Possible Roles of Kainate Receptors on GABAergic Nerve Terminals Projecting to Rat Substantia Nigra Dopaminergic Neurons

Michiko Nakamura, Il-Sung Jang, Hitoshi Ishibashi, Shigenori Watanabe, and Norio Akaike. Possible roles of kainate receptors on GABAergic nerve terminals projecting to rat substantia nigra dopaminergic neurons. J Neurophysiol 90: 1662–1670, 2003. Published June 4, 2003; 10.1152/jn.01165.2002. GABAergic afferent inputs are thought to play an important role in the control of the firing pattern of substantia nigra pars compacta (SNc) dopaminergic neurons. We report here the actions of presynaptic kainite (KA) receptors in GABAergic transmission of rat SNc dopaminergic neurons. In mechanically dissociated rat SNc dopaminergic neurons attached with native presynaptic nerve terminals, GABAergic miniature inhibitory postsynaptic currents (mIPSCs) were recorded by use of conventional whole cell patch recording mode. In the voltage-clamp condition, KA (3 μM) significantly increased GABAergic mIPSC frequency without affecting the current amplitude. This facilitatory effect of KA was not affected in the presence of 20 μM GYK152466, a selective AMPA receptor antagonist, but was completely inhibited in the presence of 20 μM CNQX, an AMPA/KA receptor antagonist. Presynaptic KA receptors on GABAergic terminals were mainly permeable to Na+ but impermeable to Ca2+ because KA-induced facilitation of mIPSC frequency was completely suppressed in either Na+-free or Ca2+-free external solutions, and in the presence of 200 μM Cd2+, a general voltage-dependent Ca2+ channel blocker. In the slice preparation, KA increased GABAergic spontaneous mIPSC frequency, but significantly suppressed evoked IPSC (eIPSC) amplitude. However, this inhibitory action on eIPSCs was reversed by 10 μM CGP58845, a selective GABA<sub>B</sub> receptor antagonist, implicating the possible involvement of GABA<sub>B</sub> autoreceptors in KA-induced modulation of GABAergic transmission. Thus presynaptic KA receptors on GABAergic nerve terminals synapsing onto SNc neurons may play functional roles contributing the fine control of neuronal excitability and firing pattern of SNc.

INTRODUCTION

It is now well established that there are three subtypes of ionotropic glutamate receptors consisting of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kainate (KA) receptors in the mammalian CNS. KA receptors play important physiological roles in synaptic transmission and also in its presynaptic regulation. At hippocampal mossy fiber synapses, KA receptors mediate a small and slow component of the glutamatergic synaptic response (Cossart et al. 2002). However, much attention has been made to elucidate the functional significance of presynaptic KA receptors as presynaptic modulators of fast synaptic transmission (for review see Frerking and Nicoll 2000; Lerma et al. 2001). For example, presynaptic KA receptor activation can modulate either glutamatergic (Casassus and Mulle 2002; Kamiya and Ozawa 2000; Schmitz et al. 2000, 2001) or GABAergic transmission (Cossart et al. 2001; Jiang et al. 2001; Kerchner et al., 2001) at various CNS regions. The substantia nigra pars compacta (SNc) as well as the ventral tegmental area possesses a dense area of dopamine-containing neurons in the CNS. SNc dopaminergic neurons are well known to be crucial in normal and pathological motor control (for review see Bunney et al. 1991). Degeneration of these neurons is primary to the etiology of Parkinson’s disease (for review see Olanow and Tatton 1999), and dysfunction of dopaminergic transmission has been implicated in the symptom as schizophrenia (Creese et al. 1976). SNc dopaminergic neurons in vivo display three different firing patterns: a pacemaker-like regular firing pattern, a random pattern, and a burst firing pattern (Grace and Bunney 1985; Tepper et al. 1995). Among them, the pacemaker-like pattern is the only one that occurs spontaneously in dopaminergic neurons recorded in vitro (Grace 1987; Kita et al. 1986), suggesting afferent inputs play an important role in the control of the firing pattern of SNc dopaminergic neurons. SNc dopaminergic neurons receive both excitatory glutamatergic afferents inputs from pedunculopontine nucleus, subthalamic nucleus, and prefrontal cortex (Futami et al. 1995; Kita and Kitai 1987; Sesack and Pickel 1992), and inhibitory GABAergic afferents from the striatum, globus pallidus, and the substantia nigra pars reticulata (SNr) (Hajós and Greenfield 1994; Oertel et al. 1981; Ribak et al. 1976; Smith and Bolam 1990). In particular, GABAergic afferents are thought to play an important role in the control of the firing pattern of SNc dopaminergic neurons because of their abundance within the SNc (Oertel et al. 1981; Ribak et al. 1976; Smith and Bolam 1990). On the other hand, neurons in the striatum, globus pallidus, and SNr, the GABAergic afferents of which innervate the SNc, express KA receptor subunit mRNAs in a high level (Bischoff et al. 1997). In addition, there is a differential expression...
pattern of KA receptor subunit mRNAs in the basal ganglia circuitry (Bischoff et al. 1997), suggesting that KA receptors may be involved in the functions associated with the basal ganglia, with a key role in the control of the dopaminergic output pathway. In the present study, therefore, we investigated whether GABAergic presynaptic nerve terminals projecting to SNc dopaminergic neurons express functional KA receptors and whether presynaptic KA receptor activation can directly modulate GABAergic synaptic transmission.

