A-type K⁺ Current of Dopamine and GABA Neurons in the Ventral Tegmental Area

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Koyama, Susumu and Sarah B. Appel. A-type K⁺ current of dopamine and GABA neurons in the ventral tegmental area. J Neurophysiol 96: 544–554, 2006; doi:10.1152/jn.01318.2005. A-type K⁺ current (I_A) is a rapidly inactivating voltage-dependent potassium current which can regulate the frequency of action potential (AP) generation. Increased firing frequency of ventral tegmental area (VTA) neurons is associated with the reinforcing effects of some drugs of abuse like nicotine and ethanol. In the present study, we classified dopamine (DA) and GABA VTA neurons, and investigated I_A properties and the physiological role of I_A in these neurons using conventional whole cell current- and voltage-clamp recording. DA VTA neurons had a mean firing frequency of 3.5 Hz with a long AP duration. GABA VTA neurons had a mean firing frequency of 16.7 Hz with a short AP duration. For I_A properties, the voltage-dependence of steady-state I_A activation and inactivation was similar in DA and GABA VTA neurons. I_A inactivation was significantly faster and became faster at positive voltages in GABA neurons than DA neurons. Recovery from inactivation was significantly faster in DA neurons than GABA neurons. I_A current density at full recovery was significantly larger in DA neurons than GABA neurons. In DA and GABA VTA neurons, latency to the first AP after the recovery from membrane hyperpolarization (repolarization latency) was measured. Longer repolarization latency was accompanied by larger I_A current density in DA VTA neurons, compared with GABA VTA neurons. We suggest that I_A contributes more to the regulation of AP generation in DA VTA neurons than in GABA VTA neurons.

INTRODUCTION

The ventral tegmental area (VTA) is thought to be critical for the mediation of reinforcing effects of drugs of abuse (Appel et al., 2004; Wise 1987). The neurons in the VTA are classified into two major neuronal subgroups: dopamine (DA) and GABA neurons (Grace and Onn 1989; Johnson and North 1992; Korotkova et al. 2003). DA and GABA neurons have been reported to occupy 70% and 30% of the neuronal populations in the VTA, respectively (Johnson and North 1992). Morphologically, DA VTA neurons have large cell somata with multipolar or bipolar dendrite arborization, whereas GABA VTA neurons have small cell somata with multipolar dendrite arborization (Brodie et al. 1999; Grace and Onn 1989; Johnson and North 1992; Korotkova et al. 2003). Electrophysiologically, DA VTA neurons have slow firing frequencies (1–8 Hz) and long action potential (AP) durations (2–4 ms), whereas GABA VTA neurons have fast firing frequencies (>5 Hz) and short AP durations <2 ms (Grace and Onn 1989; Johnson and North 1992; Korotkova et al. 2003).

A rapidly inactivating A-type K⁺ current (I_A) has been reported to regulate inter-AP intervals by damping the developing interspike depolarization and slowing the subsequent AP generation (Conner and Stevens 1971). In addition, the activation of I_A has been reported to shorten AP duration (Zhang and McBain 1995b) and prolong the latency to the first AP after the recovery from membrane hyperpolarization (Shibata et al. 2000). Although I_A has been found to be a transient outward K⁺ current, there are differences in conductance, gating kinetics and pharmacological properties (Bekkers 2000; Kanold and Manis 1999; Korngreen and Sakmann 2000; Luther et al. 2000; Saito and Isa 2000; Shibata et al. 2000; Song et al. 1998; Tkatch et al. 2000; Zhang and McBain 1995a). The diversity of I_A is thought to be due to the heterogeneity of channels that underlie I_A, Kv1.4, Kv3.4, Kv4.1, Kv4.2 and Kv4.3 channels have been reported to mediate I_A (Baldwin et al. 1991; Covarrubias et al. 1991; Schroter et al. 1991; Serodio et al. 1996; Stuhmer et al. 1989). There have been several studies which have identified I_A in midbrain DA neurons (Hahn et al. 2003; Liss et al. 2001; Silva et al. 1990; Yang et al. 2001). However, there is little information on I_A of GABA VTA neurons and no comparison of I_A in the two major cell types of the VTA, DA, and GABA neurons. It is possible that different I_A properties of DA and GABA VTA neurons may contribute to their different AP properties and firing frequencies. This information is also necessary for careful analysis of the effects of specific agents that alter I_A in the function of the VTA. In addition, since some drugs of abuse (e.g., ethanol or nicotine) increase the rate of action potential generation in DA VTA neurons, it is important to understand factors which control firing frequency in these neurons. In the present study, we used both current- and voltage-clamp whole cell recording on acutely dissociated VTA neurons. We classified DA and GABA VTA neurons based on their firing frequencies and AP parameters and then investigated I_A properties and the physiological contribution of I_A in these neurons. Part of this work has appeared previously in abstract form (Koyama and Appel 2005).

METHODS

Region specific cell dissociation

Animals used in this study were treated in strict accordance with the U.S. National Institutes for Health Guide for the Care and Use of Laboratory Animals and all experimental methods were approved by the Animal Care Committee of the University of Illinois at Chicago.

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Fisher 344 rats (14–20 days old, both genders) were decapitated, and the brain was quickly removed. Since \( I_A \) amplitude is detected at birth and increased with age, reaching 80% of maximal amplitude at 2 wk and a plateau at 3 wk in rat central neurons (Hattori et al. 2003), we used 14–20 day-old rats to examine \( I_A \). The brain was placed in the ice-cold cutting solution (in mM: 220 sucrose, 2.5 KCl, 2.4 CaCl\(_2\), 1.3 MgSO\(_4\), 1.24 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\) and 11 D-glucose), which was constantly bubbled with 95% O\(_2\)-5% CO\(_2\) at room temperature (23–25°C) for 30 min. The brain slices were then incubated in a HEPES-buffered bathing solution (see following text) containing papain (15–18 U/ml) at 32°C for 20–30 min. After papain treatment, the brain slices were further incubated in the ACSF for 20–40 min. The VTA neurons were dissociated using a vibrating stylus apparatus dispersing cells from the brain slices as previously described (Koyama et al. 2005). The cells were dispersed from the region located between the interfascicular nucleus and the medial lemniscus in the horizontal axis and between the paranigral nucleus and the red nucleus in the sagittal axis. Once the cell dissociation procedure was completed (4–7 min), the brain slice was removed from the culture dish, and the dissociated neurons settled and adhered to the bottom of the dish within 20 min.

