Effects of Methylphenidate on the Membrane Potential and Current in Neurons of the Rat Locus Coeruleus

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Ishimatsu, Masaru, Yuri Kidani, Akira Tsuda, and Takashi Akasu. Effects of methylphenidate on the membrane potential and current in neurons of the rat locus coeruleus. J Neurophysiol 87: 1206–1212, 2002; 10.1152/jn.00463.2001. Effects of methylphenidate (MPH), a therapeutic agent used in children presenting the attention deficit hyperactivity disorder (ADHD), on the membrane potential and current in neurons of the rat locus coeruleus (LC) were examined using intracellular and whole cell patch-clamp recording techniques. Application of MPH (30 μM) to artificial cerebrospinal fluid (ACSF) produced a hyperpolarizing response with amplitude of ±1 mV (n = 29). Spontaneous firing of LC neurons was blocked during the MPH-induced hyperpolarization. Superfusion of LC neurons with ACSF containing 0 mM Ca2+ and 11 mM Mg2+ (Ca2+-free ACSF) produced a depolarizing response associated with an increase in spontaneous firing of the action potential. The MPH-induced hyperpolarization was blocked in Ca2+-free ACSF. Yohimbine (1 μM) and prazosin (10 μM), antagonists for α2 and α2B/2C receptors, respectively, blocked the MPH-induced hyperpolarization in LC neurons. Tetrodotoxin (TTX, 1 μM) produced a partial depression of the MPH-induced hyperpolarization in LC neurons. Under the whole cell patch-clamp condition, MPH (30–300 μM) produced an outward current (Iout) with amplitude of ±1 mV (n = 17) in LC neurons. The Iout was blocked by Co2+ (1 mM). During prolonged application of MPH (300 μM for 45 min), the hyperpolarization gradually decreased in the amplitude and eventually disappeared, possibly because of depression of norepinephrine (NE) release from noradrenergic nerve terminals. At a low concentration (1 μM), MPH produced no outward current but consistently enhanced the outward current induced by NE. These results suggest that the MPH-induced response is mediated by NE via α2B/2C-adrenoceptors in LC neurons. Iout was associated with an increase in the membrane conductance of LC neurons. The Iout reversed its polarity at ~12 ± 6 mV (n = 8) in the ACSF. The reversal potential of Iout was changed by 54 mV per decade change in the external K+ concentration. Current-voltage relationship showed that the Iout exhibited inward rectification. Ba2+ (100 μM) suppressed the amplitude and the inward rectification of the Iout. These results suggest that the Iout is produced by activation of inward rectifier K+ channels in LC neurons.

INTRODUCTION

The locus coeruleus (LC) is a compact noradrenergic nucleus in the pons that sends extensive projections throughout the CNS (Amaral and Sinnamon 1977; Dahlström and Fuxe 1964; Moore and Bloom 1979; Ungerstedt 1971). The LC has been considered to play an important role in such brain functions as vigilance, attention, and mediation of stress response (Aston-Jones et al. 1991; Foote et al. 1980, 1983; Hobson et al. 1975; Olpe et al. 1985; Tanaka et al. 1983; Tsuda et al. 1982). Tonic activity of rat LC neurons was found to vary in association with changes in behavioral state. The highest levels of neuronal activity in the LC are observed during wakefulness, while lower firing rates occur when animals are drowsy (Aston-Jones and Bloom 1981a,b; Foote et al. 1980). It has been proposed that recurrent collaterals of the LC projections release norepinephrine (NE) onto the LC neurons themselves (Aghajanian et al. 1977). Egan et al. (1983) suggested that released NE onto other LC neurons mediates the inhibitory postsynaptic potential (IPSP) through α2-adrenoceptors. Firing rate of spontaneous action potentials, a pacemaker-like regulatory activity in the LC neurons, was reduced during the hyperpolarization induced by exogenously applied NE via α2-adrenoceptors (Williams and North 1985).