METHODS

Preparation

All experiments conformed to the guiding principles for the care and use of animals approved by the Council of the Physiological Society of Japan and all efforts were made to minimize both the number of animals used and their suffering.

Wistar rats (12–15 days old) were decapitated under pentobarbital anesthesia [50 mg/kg, intraperitoneally (ip)]. The brain was dissected and transversely sliced at a thickness of 370 µm using a microslicer (VT1000S; Leica, Nussloch, Germany). Slices containing the SNc and SNr were identified under an upright microscope (DM-SP2; Leica) and the region of the SNc was identified in the basal ganglia, with a key role in the control of the dopaminergic output pathway. In the present study, therefore, we investigated whether KA receptors on GABAergic presynaptic terminals projecting to SNc dopaminergic neurons can directly modulate KA-induced GABAergic synaptic transmission.

For the slice preparation, Wistar rats (12–15 days old) were decapitated under pentobarbital anesthesia (50 mg/kg, ip). The brain was dissected and transversely sliced at a thickness of 270 µm by using a microslicer (VT1000S; Leica) in a cold low-Na+ medium (see following text). The slices were kept in an external bath solution (see following text) saturated with 95% O2–5% CO2 at 34–35°C for ≥1 h. Thereafter the slices were transferred into a recording chamber, and the SNc and SNr were identified under an upright microscope (DM LFSA; Leica). Drug was perfused by bath application at 3–4 ml/min.

Electrical measurements

All electrical measurements were performed using the conventional whole cell patch recording mode at a holding potential (VH) of 0 mV (CEZ-2300; Nihon Kohden, Tokyo), except where indicated. Patch pipettes were made from borosilicate capillary glass (1.5 mm OD, 0.9 mm ID; G-1.5; Narishige, Tokyo) in two stages on a vertical pipette puller (PB-7; Narishige). The resistance of the recording pipettes filled with internal solution was 5–6 MΩ. Electrode capacitance and liquid junction potential were compensated, but series resistance was not.

Neurons were viewed under phase contrast on an inverted microscope (Diapath; Nikon). Current and voltage were continuously monitored on an oscilloscope (VC-6023; Hitachi) and a pen recorder (RECTI-HORIT-8K, Sanei, Tokyo), and recorded on a digital-audio tape recorder (RD-120TE; TEAC). Membrane currents were filtered at 1 kHz (E-3201A Decade Filter; NF Electronic Instruments, Tokyo), digitized at 4 kHz, and stored on a computer equipped with pCLAMP 8.02 (Axon Instruments). When recording, 10-mV hyperpolarizing step pulses (30 ms in duration) were periodically delivered to monitor the access resistance. All experiments were performed at room temperature (23–25°C), except for the slice preparation (34–35°C).

Electrical stimulation used to obtain GABAergic evoked IPSCs (eIPSCs) was performed by applying short current pulses (100 µs, 30–50 µA) at 0.1 Hz through a glass pipette (ID, 7–8 µm). The pipette was placed around the dorsomedial or peripeduncular region of the SNr, 500–700 µm distally from the recorded neurons (Hajós and Greenfield 1994) and filled with the external bath solution, using a stimulator (SEN-7203; Nihon Kohden) with an isolator unit (SS-701J; Nihon Kohden). The signals were filtered at 3 kHz and digitized at 10 kHz, and stored on a computer equipped with pCLAMP 8.0.