Electrophysiological recording

Current- and voltage-clamp whole cell recording was performed using an Axopatch-1B patch-clamp amplifier (Axon Instruments, Union City, CA). Microelectrodes were fabricated using a P-87 puller (Sutter Instrument Company, Novato, CA) from LE16 glass capillaries (Dagan, Minneapolis, MN) and heat-polished on a microforge (Narishige, Tokyo). The tip resistances of the electrodes were 1.5–3 MΩ when filled with a pipette solution (in mM: 110 K-glucuronate, 20 KCl, 4 MgATP, 0.3 NaGTP, 0.2 ethylene glycol-bis-(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid [EGTA] and 10 N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] [HEPES]; pH adjusted to 7.2 with KOH, final [K\(^+\)] = 139 mM, osmolality adjusted to 290 mOsm with sucrose). Cell capacitance was measured after the cancellation of the capacitative transients. Series resistance (3–7 MΩ) was compensated for by 80% and periodically monitored. Membrane currents and voltage were filtered at 1 kHz by a 3 dB 4-pole filter and acquired at a sampling frequency of 10 kHz. Data acquisition was performed by a DigiData 1322A interface and pClamp software version 9.0 (Axon Instruments). The dissociated VTA neurons were visualized under phase-contrast on an inverted microscope (Diaphot 300, Nikon, Tokyo). All experiments were performed at room temperature (23–25°C).

Experimental solutions

Current-clamp recording was done with a HEPES-buffered bathing solution (in mM: 145 NaCl, 2.5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES and 11 D-glucose; pH adjusted to 7.4 with NaOH, osmolality 300 mOsm). The liquid junction potentials between the pipette solution and the extracellular solutions were estimated to be 12 mV (Neher 1992) and the results have been corrected by this amount. Osmolarity of the solutions was measured by a Vapro vapor pressure osmometer (Wescor, Logan, UT).

Drug application

Drug solutions were applied via a multiple channel manifold (Becton Dickinson). Each channel of the manifold was connected to a gravity-fed reservoir with tubing (860 μm, i.d.). The output of the manifold was connected to an outflow tube (580 μm, i.d.), the tip of which was placed within 200 μm of the soma of the recorded neuron. Solution flowed continuously through one manifold channel. Application of drug solutions was controlled by opening or closing valves connected to the reservoirs.

Source of drugs and chemical agents

The drugs and chemical agents used in this study 4-aminopyridine (4-AP), CdCl\(_2\), CsCl, EGTA, HEPES, MgATP, Na\(_2\)GTP, papain and TTX were purchased from Sigma (Saint Louis, MO).

Data analysis and curve fitting

Action potentials were analyzed off-line by pClamp software version 9.0 (Axon Instruments). Data from cells containing AP amplitude <50 mV were discarded. All average values are expressed as mean ± SE. Statistical comparison to assess significant differences was done by paired or unpaired Student’s t-test. 4-AP effect on DA and GABA VTA neurons were evaluated with a two-way ANOVA. To calculate \( I_A \) current density, \( I_A \) amplitude was divided by cell capacitance. \( I_A \) inactivation was fitted by a single exponential function

\[
y = A e^{x(x - t)}
\]

where \( A \) is amplitude obtained from the beginning of the fit and \( t \) is decay time constant. To analyze the steady-state \( I_A \) activation, the current (I) was converted to conductance by the following equation

\[
G = I / (V - E_K)
\]

Where \( G \) is the conductance at the test voltage of \( V \) and \( E_K \) is the reversal potential of K\(^+\) current, which was calculated to be −100 mV by the Nernst equation. \( I_A \) conductance was normalized to the maximal conductance and fitted by the Boltzmann equation, using Origin software version 7 (Origin Lab., Northampton, MA)

\[
y = A_2 + (A_1 - A_2) / [1 + \exp(x - x_0) / dx]
\]

where \( y \) is the conductance at the test voltage of \( x \), \( A_1 \) is the minimal conductance and set to be 0, \( A_2 \) is the maximal conductance and set to be 1, \( x_0 \) is the membrane potential for half-activation and \( dx \) is the slope factor. To analyze the steady-state \( I_A \) inactivation, the currents were fitted by the Boltzmann equation.

RESULTS

Classification of DA and GABA VTA neurons

In the present study, we recorded 84 VTA neurons in whole cell current-clamp recording; 56 DA neurons and 28 GABA neurons; all neurons which we examined generated action potentials (APs) spontaneously. As shown in Table 1, DA VTA neurons had smaller AP amplitudes, wider AP half-width, more positive AP threshold potentials and more positive peak afterhyperpolarization (AHP) than GABA VTA neurons.
neurons, whereas there was no significant difference in AHP amplitude in these neurons (Table 1).

**Steady-state activation of A-type K⁺ current (IA)**

As illustrated in Fig. 1, we examined steady-state IA activation in DA and GABA VTA neurons. Two types of prepulse protocols were used to obtain IA by taking advantage of the sensitivity of IA to different voltages (Bekkers 2000; Han et al. 2003; Luther et al. 2000; Saito and Isa 2000; Song et al. 1998; Zhang and McBain 1995a; Yang et al. 2001). In the first protocol, a 500 ms-long prepulse from a VH of 92 mV to 18 mV in 10 mV increments. In the second protocol, a 500 ms-long prepulse from a VH of 92 mV to 42 mV was given before various depolarizing test voltage steps from −92 mV to 18 mV in 10 mV increments. In the second protocol, a 500 ms-long prepulse from a VH of −62 mV to −2 mV was given before various depolarizing test voltage steps from −92 mV to 18 mV in 10 mV increments. IA was obtained by subtracting the currents in the second prepulse protocol from the currents in the first prepulse protocol.