Methylphenidate (MPH; Ritalin) is the most widely used drug for the treatment of children presenting the attention deficit hyperactivity disorder (ADHD) (Hunt et al. 1984). Biochemical studies have shown that MPH, like d-amphetamine, enhances the release and/or blocks the re-uptake of NE and dopamine (DA) in mammalian brain (Axelrod 1970; Carlsson et al. 1966; Ferris et al. 1972; Hendley et al. 1972; Raiteri et al. 1974; Ross 1978). Administration of MPH decreased the rate of spontaneous firing in rat LC neurons in vitro (Lacroix and Ferron 1988; Olpe et al. 1985). However, little is known about the mechanism underlying the inhibitory action of MPH on the neuronal activity in the LC. The purpose of the present study is to determine the effects of MPH on membrane potential and neuronal excitability in LC neurons and to examine whether these actions are mediated by intrinsic NE. Preliminary findings of this work have appeared in abstract form (Kidani et al. 2000).

METHODS

Brain slices containing the LC were obtained from rats in a manner described previously (Ishimatsu and Williams 1996; Williams et al. 1984). Male Wistar rats, 150–200 g, were killed by a heavy blow to the chest, and their brains were rapidly removed and immersed for 8–10 s in a cooled artificial cerebrospinal fluid (ACSF, 4–6°C) that...
was prebubbled with 95% O2-5% CO2. Horizontal brain slices (250–300 μm in thickness) were cut with a Vibroslice (Campden Instruments) in cooled ACSF and left to recover for 1 h in oxygenated ACSF at room temperature (22–24°C). A hemisected slice was then transferred to a recording chamber and submerged in the ACSF at 32–33°C. The composition of the ACSF was as follows (in mM): 126 NaCl, 2.5 KCl, 2.4 CaCl2, 1.2 MgCl2, 21 NaHCO3, 1.2 NaHPO4, and 11 d-glucose (pH: 7.4 and 295–305 mOsm). Intracellular recordings were made with glass microelectrodes filled with 2 M KCl (tip resistance: 26–40 MΩ). Whole cell tight-seal recordings were made from LC neurons using the slice patch technique (Blanton et al. 1989; Coleman and Miller 1989). Patch pipettes were filled with the internal solution containing (mM): 130 KCl, 0.3 CaCl2, 1 MgCl2, 1 ethylene glycol-bis(β-aminoethyl ether)-N,N’,N’’,N’’-tetraacetic acid (EGTA); 2 ATP (Mg-ATP); 0.25 GTP; 10 N-(2-hydroxyethyl)pipera- 

dine-N,N-,N’-(2-ethanesulfonic acid) (HEPES) (pH 7.3 adjusted by KOH, 280 mOsm). The tip resistance of a whole cell patch-pipette was 3–5 MΩ. Voltage and current were recorded with an Axoclamp-2A amplifier and were monitored continuously with a memory oscilloscope (Nihon-Kohden, RTA-1100). During the whole cell voltage-clamping, sample frequencies were between 4.5 and 6 Hz and the amplifier gain was 0.8–2.5 nA/mV. A pClamp system (Axon Instruments) operating on a computer (PowerMac G4; Apple Computer) was used to analyze the membrane potential and current. The drugs used, GTP, EGTA, NE, yohimbine, prazosin, and tetraethylammonium (TEA) were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO). Tetrothodoxin (TTX) was purchased from Wako Pure Chemical Industries (Osaka, Japan). MPH hydrochloride was a gift from Novartis Pharma. Drugs were directly dissolved in the ACSF. Each experimental value was presented as the mean ± SE and was analyzed by unpaired Student’s t-test.