FIG. 1. Mechanically dissociated SNc neurons. A: typical example of mechanically dissociated SNc neurons. Left: phase contrast images; right: TH-immunostaining images. Note that large neuron (upper) was TH-positive, but small one (bottom) was not. B: membrane responses to injection of depolarizing and hyperpolarizing currents. Currents were delivered through patch electrode in current-clamp condition. C: typical traces recorded from SNc neurons in current-clamp condition. Note that 1 µM dopamine hyperpolarized membrane potential and decreased action potential firing rate. Insets: traces indicated in B with expanded time scale. Dotted line, −60 mV.

J Neurophysiol • VOL 90 • SEPTEMBER 2003 • www.jn.org
Data analysis

Miniature IPSCs (mIPSCs) were counted and analyzed using the MiniAnalysis program (Synaptosoft, Decatur, GA) as described previously (Jang et al. 2002). Briefly, spontaneous events were screened automatically using an amplitude threshold of 10 pA and then visually accepted or rejected based on the rise and decay times. The average values of mIPSC frequency and amplitude during the control period (5–10 min) were calculated, and the frequency and amplitude of all the events during KA application (2 min) were normalized to these values. The effect of KA was quantified as a percentage increase in mIPSC frequency compared with the control values. The interevent intervals and amplitudes of a large number of mIPSCs obtained from the same neuron were examined by constructing cumulative probability distributions and compared using Kolmogorov–Smirnov test with Stat View software (SAS Institute, Cary, NC). The amplitude of evoked IPSCs (eIPSCs) was analyzed using pCLAMP 8.0. Numerical values are provided as means ± SE using values normalized to the control. Possible differences in amplitude and frequency distribution were tested by Student’s paired 2-tailed t-test using their absolute rather than normalized values. Values of P < 0.05 were considered significant.

Immunocytochemistry

To determine whether the large neurons (>25 μm in soma diameter) used for electrophysiological recordings in the present experiments indeed belong to SNc dopaminergic neurons, we performed immunocytochemical examinations using anti-tyrosine hydroxylase antibody. Neurons were mechanically dissociated on the glass coverslips coated with polyethylenimine (PEI) in a 35-mm culture dish. After most neurons were settled down and adhered to the coverslips, each coverslip was moved to a paraformaldehyde 10% paraformaldehyde in PBS for 30 min. After treatment with 0.2% Triton-X 100 for 5 min, neurons were incubated with PBS containing rabbit anti-tyrosine hydroxylase (TH) antibody (1:200; Chemicon International) and 1% normal bovine serum for 1 h. Then neurons were incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Laboratories) for 1 h. Images of the neurons were analyzed by immunocytochemistry. Neurons were collected with a digital camera (Carl Zeiss, Germany) attached to a fluorescence microscope (Carl Zeiss) and stored in a computer using AxoVision 2.05 (Carl Zeiss). All procedures were performed at room temperature.

Solutions

The ionic composition of the incubation medium consisted of (in mM) 124 NaCl, 3 KCl, 1.5 KH₂PO₄, 24 NaHCO₃, 2.4 CaCl₂, 1.3 MgSO₄, and 10 glucose saturated with 95% O₂–5% CO₂. The pH was about 7.45. The low-Na⁺ medium consisted of (in mM) 230 sucrose, 3 KCl, 1.5 KH₂PO₄, 10 MgSO₄, 0.5 CaCl₂, 26 NaHCO₃, and 10 glucose. The standard external solution was (in mM) 150 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 Hepes. The Na⁺−free external solution consisted of (in mM) 150 N-methyl-D-glucamine-Cl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 Hepes. The Ca²⁺−free external solution consisted of (in mM) 150 NaCl, 5 KCl, 5 MgCl₂, 2 EGTA, 10 glucose, and 10 Hepes. These external solutions were adjusted to a pH of 7.4 with Tris-base. For recording mIPSCs, external solutions routinely contained 500 nM tetrodotoxin (TTX) and 50 μM n-2-amino-5-phosphonovaleric acid (APV) to block voltage-dependent Na⁺ channels and NMDA receptors, respectively, except where indicated. In the slice experiments, the external bath solution consisted of (in mM) 124 NaCl, 3 KCl, 1.5 KH₂PO₄, 24 NaHCO₃, 2 CaCl₂, 1 MgCl₂, and 10 glucose saturated with 95% O₂–5% CO₂. The pH was about 7.5. The ionic composition of the internal (patch-pipette) solution for the whole cell patch recording consisted of (in mM) 145 Cs-methanesulfonate, 5 TEA-Cl, 5 CsCl, 2 EGTA, 10 Hepes, and 4 ATP-Mg with a pH adjusted to 7.2 with Tris-base. In the current-clamp experiment, the internal solution for the nystatin-perforated patch recording consisted of (in mM) 80 K-methanesulfonate, 70 KCl, 5 MgCl₂, and 10 Hepes with pH adjusted to 7.2 with Tris-base. Nystatin was dissolved in acidified methanol at 10 mg/ml. This stock solution was diluted with the internal solution just before use to a final concentration of 100–200 μg/ml.