The first prepulse protocol activated both rapidly and slowly inactivating outward currents in a DA VTA neuron (Fig. 1A1, top). The second prepulse protocol activated only slowly inactivating outward currents in the same neuron (Fig. 1A1, bottom). The digital subtraction revealed IA in this DA VTA neuron (Fig. 1A2). On the other hand, the first prepulse protocol activated notch-like transient outward current followed with slowly inactivating outward currents in a GABA VTA neuron (Fig. 1B1, top). The second prepulse protocol activated only slowly inactivating outward currents in the same neuron (Fig. 1B1, bottom). The digital subtraction revealed IA in this GABA VTA neuron (Fig. 1B2). Figure 1C shows the average current-voltage relationship of IA in DA VTA neurons (open circles) and GABA VTA neurons (open triangles). In both VTA neurons, the current-voltage relationship of IA is linear over the range of −60 to +18 mV. Average IA amplitude of DA VTA neurons was larger than that of GABA VTA neurons. As shown in Fig. 1D, the normalized IA conductance was plotted as a function of test voltage for DA VTA neurons (open circles) and GABA VTA neurons (open triangles). These plots were fitted by a single Boltzmann function. In DA VTA neurons, the normalized IA conductance was 38.2 ± 1.2 mV and average slope factor was 12.4 ± 0.1 (n = 6). In GABA VTA neurons, the normalized IA conductance was −35.2 ± 2.1 mV and average slope factor was 11.2 ± 0.7 (n = 4). There was no significant difference in IA activation in DA and GABA VTA neurons.

### Table 1. Properties of VTA neurons

<table>
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<th>n</th>
<th>Firing Frequency, Hz</th>
<th>Amplitude, mV</th>
<th>Half-width, ms</th>
<th>Threshold, mV</th>
<th>Peak AHP, mV</th>
<th>AHP amplitude, mV</th>
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<tr>
<td>DA neurons</td>
<td>56</td>
<td>3.3 ± 0.2</td>
<td>68.5 ± 1.3</td>
<td>2.4 ± 0.1</td>
<td>−38.3 ± 0.5</td>
<td>−67.8 ± 0.7</td>
<td>29.5 ± 0.6</td>
</tr>
<tr>
<td>GABA neurons</td>
<td>28</td>
<td>16.4 ± 3.3</td>
<td>85.2 ± 2.8</td>
<td>1.3 ± 0.1</td>
<td>−42.6 ± 1.1</td>
<td>−72.2 ± 1.8</td>
<td>29.6 ± 1.3</td>
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<tr>
<td></td>
<td>P</td>
<td>&lt; 0.0001</td>
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Average values are expressed as ± SE. Statistical comparison to assess significant differences was done by Student’s t-test. Action potential (AP) amplitude was measured from AP threshold to AP peak. AP threshold was measured as a potential at which the linear fit of AP rising phase and the line fit of slow depolarizing phase just before the abrupt change of AP rising phase crossed. AP half-width was measured half way between AP threshold and AP peak. Afterhyperpolarization (AHP) amplitude was measured from threshold to AHP peak. DA, dopamine; n, number of neurons; N.S., no significant difference; VTA, ventral tegmental area.

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**Fig. 1. Steady-state activation of A-type K⁺ current (IA).**

A1: outward currents of a DA VTA neuron in the first prepulse protocol. (bottom) Outward currents of the same DA VTA neuron in the second prepulse protocol. A2: IA obtained by digital subtraction of the outward currents in the second protocol from the outward currents in the first protocol. B1: (top) outward currents of a GABA VTA neuron in the first prepulse protocol. B2: (top) outward currents of the same GABA VTA neuron in the second prepulse protocol. B2: IA obtained by digital subtraction of the outward currents in the second protocol from the outward currents in the first protocol. C: average current amplitude plotted as a function of test voltage for 6 DA VTA neurons (open circles) and 4 GABA VTA neurons (open triangles). D: average normalized IA conductance plotted as a function of test voltage for DA VTA neurons (open circles) and GABA VTA neurons (open triangles) from C. Continuous lines represent curve fittings by a single Boltzmann function.
Activation and inactivation of $I_A$

As illustrated in Fig. 2, we examined $I_A$ activation and inactivation. Because the short depolarizing prepulse effectively activates $I_A$ but minimizes delayed rectifier K$^+$ current activation (Bekkers 2000), we used two types of short prepulse protocols to obtain $I_A$. In the first protocol, a 100 ms-long prepulse from a $V_{th}$ of $-62$ mV to $-102$ mV was given before various depolarizing test voltage steps from $-52$ mV to 8 mV in 10 mV increments. In the second protocol, a 100 ms-long prepulse from a $V_{th}$ of $-62$ mV to $-42$ mV was given before various depolarizing test voltage steps from $-52$ mV to 8 mV in 10 mV increments. $I_A$ was obtained by subtracting the currents in the second prepulse protocol from the currents in the first prepulse protocol.

Figure 2A shows $I_A$ activation in a DA VTA neuron (Fig. 2A1) and a GABA VTA neuron (Fig. 2A2). Figure 2A3 shows average $I_A$ time-to-peak plotted as a function of test voltage for 5 DA VTA neurons (open circles) and 7 GABA VTA neurons (open triangles). DA VTA neurons had significantly slower time-to-peak than GABA VTA neurons at each voltage ($P < 0.05$). Figure 2B shows $I_A$ inactivation in the DA VTA neuron in A1 (Fig. 2B1) and the GABA VTA neuron in A2 (Fig. 2B2). In both DA and GABA VTA neurons, $I_A$ inactivation time constants were well fitted by a single exponential function. Figure 2B3 shows average $I_A$ inactivation time constants plotted as a function of test voltage for 5 DA VTA neurons (open circles) and 7 GABA VTA neurons (open triangles). DA VTA neurons had significantly slower inactivation time constant than GABA VTA neurons at each voltage ($P < 0.01$). The plots of $I_A$ inactivation time constants show weak voltage-dependency in DA VTA neurons and strong voltage-dependency in GABA VTA neurons. It should be noted that DA VTA neurons had a larger cell capacitance ($28.2 \pm 1.2 \text{ pF, } n = 43$) than GABA VTA neurons ($22.4 \pm 1.0 \text{ pF, } n = 26$) in cells which capacitance was measured ($P < 0.001$).