RESULTS

MPH-induced hyperpolarization in LC neurons

LC neurons showed tonic firing of spontaneous action potentials with a frequency of 0.5–3 Hz, when impaled by a microelectrode. Bath application of MPH (30 μM) for 1–10 min caused a hyperpolarizing response (Fig. 1A) in 29 out of 32 neurons. In the remaining three neurons, MPH produced no changes in membrane potential. The firing of spontaneous action potentials was blocked during the hyperpolarization induced by MPH. The membrane potential and spontaneous action potentials recovered within 15–20 min after withdrawal of MPH from the superfusing solution. Properties of the MPH-induced hyperpolarization were analyzed at −60 mV, a membrane potential level at which the spontaneous action potentials were blocked. Electrototoxic potentials produced by injection of hyperpolarizing current pulses with duration of 200 ms were depressed during the MPH-induced hyperpolarization (Fig. 1Ab). These results indicate that MPH decreases input resistance of LC neurons. When MPH (30 μM) was first applied to the ACSF, LC neurons showed hyperpolarization with amplitude of 12.0 ± 1.4 mV (n = 29) at −60 mV. The effect of MPH was use dependent: the MPH-induced hyperpolarization was diminished when the drug was repeatedly applied at an interval of 30–40 min. The amplitude of the hyperpolarization produced by the second application of MPH (30 μM) was 3 ± 2 mV (n = 12; Fig. 1Bb). Therefore the hyperpolarization produced by the first application of MPH was used for data analysis in the following study. Consequently, in the experiments testing the effect of drugs on the MPH response, the control and test neurons were two different sets. Figure 1C shows the voltage-current relationships (V-I curves) taken before and during the application of MPH (30 μM). In the presence of MPH, the V-I curve decreased in its slope and intersected the control curve at −107 mV. Pooled data showed that the reversal potential of the MPH-induced hyperpolarization was −91 ± 4 mV (n = 5). It has been shown that synaptic transmission in the LC was blocked in an ACSF containing 0 mM Ca2+ and 11 mM Mg2+ (Ca2+-free ACSF) (Egan et al. 1983). Figure 2A shows the effect of MPH (30 μM) on the membrane potential of LC neurons in the Ca2+-free ACSF. When the external solution was switched to the Ca2+-free ACSF, LC neurons exhibited a slow depolarization (10 ± 2 mV, n = 6) associated with an increase in the firing rate of spontaneous action potentials. Application of MPH (30 μM) to the Ca2+-free ACSF produced no obvious hyperpolarizing response. Pooled data showed that the MPH-induced hyperpolarization was almost completely blocked in an ACSF containing 0 mM Ca2+ (Egan et al. 1983). Figure 2B shows the effect of MPH (30 μM) on the membrane potential of LC neurons in the Ca2+-free ACSF. When the external solution was switched to the Ca2+-free ACSF, LC neurons exhibited a slow depolarization (10 ± 2 mV, n = 6) associated with an increase in the firing rate of spontaneous action potentials. Application of MPH (30 μM) to the Ca2+-free ACSF produced no obvious hyperpolarizing response. Pooled data showed that the MPH-induced hyperpolarization was almost completely blocked in an ACSF containing 0 mM Ca2+ (Egan et al. 1983).
show that the amplitude of the MPH (30 μM) induced hyperpolarization was 8 ± 1 mV (n = 7) in the TTX (1 μM)-containing ACSF (Fig. 2C).

**Contribution of NE to the MPH-induced hyperpolarization**

MPH has been reported to enhance the release of NE and/or inhibit its re-uptake system in central neurons (Axelrod 1970; Carlsson et al. 1966; Ferris et al. 1972; Hendley et al. 1972; Raiteri et al. 1974; Ross 1978). We examined whether NE mediates the hyperpolarization induced by MPH in LC neurons. Bath application of yohimbine (1 μM), an α2-adrenoceptor antagonist, produced a depolarizing response with amplitude of 6 ± 1 mV (n = 6) in LC neurons. Figure 3A shows the effect of yohimbine (1 μM) on the MPH-induced hyperpolarization in an LC neuron. Addition of MPH (30 μM) to yohimbine-containing ACSF for 10 min produced no visible hyperpolarization in this LC neuron. Pooled data showed that the amplitude of MPH-induced hyperpolarization was significantly depressed by yohimbine (1 μM; Table 1). It has been shown that prazosin (10 μM), an α2-adrenoceptor blocker, also antagonizes NE-induced outward current in dissociated LC neurons (Arima et al. 1998). In the present study, the effect of prazosin on the MPH-induced hyperpolarization was examined in LC neurons. Bath application of prazosin (10 μM) produced