Drugs

The drugs used in the present study were TTX, bicuculline, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), AP5, NMDA, AMPA, nystatin, GYKI52466, (RS)-2-amino-3(3-hydroxy-5-tert-butylisoxazol-4-yl)propionic acid (ATPA), KA, dopamine, EGTA, (±)-4-(4-amino phenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphenalazine (SYM2206), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), (±)-α-methyl-4-carboxyphenylglycine (MCPG), ATP-Mg (from Sigma, St. Louis, MO), (2S)-(−)(1S)-(−)(3,4-dihydroxyphenethyl)ethylamino-2-hydroxypropylphenyl(methyl)phosphinic acid (CGP55845), and N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM-251) (from Tocris, UK). All solutions containing drugs were applied using the “Y-tube” system for rapid solution exchange (Akaike and Harata 1994), except for the slice preparation.

RESULTS

Mechanically dissociated SNc neurons

After brief mechanical dissociation of the SNc region, we found large (>25 μm in somatic diameter) and small neurons (15–20 μm) (Fig. 1A). Large neurons displayed a variety in the shape of the somata including fusiform, triangular, and multipolar forms, and were comparable to those previously described types (Grace and Onn 1989; Katayama et al., 2003; Lacey et al. 1989; Uchida et al. 2000). To confirm whether these large neurons dissociated mechanically belong to SNc dopaminergic neurons, we examined immunoreactivity against tyrosine hydroxylase (TH), a marker of dopaminergic neurons, and electrophysiological membrane properties. As shown in Fig. 1A, large neurons were TH-positive, whereas small ones were TH-negative. In the current-clamp condition, large neurons fired spontaneously in a very regular pacemaker fashion without bursting activity (Fig. 1, B and C). Membrane responses to injection of hyperpolarizing currents were distinct in inward rectification, which is one of the distinctive physiological membrane properties of SNc neurons (Fig. 1B). Because SNc dopaminergic neurons are known to express dopamine D₂-like receptors (Uchida et al. 2000), we also tested the effects of dopamine on SNc neurons. As shown in Fig. 1C, dopamine (1 μM) hyperpolarized SNc neurons and significantly reduced action potential firing rate. All of such immunocytochemical and electrophysiological properties were indistinguishable from those described previously in vitro as identified dopaminergic neurons (Grace and Onn 1989; Katayama et al. 2003; Lacey et al. 1989; Uchida et al. 2000). Therefore we used large neurons in all subsequent experiments.

In the presence of 500 nM TTX and 50 μM AP5, the spontaneous outward miniature postsynaptic currents were recorded from mechanically dissociated SNc neurons at a V₉₀ of 0 mV. These currents were completely and reversibly blocked.
by 3 μM bicuculline (Fig. 2A). Figure 2B shows typical events at various $V_H$ values and their $I$–$V$ relationship. The reversal potential of the miniature currents, as estimated from the $I$–$V$ relationship, was $-68.1$ mV ($n = 4$), which was almost identical to the theoretical Cl$^-$ equilibrium potential ($E_{Cl}$) of $-69.9$ mV calculated by the Nernst equation using extra- and intracellular Cl$^-$ concentrations ($161$ and $10$ mM, respectively). These results indicate that the spontaneous postsynaptic currents are GABAergic mIPSCs.