Steady-state inactivation of $I_A$

As illustrated in Fig. 3, we examined the steady-state $I_A$ inactivation in DA and GABA VTA neurons. One second long conditioning voltage steps from $-122$ mV to $-32$ mV (in 10 mV increments) were given from a $V_{th}$ of $-62$ mV before a test step to $-27$ mV. There was no significant difference in time-to-peak value at $-22$ mV between subtracted and unsubtracted raw currents. For DA VTA neurons, the time-to-peak was $3.1 \pm 0.2 \text{ ms using subtracted currents and } 3.2 \pm 0.2 \text{ ms using raw currents (} P > 0.3, n = 5 \text{). For GABA VTA neurons, time-to-peak was } 2.3 \pm 0.2 \text{ ms using subtracted currents and } 2.5 \pm 0.2 \text{ ms using raw currents (} P > 0.05, n = 7 \text{).}$ We used raw currents for analyzing the steady-state inactivation of $I_A$. The depolarizing conditioning voltage steps decreased $I_A$ amplitude in a DA VTA neuron (Fig. 3A1) and a GABA VTA neuron (Fig. 3A2). The peak amplitude of $I_A$ was measured and normalized after subtracting a residual outward current. As shown in Fig. 3B, normalized $I_A$ amplitude was plotted as a function of conditioning voltage for DA VTA neurons (open circles) and GABA VTA neurons (open triangles). These plots were well fitted by a single Boltzmann function. In DA VTA neurons, average half-inactivating voltage ($V_{inact,0.5}$) was $-84.5 \pm 1.6 \text{ mV and average slope factor was } 5.7 \pm 0.6 (n = 7)$. In GABA VTA neurons, average $V_{inact,0.5}$ was $-85.2 \pm 1.2 \text{ mV and average slope factor was } 6.4 \pm 0.2 (n = 9)$. There was no significant difference in $V_{inact,0.5}$ ($P > 0.3$) or in slope factor ($P > 0.7$) between DA and GABA VTA neurons. We further examined steady-state $I_A$ inactivation in a test solution contained normal Ca$^{2+}$ concentration (2 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$, see MATERIALS). Normalized $I_A$ amplitude was plotted as a function of conditioning voltage for DA VTA neurons (filled circles) and GABA VTA neurons (filled triangles) (Fig. 3B). The plots were well fitted by a single Boltzmann function. In DA VTA neurons, average $V_{inact,0.5}$ with normal Ca$^{2+}$ test solution was $-74.5 \pm 2.7 \text{ mV and average slope factor was } 6.8 \pm 0.3 (n = 4)$. In GABA VTA neurons, average $V_{inact,0.5}$
peak current amplitude as a function of time was made (Fig. 4B1) from the DA VTA neuron shown in Fig. 4A1 (top); recovery time constant was 41.7 ms being fitted by a single exponential function. Similarly, we plotted \( I_A \) peak current amplitude as a function of time (Fig. 4B2) from the GABA VTA neuron shown in Fig. 4A1 (bottom); recovery time constant was 220.9 ms being fitted by a single exponential function. Figure 4C shows average \( I_A \) recovery time constants in DA and GABA VTA neurons. The average \( I_A \) recovery time constant of DA VTA neurons was 49.5 ± 4.3 ms (n = 9) and that of GABA VTA neurons was 306.0 ± 29.8 ms (n = 8); these values were significantly different (\( P < 0.001 \)). Figure 4D shows average \( I_A \) density at full recovery in DA and GABA VTA neurons. The average \( I_A \) current density of DA VTA neurons was 67.8 ± 18.0 pA/pF (n = 9) and that of GABA VTA neurons was 14.4 ± 2.5 pA/pF (n = 8); these values were significantly different (\( P < 0.05 \)).

4-aminopyridine (4-AP) sensitivity of \( I_A \) in VTA neurons

Because sensitivity to 4-AP is characteristic of each \( I_A \) subtype (Bekkers 2000; Hahn et al. 2003; Kanold and Manis 1999; Kornigreen and Sakmann 2000; Liss et al. 2001; Luther et al. 2000; Saito and Isa 2000; Shibata et al. 2000; Song et al., 1998; Tkatch et al., 2000; Zhang and McBain 1995-a), we examined \( I_A \) sensitivity to 4-AP. Figure 5A shows 4-AP effect on \( I_A \) in a DA VTA neuron (top) and a GABA VTA neuron (bottom). Although 4-AP reduced \( I_A \) amplitude in a concentration-dependent manner in both neurons, \( I_A \) of the GABA neuron was more sensitive to 4-AP than that of the DA VTA neuron. Figure 5B shows average % inhibition of \( I_A \) by 4-AP in DA VTA neurons (open circles) and GABA VTA neurons (open triangles). In DA VTA neurons, 4-AP inhibited \( I_A \) by 14.2 ± 3.8% at 0.3 mM, 23.7 ± 2.4% at 1 mM and 37.8 ± 3.4% at 3 mM (n = 6). In GABA VTA neurons, 4-AP inhibited \( I_A \) by 34.5 ± 5.6% at 0.3 mM, 70.3 ± 7.5% at 1 mM and 78.5 ± 7.7% at 3 mM (n = 4). \( I_A \) sensitivity to each concentration of 4-AP was significantly different between DA and GABA VTA neurons (\( P < 0.05 \)).

