of 7 neurons (**P < 0.001, 2-tailed paired Student’s t-test). D: successive sample traces of mIPSCs recorded in the continuous presence of TTX (1 μM) and α-LTX (0.3 nM) before (top), during (middle), and after (bottom) perfusion of 100 μM AP4. E: in the cell shown in D, no significant changes were observed after perfusion of AP4 in the interevent interval (P > 0.33, K-S test) or in the amplitude distribution (P > 0.31, K-S test). F: successive sample traces of mIPSCs recorded in the continuous presence of TTX (1 μM) and α-LTX (0.3 nM) before (top), during (middle), and after (bottom) perfusion of 10 μM baclofen. G: in the cell shown in F, baclofen increased mIPSCs interevent interval distribution (P < 0.0001, K-S test) and reduced their amplitude (P < 0.0001, K-S test). Insets in E and G: group analysis histograms (means ± SE) of mIPSCs amplitude (left) and interevent interval (right) from 10 (E) and 9 (G) neurons (**P < 0.02, ****P < 0.001, 2-tailed paired Student’s t-test).
Most of the eight cloned mGluR subtypes (Conn and Pin 1997) are distributed throughout all areas of the basal ganglia and regulate cell excitability and synaptic transmission at excitatory and inhibitory synapses (Rouse et al. 2000). In situ hybridization and immunohistochemistry studies has demonstrated the presence of group III mGluRs in the SNC of the mGluR7 subtype, located onto striatonigral presynaptic terminals (Kosinski et al. 1999). Indeed the high concentration of the selective group III agonist AP4 used in our investigation to obtain a maximal response is indicative of this mGluR subtype (Conn and Pin 1997). In the striatopallidal projection, AP4 does also presynaptically depress GABAAergic transmission acting on higher affinity mGluR4 of the same group III mGluR family (Valenti et al. 2003). Interestingly though, mIPSCs are depressed by AP4 in this area, suggesting that different subtypes of the same receptor family use separate mechanism of action to depress neurotransmission.

Differently from group III mGluRs, we have shown that stimulation of GABA_B receptors resulted in presynaptic inhibition of GABA release in all experimental conditions implemented. In particular, the Ca^{2+}-independent release of neurotransmitter induced by α-LTX (Capogna et al. 1996a,b) was significantly inhibited by baclofen, and the reduction of mIPSC frequency due to this toxin was not significantly different from that observed in the presence of cadmium (Fig. 6). In addition, we have shown that baclofen reduced the spontaneous release of GABA induced by the ionophore ionomycin, by a similar extent to that observed in the presence of cadmium (Fig. 6). Because ionomycin promotes a VCDC-independent, though Ca^{2+}-dependent, transmitter release (Capogna et al. 1996a,b), we can conceivably hypothesize that a direct interference with the exocytotic process may also account for presynaptic inhibition of evoked, Ca^{2+}-dependent, GABA release by GABA_B receptors in these synapses.

Our results confirm and further extend a previous report on inhibition of cadmium-resistant mIPSCs by baclofen, in embryonic midbrain cultures (Jarolimek and Misgeld 1992; Rohrbacher et al. 1997), moreover, a similar effect of inhibition of ionomycin-induced mIPSCs has been observed in embryonic cultures of the ventral tegmental area, after stimulation of mu-opioid receptors (Bergevin et al. 2002). Presynaptic inhibition by GABA_B receptors acting on the release machinery has already been described in several other areas of the CNS (Capogna et al. 1996b; Kolaj et al. 2004; Scanziani et al. 1992), although mechanisms of reduction of VCDC conductance has similarly been proposed (Chen and van den Pol 1998; Isaacs 1998; Takahashi et al. 1998; Wu and Saggau 1995), alone or in combination with impairment of the exocytotic process (Dittman and Regehr 1996).

Functional considerations

If we take into account the whole circuitry rather than single evoked synaptic responses, we may predict different outcomes resulting from a presynaptic inhibition targeting VCDCs or the exocytotic machinery. First of all, VCDCs are subject to a voltage-dependent relief from G protein mediated inhibition (Brody and Yue 2000), therefore the efficacy of presynaptic inhibition targeting VCDCs is highly sensitive to the level of presynaptic terminal polarization as well as to specific patterns of presynaptic firing (Brody et al. 1997; Reid et al. 2003). In the context of presynaptic inhibition, it is evident that the more sensitive the release machinery is to a presynaptic stimulus the more important will be the contribution of the presynaptic inhibition to the final outcome. Therefore, it can be expected that the level of effects of a presynaptic stimulus is lower in a context where the contribution of postsynaptic events is high.
contrast, presynaptic inhibition targeting the exocytotic machinery is largely independent from the level of presynaptic activity. Moreover, it causes a parallel inhibition of signals actively generated by coded afferent inputs and of spontaneous signals randomly generated by the passive fusion of vesicles into the presynaptic membrane, hence differently affecting the signal-to-noise ratio. In addition, presynaptic inhibition of spontaneous neurotransmitter release, particularly at GABAergic synapses, can reduce the tonic activation of postsynaptic receptors, thus affecting network excitability (Semyanov et al. 2004). Therefore presynaptic GABA<sub>B</sub> receptors and group III mGluRs may differently modulate GABAergic inputs to DA neurons of the SNC. Although GABA<sub>B</sub> receptors should exert a simple feed-back auto-inhibition of any form of GABAergic response, stimulation of group III mGluRs by extrinsic glutamate afferent should selectively depress actively generated signals. In addition, specific codes of GABAergic signaling may display different sensitivities to this glutamate-mediated hetero-inhibition.

The firing activity of midbrain DA neurons, and consequently the release of DA to target areas, is highly sensitive to tonic inhibition by GABAergic afferents (Celada et al. 1999; Paldini and Tepper 1999; Paladini et al. 1999). Therefore the characterization of group-III-mediated presynaptic inhibition reported here, in relation to that of GABA<sub>B</sub> receptors, provides new insights into the role of these receptors in the processing of information in the SNC and their potential use as targets for the pharmacological treatment of Parkinson’s disease (Rouse et al. 2000; Valenti et al. 2003).

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