**Effect of KA on GABAergic mIPSCs**

To elucidate whether GABAergic presynaptic nerve terminals projecting to SNc dopaminergic neurons express functional KA receptors, and whether presynaptic KA receptor activation can directly modulate GABAergic synaptic transmission, first we observed the effect of exogenously applied KA on GABAergic mIPSCs. In the majority of SNc neurons tested (62 out of 76 cells; 82%), 3 μM KA increased GABAergic mIPSC frequency. In 14 neurons in which this effect was fully analyzed, KA increased the mean mIPSC frequency to $222 \pm 14\%$ of the control ($P < 0.01$), without affecting the mean mIPSC current amplitude ($108 \pm 4\%$ of the control, $P = 0.09$) (Fig. 3, A and B insets). The KA-induced increase in mIPSC frequency sustained throughout the period of application and was rapidly reversed on washout (Fig. 3A). In addition, as shown in Fig. 3B, KA (3 μM) significantly shifted the distribution of inter-event interval to the left, but did not affect the amplitude of mIPSCs. Taken together, these results suggest that KA acts presynaptically to increase spontaneous GABA release onto SNc neurons.

To quantify KA-induced presynaptic responses, we examined the effects of the repeated applications of KA on GABAergic mIPSC frequency. The results might be important particularly for determining concentration–response relationships or analyzing the pharmacological properties of KA receptor subtypes. KA-induced presynaptic responses were reproducible during the repeated applications with a time interval of 10 min (first: $232 \pm 37\%$; second: $216 \pm 37\%$; third: $212 \pm 49\%$, $n = 4$). Figure 3C shows the concentration–response relationship for both the KA-induced increase in mIPSC frequency. KA even at lower concentrations significantly increased mIPSC frequency (1 μM: $181 \pm 23\%$; 3 μM: $215 \pm 38\%$; 10 μM: $173 \pm 22\%$). We also tested the effect of ATPA, a potent agonist of KA receptor containing GluR5 subunit, on GABAergic mIPSCs, and found that ATPA had little effect on mIPSC frequency (Fig. 3C).

**Pharmacological properties of presynaptic KA receptors**

KA can activate both KA and AMPA receptors (for review see Lerma et al. 2001). To investigate the receptor subtypes involved in the facilitatory action of KA on GABAergic mIPSCs, we tested the effects of GYKI52466 or SYM2206, selective AMPA receptor antagonists, and CNQX, a nonselective AMPA/KA receptor blocker. In the presence of 20 μM GYKI52466, 3 μM KA increased mIPSC frequency to $221 \pm 18\%$ of the control ($P < 0.05$, $n = 5$; Fig. 4, A and B). This facilitation ratio was almost identical to that induced by KA in the absence of GYKI52466. In addition, KA also increased mIPSC frequency in the presence of 30 μM SYM2206 (198 ± 39% of the control, $n = 4$; data not shown), thereby indicating that AMPA receptors are not involved in the facilitatory actions of mIPSCs. On the other hand, KA-induced facilitation of mIPSC frequency was completely suppressed in the presence of 20 μM CNQX to $111 \pm 23\%$ of the control ($P = 0.89$, $n = 5$; Fig. 4, A and B). In a subset of experiments, we also tested the effects of AMPA and NMDA on GABAergic mIPSCs. Neither AMPA nor NMDA (0.1, 1 and 10 μM, $n = 3$, respectively) affected GABAergic mIPSCs (data not shown).

**Ca$^{2+}$-impermeable KA receptors modulate spontaneous GABA release**

Next, we studied the mechanisms involved in the KA-induced increase in mIPSC frequency. The application of 200 μM Ca$^{2+}$, a general voltage-dependent Ca$^{2+}$ channel (VDCC) blocker, itself reduced the frequency (32 ± 9%, $n = 7$, $P < 0.01$) and amplitude (59 ± 6%, $n = 7$, $P < 0.01$) of mIPSCs. In the presence of Ca$^{2+}$, KA action on mIPSC frequency (Fig. 5Aa) was completely abolished to $113 \pm 12\%$ of the control ($n = 7$, $P > 0.05$; Fig. 5Aa). We also examined the effect of Ca$^{2+}$-free external solution on KA-induced facilitation of mIPSC frequency. In a Ca$^{2+}$-free external solution, both frequency and amplitude of mIPSCs were greatly reduced (to $25 \pm 6\%$, $n = 5$, $P < 0.01$, or to $63 \pm 2\%$, $n = 5$, $P < 0.01$, respectively) (Fig. 5Ab). The facilitatory effect of KA on mIPSC frequency was also completely inhibited to $106 \pm 12\%$ of the Ca$^{2+}$-free condition ($n = 5$, $P = 0.79$; Fig. 5, Ab and B). These results suggest that KA-induced facilitation of mIPSC
frequency is mediated by the Ca^{2+} influx from the extracellular space by VDCC activation or directly through KA receptors. To distinguish these two possibilities, we observed the effect of Na^{+}-free external solution on KA-induced facilitation of mIPSC frequency. In a Na^{+}-free external solution, GABAergic mIPSC frequency slightly increased, but the effect was not statistically significant (147 ± 35%, n = 6, P = 0.29). In a Na^{+}-free external solution, the facilitatory effect of KA on mIPSC frequency was not observed (to 97 ± 5% of the control, P = 0.85, n = 6) (Fig. 5, Ac and B).