Effect of cadmium (\( \text{Cd}^{2+} \)) on \( I_A \) steady-state inactivation in VTA neurons

Another pharmacological characteristic of \( I_A \) has been reported to be \( \text{Cd}^{2+} \) modulation of voltage-dependency in steady-state activation and inactivation (Song et al. 1998; Kuo and Chen 1999; Tkatch et al. 2000). As illustrated in Fig. 6, we examined 200 \( \mu \)M \( \text{Cd}^{2+} \) effect on steady-state \( I_A \) inactivation. \( \text{Cd}^{2+} \) reduced \( I_A \) amplitude in a DA VTA neuron (Fig. 6A1, top). In this neuron, 200 \( \mu \)M \( \text{Cd}^{2+} \) shifted \( V_{\text{iact, 0.5}} \) in a positive direction by 20.2 mV without affecting slope factor (4.9 in control, 4.6 with \( \text{Cd}^{2+} \)) (Fig. 6A1, bottom). Figure 6A2 summarizes \( \text{Cd}^{2+} \) effect on steady-state \( I_A \) inactivation in 5 DA VTA neurons. \( \text{Cd}^{2+} \) significantly shifted \( V_{\text{iact, 0.5}} \) in a positive direction (-85.7 ± 1.2 mV in control, -63.2 ± 1.8 mV with \( \text{Cd}^{2+} \); \( P < 0.01 \)) (Fig. 6A2, left) without significant effect on slope factor (5.0 ± 0.2 mV in control, 5.8 ± 0.5 with \( \text{Cd}^{2+} \); \( P > 0.1 \)) (Fig. 6A2, right). Similarly, \( \text{Cd}^{2+} \) reduced \( I_A \) amplitude in a GABA VTA neuron (Fig. 6B1, top). In this neuron, 200 \( \mu \)M \( \text{Cd}^{2+} \) shifted \( V_{\text{iact, 0.5}} \) in a positive direction by 23.6 mV.

FIG. 3. Steady-state inactivation of \( I_A \); \( I_A \) in a DA VTA neuron. (inset) Recording of \( I_A \) from the same neuron on a faster time scale. \( A \), \( I_A \) in a GABA VTA neuron. (inset) Recording of \( I_A \) from the same neuron on a faster time scale. \( B \): average normalized peak amplitudes of \( I_A \) plotted as a function of conditioning voltage for 7 DA VTA neurons (open circles) and 9 GABA VTA neurons (open triangles). In a test solution containing normal \( \text{Ca}^{2+} \) concentration (2 mM), average normalized peak amplitudes of \( I_A \) were also plotted as a function of conditioning voltage for 4 DA VTA neurons (filled circles) and 4 GABA VTA neurons (filled triangles). Continuous lines represent curve fittings by a single Boltzmann function. Bolzmann curves of steady-state \( I_A \) activation for DA VTA neurons (continuous line) and GABA VTA neurons (dashed line) obtained in Fig. 1D are superimposed.

with normal \( \text{Ca}^{2+} \) test solution was \(-75.3 ± 2.9 \text{ mV} \) and average slope factor was \(5.9 ± 0.2 \) (n = 4).

\( I_A \) recovery from inactivation and \( I_A \) current density at full recovery

As illustrated in Fig. 4, we examined \( I_A \) recovery from inactivation in DA and GABA VTA neurons. Figure 4A1 shows \( I_A \) recovery from inactivation in a DA neuron (top) and a GABA VTA neuron (bottom). Membrane potential was hyperpolarized from a \( V_H \) of \(-42 \text{ mV} \) to \(-92 \text{ mV} \) in various durations (from 5 ms to 3000 ms) before the membrane potential was returned to \(-42 \text{ mV} \) (Fig. 4A1). \( I_A \) current amplitude of the DA VTA neuron was larger than that of the GABA VTA neuron. Figure 4A2 shows the early part of \( I_A \) recovery from the same DA VTA neuron (top) and GABA VTA neuron (bottom). \( I_A \) recovery from inactivation was faster in the DA VTA neuron than the GABA neuron. A plot of \( I_A \) recovery from inactivation in a DA VTA neuron. Figure 4B shows the early part of \( I_A \) recovery from the same DA VTA neuron (top) and GABA VTA neuron (bottom). \( I_A \) recovery from inactivation was faster in the DA VTA neuron than the GABA neuron. A plot of \( I_A \) recovery from inactivation in a DA VTA neuron. Figure 4B shows the early part of \( I_A \) recovery from the same DA VTA neuron (top) and GABA VTA neuron (bottom). \( I_A \) recovery from inactivation was faster in the DA VTA neuron than the GABA neuron.
were injected. Figure 7 summary shows Cd(2+) effect on steady-state IA inactivation in 4 GABA VTA neurons. Cd(2+) significantly shifted V_{inact,0.5} in a positive direction (-13.0 ± 2.2 mV in control, -61.0 ± 2.3 mV with Cd(2+); P < 0.01) (Fig. 6B_b, left) without significant effect on slope factor (6.0 ± 0.3 in control, 6.2 ± 0.8 with Cd(2+); P > 0.7) (Fig. 6B_b, right).

**Repolarization latency and IA in VTA neurons**

We examined whether IA contributed to repolarization latency, which is designated as the duration from the end of hyperpolarizing current step to the first AP. In whole cell current-clamp recording, 1 s long hyperpolarizing current pulses, amplitude of which was -30, -50, -70, and -90 pA, were injected. Figure 7A_f shows a DA VTA neuron with a prominent time-dependent inward rectification (voltage-sag) during strong hyperpolarizing current injection followed by long repolarization latency. Figure 7A_g shows a GABA VTA neuron with a smaller voltage-sag during strong hyperpolarizing current injection followed by very short repolarization latency. Figure 7A_h shows the average repolarization latency of each hyperpolarizing current in DA and GABA VTA neurons. In DA VTA neurons, average repolarization latency was 692.2 ± 131.5 ms at -90 pA, 712.7 ± 133.5 ms at -70 pA, 714.1 ± 147.8 ms at -50 pA and 636.5 ± 121.6 ms at -30 pA (Fig. 7A_f, open circles, n = 13). In GABA VTA neurons, average repolarization latency was 14.0 ± 3.6 ms at -90 pA, 13.5 ± 3.0 ms at -70 pA, 15.2 ± 3.5 ms at -50 pA and 13.5 ± 2.8 ms at -30 pA (Fig. 7A_f, open triangles, n = 6). Repolarization latency at each hyperpolarizing current was significantly different between DA and GABA VTA neurons (P < 0.05).