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The number of experiments is shown in parentheses. All data are represented by means ± SE. MPH, methylphenidate. *Statistical significance obtained by unpaired Student’s t-test (P < 0.01), of the difference from control.
Effects of MPH on the membrane current in LC neurons

LC neurons were voltage-clamped at −60 mV with a whole cell configuration. Bath application of MPH (30–300 μM) caused an outward current (I_{MPH}) in 22 of 24 LC neurons (Fig. 4A). In the remaining two neurons, MPH did not change the holding current and membrane conductance. The I_{MPH} reached its maximum amplitude within 3 min after beginning of the application of MPH (30 μM) and recovered within 20 min after withdrawal of MPH. At a concentration of 0.1–1 μM, MPH produced no detectable response in LC neurons (n = 5). MPH (30 μM) produced the outward current with amplitudes of 110 ± 6 pA (n = 17). However, when the concentration of MPH was increased to 100–300 μM, the amplitude of I_{MPH} was almost the same as those produced by 30 μM MPH (Fig. 4B). Interestingly, the I_{MPH} appeared to be transient during a continuous application of MPH. Figure 4B shows the time course of the outward current during a prolonged application of MPH (300 μM) in an LC neuron. The I_{MPH} declined within 20 min even in the presence of MPH. To examine the sensitivity of α₂-adrenoceptor, NE (30 μM) was applied to a neuron where the I_{MPH} had been suppressed by a prolonged application of MPH (300 μM). NE (30 μM) produced an outward current with amplitude of 72 ± 5 pA (n = 8), even in the presence of MPH (300 μM). It has been shown that no detectable desensitization of α₂-adrenoceptors occurs during a continuous application of NE to LC neurons (Surprenant and Williams 1987; Williams et al. 1985). Supporting these reports, the amplitude of the I_{NE} was maintained as long as NE (30 μM) was present in the superfusing solution for 45 min (Fig. 4C). These results suggest that the decline of the I_{MPH} is due to a decrease in the release of NE from noradrenergic nerve terminals.

Cocaine and desmethylimipramine, nonselective re-uptake inhibitors for catecholamines, have been shown to enhance the response to exogenous NE in LC neurons (Egan et al. 1983; Surprenant and Williams 1987). The effect of MPH on the outward current induced by exogenously applied NE was examined by whole cell patch-clamp techniques. In the control, NE (0.1–1 μM) produced no detectable outward current in the ACSF. When NE (10 μM) was added to the normal ACSF for 1–5 min, LC neurons showed outward current with amplitude of 51 ± 3 pA (n = 8). NE, at a concentration of 100 μM, produced an outward current with amplitude similar to that produced by 10 μM NE. Application of MPH (1 μM) did not produce any change in the membrane current and conductance of LC neurons. In the same cells, NE (1 μM) produced the outward currents with amplitude of 47 ± 4 pA (n = 8) in the presence of MPH (1 μM; Fig. 5). MPH (1 μM) also enhanced the outward current produced by NE (10–100 μM) in LC neurons (Fig. 5). The effect of MPH (1 μM) in enhancing the NE-induced response was reversible. The I_{NE} was restored, when LC neurons were superfused with the recovery solution for 20 min.

**MPH activates inward rectifier K⁺ channels in LC neurons**

Current-voltage relationships (I-V curves) were constructed by step command potentials with duration of 200 ms (Fig. 6A). MPH (30 μM) increased the amplitude of currents produced by step command potentials, indicating that the membrane conductance of LC neurons was increased by MPH (Fig. 6A). The component of current activated by MPH (net I_{MPH}) was obtained by digital subtraction of the control I-V curve taken in the ACSF from that recorded in the presence of MPH (30 μM). The net I_{MPH} showed inward rectification in LC neurons (Fig. 6B). The I_{MPH} reversed polarity at −102 ± 6 mV (n = 8) in the ACSF (containing 2.5 mM K⁺; Fig. 6B). The reversal potential