**Effect of KA on action potential-dependent GABA release**

To elucidate a physiological significance of the present finding, we observed the effect of KA on the mIPSCs and evoked GABAergic IPSCs (eIPSCs), by use of slice preparation. Because SCs dopaminergic neurons receive GABAergic afferents from the striatum, globus pallidus, and the axon collaterals of the SNr (Hajós and Greenfield 1994; Oertel et al. 1981; Ribak et al. 1976; Smith and Bolam 1990), the stimulus pipette was placed around the SNr. We found that bath application of KA increases spontaneous IPSC frequency dose-dependently (0.3 μM: 123 ± 18%; 1 μM: 251 ± 22%; 3 μM: 283 ± 37% of the control, n = 6, respectively). However, KA (0.3 to 3 μM) significantly reduced eIPSC amplitude in a dose-dependent manner (0.3 μM: 75 ± 5%; 1 μM: 60 ± 7%; 3 μM: 44 ± 9% of the control, n = 6, respectively; Fig. 6A). The inhibitory action of KA on eIPSC amplitude is probably not attributable to either vesicle depletion or the postsynaptic mechanism, given that during the KA application, a sustained increase in spontaneous IPSC frequency was observed and the amplitude distribution of spontaneous IPSC was not affected (data not shown). We also examined the effect of KA on GABAergic mIPSCs in the presence of TTX, and found that KA (3 μM) also increases mIPSC frequency to 173 ± 25% of the control (n = 4, P < 0.05; Fig. 6B), thereby indicating that KA acts on presynaptic nerve terminals of GABAergic neurons that innervate SCs.

The fact that kainate at lower concentrations suppressed the amplitude of eIPSCs tempted us to examine the possible indirect actions by metabotropic receptors, including GABA_A receptors, as suggested by a recent study (Kerchner et al. 2001).
Interestingly, in the presence of 10 μM CGP55845, a selective GABA<sub>B</sub> receptor antagonist, KA at lower concentrations slightly increased eIPSC amplitude (0.3 μM: 120 ± 5%, P < 0.05; 1 μM: 113 ± 7% of the control, P = 0.18, n = 6, respectively; Fig. 7, A and B). However, 3 μM KA suppressed eIPSC amplitude (to 82 ± 8% of the control, n = 6, P < 0.05), although the extent of inhibition was relatively small (Fig. 7, A and B). In another set of experiments, we also tested the possible involvement of other metabotropic receptors, such as adenosine A<sub>1</sub> receptors, metabotropic glutamate receptors, and cannabinoid CB1 receptors, in KA receptor-mediated modulation of GABAergic eIPSCs. In two neurons tested, further application of a cocktail of CGP55845 (10 μM); DPCPX (100 nM), a selective A<sub>1</sub> receptor antagonist; MCPG (1 mM), a nonselective metabotropic glutamate receptor antagonist; and AM-251 (10 μM), a selective CB1 receptor antagonist, hardly changed the biphasic effects of KA on eIPSCs (Fig. 7C). These results suggest that GABA released by the excitation of GABAergic nerve terminals during KA receptor activation might also act on presynaptic GABA<sub>B</sub> autoreceptors to inhibit the release of GABA.