To activate IA at the range of subthreshold membrane potentials in voltage-clamp configuration, 1 s-long hyperpolarizing voltage step was given from a VH of -42 mV to -92 mV and the membrane potential was returned to -42 mV. Figure 7B_f shows large IA in a DA VTA neuron (top). In this neuron, IA inactivation time constant was 32.2 ms being fitted by a single exponential function (Fig. 7B_f, bottom). Figure 7B_g shows small IA in a GABA VTA neuron (top). In this neuron, IA inactivation time constant was 14.6 ms being fitted by a single exponential function (Fig. 7B_g, bottom). Figure 7B_h shows the relationship between IA current density at -42 mV and repolarization latency induced by the hyperpolarizing current of -90 pA in DA and GABA VTA neurons. DA VTA neurons had an average IA current density of 92 ± 9.5 pA/pF and an average repolarization latency of 663.9 ± 147.1 ms (n = 9). GABA VTA neurons had an average IA current density of 24.8 ± 4.4 pA/pF and an average repolarization latency of 16.0 ± 3.7 ms (n = 5).
VTA neuron before and after treatment with 0.3, 1 and 3 mM 4-AP. Figure 8

A neurons. Figure 8 train of spikes with the delay of spike generation in DA VTA neurons was calculated as $\left[ \frac{V_{\text{peak}} \text{ of } 30 \text{ to } 120 \text{ ms (Fig. 8C).}}{\text{V}_{\text{m}} \text{ of } -42 \text{ mV to } -92 \text{ mV; and the membrane potential was returned to } -42 \text{ mV. Dashed lines indicate initial baselines. } I_A \text{ amplitude was measured from the initial baseline to } I_A \text{ peak.}} \right]$ $I_A$ inhibition rate by 4-AP for 6 DA VTA neurons (open circles) and 4 GABA VTA neurons (open triangles). Percent inhibition in $I_A$ was calculated as $\left[ \frac{I_{A,\text{control}} - I_{A,4-AP}}{I_{A,\text{control}}} \right] \times 100$. When 4-AP was applied at concentration more than 1 mM, the osmolarity was adjusted by reducing the concentration of extracellular NaCl.

Time- and voltage-dependent spike generation delay in DA VTA neurons

As shown in Fig. 8, depolarizing current injection induced a train of spikes with the delay of spike generation in DA VTA neurons. Figure 8A shows membrane response to depolarizing current (70 pA) injection at various times after spontaneous AP generation in a DA VTA neuron. When depolarizing current pulse was initiated at relatively positive membrane potential after recovery from AHP, the delay to the first spike was short (Fig. 8A, top). When depolarizing current pulse was initiated just after spontaneous AP near the peak of AHP, the delay to the first spike remained short (Fig. 8A, middle). By contrast, when depolarizing current pulse was initiated during the AHP of spontaneous AP with enough time for membrane hyperpolarization, the delay to the first spike was long (Fig. 8A, bottom). We recorded 15 trials of depolarizing current pulse injection in DA VTA neurons and obtained the total of 75 data points from 5 neurons. The graph in Fig. 8B shows the relationship between the membrane potential at which depolarizing current was injected and the delay to the first spike. The delay to the first spike became longer at membrane potentials negative to $-55 \text{ mV}$ (Fig. 8B). The graph in Fig. 8C is the relationship between the duration from AP to the initiation of depolarizing current injection and the delay to the first spike. The longest delay is detected between 30 to 120 ms (Fig. 8C).

DISCUSSION

In DA and GABA VTA neurons, a rapidly activating and inactivating outward current, which is consistent with $I_A$, was detected. Although $I_A$ had similar voltage-dependency for steady-state activation and inactivation in DA and GABA VTA neurons, we found some differences in $I_A$ properties and the physiological contribution of $I_A$ to spontaneous action potential generation in these neurons.

Biophysical properties of $I_A$ in VTA neurons

There was no significant difference in half-activating and half-inactivating voltages and slope factors for $I_A$ between DA and GABA VTA neurons. In our study on steady-state $I_A$ inactivation, half-inactivating voltage was $-85 \text{ mV}$ in Ca$^{2+}$-free test solution and $-75 \text{ mV}$ in normal Ca$^{2+}$-containing test solution. Extracellular 2 mM Ca$^{2+}$ shifted steady-state $I_A$ inactivation to a positive direction by 10 mV. This observation in our study is consistent with previous studies on midbrain DA neurons showing that half-inactivating voltage is $-79$ to $-64 \text{ mV}$ in the extracellular solutions containing normal Ca$^{2+}$ concentration (Hahn et al. 2003; Liss et al. 2001). Previous studies on $I_A$ of midbrain DA neurons have reported that half-activating voltage is $-27$ to $-25 \text{ mV}$ (Hahn et al., 2003; Liss et al., 2001). In our present study on DA VTA neurons, half-activating voltage was $-38 \text{ mV}$. Although there is 10 mV difference in half-activating voltage between previous and our studies, this difference is likely due to the different concentration of extracellular Ca$^{2+}$; the extracellular solutions in previous studies contained normal Ca$^{2+}$ concentration (Hahn et al. 2003; Liss et al. 2001) and we used a Ca$^{2+}$-free test solution to block voltage-dependent Ca$^{2+}$ currents (see METHODS).