*FIG. 4. Whole cell patch-clamp recordings of the outward currents produced by MPH (30 and 300 μM) and norepinephrine (NE; 30 μM) in LC neurons. A: example of the outward current (I_{MPH}) produced by MPH (30 μM). B: time course of the I_{MPH} obtained by a continuous application of MPH (300 μM) for 45 min. NE (30 μM) was applied to the external solution containing MPH (300 μM). The periods of application of these drugs are indicated by solid horizontal bars. C: steady outward current induced by continuous application of NE (30 μM) for 45 min. B and C were taken from the same neuron.*

*FIG. 5. Effect of MPH (1 μM) on the outward current induced by NE (0.1–100 μM) in an LC neurons. Left and right: taken before and 15 min after application of MPH (1 μM), respectively. The holding membrane potential was −60 mV. Horizontal bars indicate the period of the application of NE.*
cell patch-clamp condition, MPH (30–300 μM) caused an outward current in a great majority of LC neurons. It has been shown that synaptic transmission in the LC is blocked in the Ca\(^{2+}\)-free ACSF (Egan et al. 1983). We examined whether or not MPH directly produced the hyperpolarization (and outward current) in rat LC neurons. The MPH-induced hyperpolarization was blocked, when LC neurons were superfused with the Ca\(^{2+}\)-free ACSF. Furthermore, Co\(^{2+}\) (1 mM), a nonselective Ca\(^{2+}\) channel blocker, also strongly depressed the \(I_{\text{MPH}}\) in LC neurons. These results suggest that a neurotransmitter secondary mediates the hyperpolarization (and the outward current) induced by MPH in LC neurons. MPH, like d-amphetamine, has been reported to enhance the release and/or to block the re-uptake of NE and DA in mammalian brain (Axelrod 1970; Carlsson et al. 1966; Ferris et al. 1972; Hendley et al. 1972; Raiteri et al. 1974; Ross 1978). Electrophysiological studies have shown that NE produces a hyperpolarizing response mediated by \(\alpha_2\)-adrenoceptors in LC neurons (Aghajanian and VanderMaulen 1982; Arima et al. 1998; Egan et al. 1983; Williams and Marshall 1987). The present study clearly showed that NE mediates the hyperpolarization induced by MPH because the MPH-induced hyperpolarization was strongly depressed by yohimbine, an antagonist for \(\alpha_2\)-adrenoceptors (1 μM). Prazosin (10 μM), which blocks \(\alpha_2B/\alpha_2C\)-adrenoceptors in cultured LC neurons (Arima et al. 1998), also depressed the MPH-induced hyperpolarization. The prazosin-induced depression of the \(I_{\text{MPH}}\) may not be mediated by \(\alpha_1\)-adrenoceptors in rat LC neurons because the \(\alpha_1\)-adrenoceptor has been shown to mediate a depolarizing response at early stages of development, but it is almost absent in adult rats (Williams and Marshall 1987). We suggest that the MPH-induced hyperpolarization is mediated by activation of \(\alpha_2B/\alpha_2C\)-adrenoceptor subtypes in LC neurons of adult rats.

The ionic mechanism underlying the \(I_{\text{MPH}}\) was examined in LC neurons. The \(I_{\text{MPH}}\) was associated with an increase in the membrane conductance. The \(I_{\text{MPH}}\) reversed polarity at a membrane potential that was close to the equilibrium potential for K\(^+\). The reversal potential of the \(I_{\text{MPH}}\) changed by 54 mV per decade change in the external K\(^+\) concentration as predicted by the Nernst equation for K\(^+\). These results indicate that \(I_{\text{MPH}}\) is carried exclusively by K\(^+\). Current-voltage relationship showed that the \(I_{\text{MPH}}\) had a characteristic inward rectification in most LC neurons. Ba\(^{2+}\) (100 μM), a selective blocker for the inward rectifier K\(^+\) current (North 1989) reduced not only the amplitude but also the inward rectification of the \(I_{\text{MPH}}\). These results suggest that MPH activates the inward rectifier K\(^+\) channels of LC neurons. These electrophysiological properties of the \(I_{\text{MPH}}\) are comparable to those of the \(I_{\text{NE}}\) in LC neurons (Arima et al. 1998; Egan et al. 1983).