**DISCUSSION**

**GABAergic mIPSCs in mechanically dissociated SNC neurons**

In the present study, we isolated single SNC neurons without using any enzymes. These mechanically isolated neurons showed similar morphological, immunocytochemical, and electrophysiological properties of SNC dopaminergic neurons described previously (Grace and Onn 1989; Katayama et al. 2003; Lacey et al. 1989; Uchida et al. 2000). Although...
solution suggest the possible involvement of Ca\textsuperscript{2+} influx from the external solution after the blockade of Na\textsuperscript{+} channels. One possible explanation for this phenomenon would be that GABAergic nerve terminals in mechanically dissociated SNc neurons may be depolarized membrane potentials to some extent. Because of the membrane depolarization of the nerve terminals, it is reasonable to assume that the activation of VDCCs may occur spontaneously, which may in turn result in the spontaneous and synchronous GABA release. Thus spontaneous synaptic events that were observed in Ca\textsuperscript{2+}-free external solution in the present experiments may correspond to the classical miniature currents in the previous studies (Capogna et al. 1993; Scanziani et al. 1992). Alternatively, the synaptic events observed in the present preparation might be Ca\textsuperscript{2+}-dependent mIPSCs. In fact, Ca\textsuperscript{2+}-dependent mIPSCs have been also reported in central neurons (Doze et al. 1995; Soltész and Mody 1995). However, further studies are warranted to understand the reasons for the dependency of mIPSCs on extracellular Ca\textsuperscript{2+} and/or VDCCs.

**GABAergic presynaptic nerve terminals express functional KA receptors**

There is much convincing evidence that KA by presynaptic ionotropic KA receptors potentiates spontaneous inhibitory synaptic transmission (Cossart et al. 2001; Kerchner et al. 2001; Mulle et al. 2000). For example, in hippocampal CA1 interneurons, KA increases mIPSC frequency without affecting their amplitude (Cossart et al. 2001) and causes an increase in mIPSC frequency by activating presynaptic ionotropic KA receptors in the spinal cord dorsal horn (Kerchner et al. 2001). The present results also provide an example that presynaptic ionotropic KA receptors are present on GABAergic nerve terminals projecting to SNc dopaminergic neurons, and that their activation increases the probability of spontaneous GABA release. Several lines of evidence support this conclusion that presynaptic ionotropic KA receptors are responsible for the KA-induced increase of mIPSC frequency. First, KA at lower concentrations increased GABAergic mIPSC frequency without affecting the current amplitude, suggesting that KA acts presynaptically to increase spontaneous GABA release. Second, KA action on mIPSC frequency was not affected by either GYKI52466 or SYM2206, selective AMPA receptor blockers, but completely blocked by CNQX, a nonelective AMPA/KA receptor blocker. In addition, exogenously applied AMPA or NMDA did not affect GABAergic mIPSC frequency. Third, the KA-induced increase in mIPSC frequency was completely suppressed in either Na\textsuperscript{+}-free or Ca\textsuperscript{2+}-free external solution, supporting the involvement of an ionic mechanism in KA-mediated action. Finally, dissociated neurons used in this study have cell-free presynaptic nerve terminals, although it is still unclear whether a short portion of the axon was attached to presynaptic nerve terminals (see also Akaike et al. 2002).

The family of KA receptors is composed of 5 different genes that code for the subunit GluR5/6/7 and KA1/2 (Bettler and Mulle 1995; Chittajallu et al. 1999). The subunit composition of KA receptors could be very diverse, given the large combinatorial possibilities suggested by KA receptor subunit mRNA distribution in the CNS (Bischoff et al., 1997; Wisden and Seeburg 1993), as well as functional expression studies of recombinant receptors (Chittajallu et al. 1999; Lerma et al. 1999).
2001). In the present experiments, ATPA failed to increase spontaneous GABA release, thereby indicating that presynaptic KA receptors on GABAergic terminals projecting to SNC dopaminergic neurons might not contain GluR5 subunit. This view is supported by the previous observations that most GABAergic neurons, which innervate SNC dopaminergic neurons, express both GluR6 and KA2, but not GluR5, subunit mRNAs (Bischoff et al. 1997).

Mechanisms involved in the KA-induced increase in spontaneous GABA release

It seems that there are several possible explanations for the KA-induced increase in GABAergic mIPSC frequency. First, activation of Na\(^+\)-permeable, but Ca\(^{2+}\)-impermeable, KA receptors might cause Na\(^+\) influx into presynaptic terminals and depolarize presynaptic membrane, and thus lead to VDCC activation (Kerchner et al. 2001). Second, activation of Ca\(^{2+}\)-permeable KA receptors might directly increase the [Ca\(^{2+}\)]\(_{\text{in}}\) within the terminals (Cossart et al. 2001; Kohler et al. 1993). In the present study, however, the KA-induced increase in mIPSC frequency was completely suppressed in Na\(^+\)-free or Ca\(^{2+}\)-free external solutions, and in the presence of Cd\(^{2+}\), suggesting that Ca\(^{2+}\) influx through VDCCs from extracellular space is closely related to KA action. Thus it seems the the most plausible mechanism involved is that activation of the presynaptic KA receptors leads to direct presynaptic depolarization by permitting Na\(^+\) influx, and the depolarization causes VDCC activation to increase the Ca\(^{2+}\) influx.