$I_A$ activation and inactivation were significantly faster in GABA VTA neurons than DA VTA neurons. In addition, $I_A$ inactivation was more voltage-dependent in GABA VTA neurons than DA VTA neurons. Previous studies on midbrain DA neurons have reported that $I_A$ inactivation time constant is 20 to 53 ms and $I_A$ inactivation does not exhibit prominent voltage-dependency (Hahn et al. 2003; Liss et al. 2001; Silva et al. 1990). In our present study on DA VTA neurons, $I_A$ inactivation time constant was 24 to 29 ms and exhibited weak voltage-dependency, and this is consistent with previous studies (Hahn et al. 2003; Liss et al. 2001). In GABA VTA neurons, $I_A$ inactivation was faster at membrane potentials positive to the AP threshold of these neurons. $I_A$ has been reported to contribute to AP duration (Zhang and McBain 1995b), but shortening of the AP duration due to $I_A$ may be relatively small in GABA VTA neurons. One factor to be considered is the difference in cell capacitance, which could contribute to the apparent difference in time-to-peak between DA and GABA VTA neurons.
The time constant of $I_A$ recovery was significantly faster in DA VTA neurons than GABA VTA neurons. Previous studies on midbrain DA neurons have reported that the $I_A$ recovery time constant is 20 to 30 ms (Hahn et al. 2003; Liss et al. 2001; Silva et al. 1990). In our present study, the $I_A$ recovery time constant was 50 ms in DA VTA neurons. The difference in $I_A$ recovery time constant may be due to different intracellular Ca$^{2+}$/H$11001$ buffering ability that is determined by the composition of Ca$^{2+}$/H$11001$ and EGTA in pipette solutions used. Intracellular Ca$^{2+}$/calmodulin-dependent protein kinase can regulate the rate of $I_A$.

**FIG. 6.** Cd$^{2+}$ effect on the steady-state $I_A$ inactivation. $A_1$, (top) $I_A$ of a DA VTA neuron before (left) and after (right) treatment with Cd$^{2+}$. (bottom) Normalized $I_A$ amplitude as a function of conditioning voltage before (open circles) and after (filled circles) treatment with Cd$^{2+}$. Continuous lines represent curve fittings by a single Boltzmann function. $A_2$, Cd$^{2+}$ effect on average $V_{max,0.5}$ (left) and average slope factor (right) in 5 DA VTA neurons. $B_1$, (top) $I_A$ of a GABA VTA neuron before (left) and after (right) treatment with Cd$^{2+}$. (bottom) Normalized $I_A$ amplitude plotted as a function of conditioning voltage before (open triangles) and after (filled triangles) treatment with Cd$^{2+}$ in the same GABA VTA neuron. Continuous lines represent curve fittings by a single Boltzmann function. $B_2$, Cd$^{2+}$ effect on average $V_{max,0.5}$ (left) and average slope factor (right) in 4 GABA VTA neurons. $I_A$ was obtained by the same voltage protocol shown in Fig. 3A. The asterisks indicate significant difference on paired Student’s t-test ($P < 0.01$).

**FIG. 7.** Repolarization latency and $I_A$ in VTA neurons. $A_1$: membrane response to hyperpolarizing current injection in a DA VTA neuron. A series of four 1 s long hyperpolarizing current pulses (−30, −50, −70 and −90 pA) were injected. Continuous current was not injected. $A_2$: membrane response to hyperpolarizing current injection in a GABA VTA neuron. $A_3$: average repolarization latency in DA VTA neurons (open circles, $n = 13$) and GABA VTA neurons (open triangles, $n = 6$). $B_1$: top: large $I_A$ in a DA VTA neuron at −42 mV. Bottom: recording of $I_A$ from the same neuron on a faster time scale. $B_2$: top: small $I_A$ in a GABA VTA neuron at −42 mV. Bottom: recording of $I_A$ from the same neuron on a faster time scale. $B_3$: relationship between $I_A$ density evoked at −42 mV and repolarization latency induced by the hyperpolarizing current pulse of −90 pA in DA neurons (open circles, $n = 9$) and GABA neurons (open triangles, $n = 5$).
large $I_A$ is critical for the regulation of spontaneous firing frequency in substantia nigra DA neurons (Liss et al. 2001). In GABA VTA neurons, because $I_A$ recovery time constant was longer than inter-AP interval and $I_A$ current density at full recovery was small, $I_A$ may be less important for the regulation of spontaneous firing in these neurons than midbrain DA neurons.

**Pharmacology of $I_A$ in VTA neurons**

$I_A$ of DA and GABA VTA neurons was sensitive to block by 4-AP. Previous studies on $I_A$ of midbrain DA neurons have reported that 4 to 10 mM 4-AP blocks $I_A$ (Hahn et al. 2003; Liss et al. 2001; Silva et al. 1990; Yang et al. 2001). $I_A$ was more sensitive to 4-AP in GABA VTA neurons than DA VTA neurons. In the CNS, it has been reported that there are neurons which exhibit $I_A$ with higher 4-AP sensitivity (IC$_{50}$ 1 to 1.8 mM) (Saito and Isa 2000; Song et al. 1998; Zhang and McBain 1995a) and neurons which exhibit $I_A$ with lower 4-AP sensitivity (IC$_{50}$ 2.4 to 4.2 mM) (Kornigreen and Sakmann 2000; Saito and Isa 2000).

Extracellular Cd$^{2+}$ shifted steady-state $I_A$ inactivation to a positive direction in DA and GABA VTA neurons. This effect of Cd$^{2+}$ could cause the activation of $I_A$ at more positive membrane potentials and could decrease the excitability of DA and GABA VTA neurons. Among divalent cations, Cd$^{2+}$, Co$^{2+}$ and Zn$^{2+}$ have been reported to shift the voltage-dependency of steady-state $I_A$ inactivation in a positive direction (Kuo and Chen 1999; Silva et al. 1990; Song et al. 1998). In substantia nigra DA neurons, both 200 μM Cd$^{2+}$ and 5 mM Co$^{2+}$ have been reported to shift the voltage dependence of steady-state $I_A$ inactivation in a positive direction by 30 mV (Silva et al. 1990).