It has been suggested that recurrent collaterals of the projections of noradrenergic neurons release NE on to the LC neurons themselves (Aghajanian et al. 1977). Spontaneous release of NE onto the somatodendritic membrane of other LC neurons produces inhibitory responses (Egan et al. 1983). Chemical studies have shown that tonic release of NE from rat LC neurons is increased when animals are exposed to continuous stressor (Iida et al. 1985; Tanaka et al. 1983; Tsuda et al. 1982). Tonic release of NE on LC neurons produces a hyperpolarization associated with increased potassium conductance via \(\alpha_2\)-adrenoceptor. The present study showed that superfusion of LC neurons with the Ca\(^{2+}\)-free ACSF resulted in a

**DISCUSSION**

The present study showed that MPH (30 μM) produced a hyperpolarizing response associated with a block of the spontaneous firing of action potentials in LC neurons. Under whole

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depolarization associated with an increase in the frequency of spontaneous action potentials. Yohimbine also produced a depolarizing response in LC neurons. Furthermore the inhibition of NE-re-uptake by MPH resulted in the hyperpolarization of LC neurons. These findings support the assumption that tonic release of NE from adrenergic nerve terminals regulates the membrane excitability of LC neurons. The present study showed, however, that TTX (1 μM) did not completely block the MPH-induced hyperpolarization, although it blocked the spontaneous firing of the action potential in LC neurons. A TTX-independent mechanism might underlie the MPH-induced NE release in the rat LC.

It has been demonstrated that cocaine and desmethylimipramine, irreversible NE re-uptake inhibitors, markedly enhance the response to exogenous NE in LC neurons (Surprenant and Williams 1987). The present study showed that MPH, at a concentration of 1 μM, produced no outward current but reversibly enhanced the I_{NE} in LC neurons. In addition, the present study showed that the MPH-induced hyperpolarization ran down, when a brief application of MPH (30 μM) was repeated in LC neurons. Furthermore, the I_{MPH} declined during a continuous application of MPH (300 μM) for 20 min. The decrease in the I_{MPH} amplitude may not be due to loss of the sensitivity of α2-adrenoceptors at postsynaptic membrane because application of NE (30 μM) clearly produced an outward current in neurons in which the I_{MPH} had been suppressed by prolonged exposure to MPH. Furthermore detectable desensitization of α2-adrenoceptors did not occur during a continuous application of NE to LC neurons (see also Surprenant and Williams 1987; Williams et al. 1985). These results suggest that the release of NE is depressed during a continuous exposure to MPH of LC neurons. Because MPH has been known to facilitate the release of NE in mammalian central neurons (Axelrod 1970; Carlsson et al. 1966; Ferris et al. 1972; Hendley et al. 1972; Raiteri et al. 1974; Ross 1978), the elevated levels of NE may inhibit the release of NE via presynaptic α2-autoreceptors. Alternatively, excess release of NE by prolonged application of MPH may result in the depletion of NE at noradrenergic nerve terminals.

The LC has been known to correlate with the level of vigilance and attention in mammals (Aston-Jones and Bloom 1981b; Aston-Jones et al. 1991; Foote et al. 1980; Hobson et al. 1975). Extracellular recordings showed that intravenous or intraperitoneal administration of MPH decreased firing rate of spontaneous activity in rat LC neurons (Lacroix and Ferron 1988; Olpe et al. 1985). The present study has shown that the MPH-induced inhibition of the firing activity of LC neurons is due to the hyperpolarization of the postsynaptic membrane. Electrical stimulation of noradrenergic nerves or administration of NE increases the discrimination of incoming external stimuli by reducing the background neuronal activity, therefore augmenting the signal-to-noise ratio (Foote et al. 1975; Segel 1985; Waterhouse et al. 1980). We suggest that the therapeutic effect of MPH on children presenting the ADHD is correlated with the enhancement of the action of NE in depressing the firing activity of LC neurons.

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J Neurophysiol • VOL 87 • MARCH 2002 • www.jn.org