Biphasic modulation of action potential-dependent GABA release by presynaptic KA receptors

It is rather surprising that KA at lower concentrations inhibits GABAergic eIPSC amplitude, as previous studies have reported that KA at nanomolar concentration facilitates glutamate release from mossy fiber or parallel fiber terminals (Delaney and Jahr 2002; Kamiya and Ozawa 2000; Schmitz et al. 2000, 2001). On the other hand, it was reported that synaptically released GABA in the presence of KA acts GABA\(_B\) autoreceptors to inhibit action potential–dependent GABA release (Kerchner et al. 2001). In the present experiments, we found that KA-induced inhibition of GABAergic eIPSCs is suppressed in the presence of CGP55845, a GABA\(_B\) receptor antagonist, thereby indicating that synaptically released GABA during KA receptor activation acts presynaptic GABA\(_B\) autoreceptor to inhibit GABA release. These actions of GABA on GABA\(_B\) autoreceptors may mask the facilitatory action of KA on spontaneous GABA release at lower concentrations. It should be mentioned, however, that after the blockade of GABA\(_B\) receptors, KA shows biphasic modulation of action potential–dependent GABA release, that is, facilitation and inhibition at lower and higher concentrations, respectively. These biphasic effects might be attributed to the extent of presynaptic depolarization induced by KA. The recent reports have demonstrated that KA at nanomolar concentration elicits mild presynaptic depolarization and facilitates neurotransmitter release by increasing the Ca\(^{2+}\) influx into presynaptic terminals as well as the excitability of presynaptic volleys. On the contrary, strong presynaptic depolarization attributed to axonal and/or terminal KA receptor activation might inactivate voltage-dependent Na\(^+\) and/or Ca\(^{2+}\) channels or membrane shunting, thus resulting in the inhibition of action potential–dependent transmitter release (Kamiya and Ozawa 2000; Kamiya et al. 2002; Schmitz et al. 2000, 2001).

Physiological significance

SNC dopaminergic neurons receive both excitatory (Futami et al. 1995; Kita and Kitai 1987; Sesack and Pickel 1992) and inhibitory afferent inputs, but the majority (>90%) of the afferents to SNC dopaminergic neurons appear to be inhibitory GABAergic inputs because most of afferent terminals are immunostained for glutamate decarboxylase, a GABA synthesizing enzyme (Oertel et al. 1981; Ribak et al. 1976; Smith and Bolam 1990). These GABAergic afferents have a crucial role in controlling the firing activity of dopaminergic neurons, as local administration of GABA\(_A\) receptor antagonists in vivo increases the number of neurons that fire in the burst mode (Celada et al. 1999; Tepper et al. 1995) or shifts single neurons from a regular or random firing pattern to burst firing mode (Paladini and Tepper 1999). These findings suggest that the GABAergic system might be involved in the regulation of motor functions as well as the firing patterns of SNC dopaminergic neurons. On the other hand, the hyperactivity of the subthalamic nucleus, which leads to glutamate excitotoxicity in the SNC, might play a pivotal role in the pathophysiology of Parkinson’s disease (Blandini et al. 2000). In addition, large increases in extracellular glutamate concentration could occur in the hypoxic/ischemic damage (Rothman and Olney 1986). It would be interesting to determine whether presynaptic KA receptors can be activated by synaptically released glutamate in such pathophysiological conditions.

The authors thank Dr. T. Nishitani for teaching immunocytochemical methods and providing drugs and Dr. M. Noda for letting us use computer software for immunocytochemistry.

DISCLOSURES

This work was supported by Grants-in-Aid for Scientific Research (No. 13307003) from The Ministry of Education, Science and Culture, Japan, and The Japan Health Sciences Foundation Grant 21279, Research on Brain Science for N. Akaike.

REFERENCES


Smith AD and Bolam JP. The output neurons and the dopaminergic neurons of the substantia nigra receive a GABA-containing input from the globus pallidus in the rat. J Comp Neurol 296: 47–64, 1990.