**Possible involvement of Kv4.3 channels and auxiliary modulation**

Specific types of Kv channels are likely to be involved in DA and GABA VTA neurons, and the identification of the specific type of Kv channel is based on their $I_A$ properties, such as recovery time constant < 1 s, half-activating voltage of −38 to −35 mV and Cd$^{2+}$ modulation of the voltage-dependency of steady-state inactivation. These characteristics of $I_A$ in DA and GABA VTA neurons are consistent with Kv4 channels (An et al. 2000; Decher et al. 2004; Patel et al. 2004; Wickenden et al. 1999). It seems unlikely that Kv1.4 channels are involved in DA and GABA VTA neurons. Kv1.4 channels have been reported to have a long recovery time constant more than 5 s and are resistant to the effect of Cd$^{2+}$ on the voltage-dependency of steady-state inactivation (Wickenden et al. 1999). Of the three members of the Kv4 channel family (Kv4.1, Kv4.2, and Kv4.3) that could be responsible for $I_A$, it seems likely that Kv4.3 channels are involved in DA and GABA VTA neurons. An anatomical study has reported that the midbrain expresses Kv4.3 gene transcripts selectively (Serodio and Rudy 1998). In addition, both midbrain DA and GABA neurons have been reported to express Kv4.3 as a subunit mRNA (Hahn et al. 2003; Liss et al. 2001). By contrast, midbrain did not express Kv4.1 and Kv4.2 gene transcripts (Serodio and Rudy 1998) and neither Kv4.1 nor Kv4.2 a subunit mRNA was detected in midbrain DA neurons (Liss et al. 2001). Kv3.4 channels have

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**FIG. 8.** Delay of spike generation in DA VTA neurons. A: a depolarizing current pulse (+70 pA) was injected at various times after a spontaneous AP; a train of spikes were induced with the delay to the first spike in the train. (top) Depolarizing current was injected from relatively positive membrane potential after recovery from AHP. (middle) Depolarizing current was injected just after a spontaneous AP at time near AHP peak. (bottom) Depolarizing current was injected during the AHP of a spontaneous AP. Arrows indicate the delay to the first spike. A: plots of the delay to the first spike as a function of membrane potential at which depolarizing current pulse was injected. B: plots of the delay to the first spike as a function of the duration from a spontaneous AP to the initiation of depolarizing current pulse on the logarithmic scale. All data points were obtained from 5 DA VTA neurons. For each neuron, 15 trials of depolarizing current injection at various times after spontaneous AP generation were performed.
been reported to be very sensitive to 4-AP with an IC_{50} of 20–500 μM and have a half-activating voltage of +10 mV (Rudy and McBain 2001). It is unlikely that Kv3.4 channels are involved in DA VTA neurons. Although I_A of GABA VTA neurons was sensitive to 4-AP as similar extent as Kv3.4 channels, V_{act}, 0.5 of I_A in these neurons is more negative than that of Kv3.4 channels by 45 mV. Therefore it is also unlikely that Kv3.4 channels contribute to I_A in GABA VTA neurons.

Although I_A of DA and GABA VTA neurons had similar voltage-dependency for steady-state activation and inactivation, there were differences in kinetics, recovery time constant and current density at full recovery. It is possible that these different I_A properties in DA and GABA VTA neurons may be due to the auxiliary modulation of I_A, rather than a difference in the channel types. Potassium channel interacting proteins (KChIPs), which are Ca^{2+}-binding proteins, have been reported to regulate Kv4.3 channel properties by increasing inactivation time constant, accelerating recovery from inactivation and augmenting current density (An et al. 2000; Decher et al. 2004; Nadal et al. 2001; Patel et al. 2004). Furthermore, it has been reported that several types of KChIP isoforms contribute to different Kv4.3 channel properties (Decher et al. 2004; Nadal et al. 2001; Patel et al. 2004). Thus different types of KChIP isoforms may modulate Kv4.3 channels involved in DA and GABA VTA neurons and contribute to the heterogeneity of I_A in these neurons. Since intracellular Ca^{2+} buffering affect I_A kinetics through KChIPs (Patel et al. 2002), future studies could determine whether different Ca^{2+} buffering conditions alter the channel kinetics in a predictable manner in one or both types of DA and GABA VTA neurons.

Physiological implication

Considering the biophysical characteristics of I_A and spontaneous firing properties in DA VTA neurons, we suggest that I_A can contribute to the spontaneous firing of these neurons. Under the low calcium conditions used in these experiments, analysis of steady-state inactivation in DA VTA neurons indicates that about 5–10% of I_A is available for activation at the AHP peak (-68 mV). By the analysis of steady-state inactivation in DA VTA neurons under more physiological conditions in which the extracellular solution contains 2 mM Ca^{2+}, 608 pA I_A (25% of maximal I_A) is available for activation at AHP peak (-68 mV) in these neurons. Since average I_A time constant for recovery from inactivation was 50 ms and average spontaneous firing frequency was 3.3 Hz (duration between APs, 303 ms) in DA VTA neurons, the membrane remains hyperpolarized for sufficient time for I_A to recover from inactivation during the inter-spike interval in these neurons. In addition, I_A current density at full recovery was large in DA VTA neurons, suggesting that the maximal magnitude of I_A can be activated during the depolarizing phase following AHP. In substantia nigra DA neurons, it has been reported that I_A charge density is negatively correlated with spontaneous firing frequency and a Kv4 channel blocker, heptapodatoxin, increases spontaneous firing frequency in a concentration-dependent manner (Liss et al. 2001). Thus I_A is thought to be important for the regulation of spontaneous firing frequency in midbrain DA neurons. On the other hand, we suggest that I_A probably does not affect the spontaneous firing of GABA neurons. Since the average I_A recovery time constant from inactivation was 306 ms and the average spontaneous firing frequency was 16 Hz (duration between APs, 63 ms) in GABA VTA neurons, the membrane is not hyperpolarized for sufficient time for recovery of I_A from inactivation between APs in these neurons.

When hyperpolarizing currents were injected, DA VTA neurons exhibited delay to the initiation of the first spike in current-clamp recording. In addition, the same DA VTA neurons exhibited large I_A current density in voltage-clamp configuration. In DA VTA neurons, since I_A had rapid activation (5 ms) and large magnitude with the inactivation time constant of about 30 ms at subthreshold membrane potentials, I_A could contribute as a hyperpolarizing driving force to delay to the initiation of the first spike. Conversely, low-threshold Ca^{2+} current (T-type Ca^{2+} current) (Wolfart and Roeppe 2002) and I_t tail current can contribute as a depolarizing driving force at recovery of membrane hyperpolarization in midbrain DA neurons (Neuhoff et al. 2002). However, the activation of T-type Ca^{2+} current is 23 ms (Wolfart and Roeppe 2002) and the activation of I_t is 843–1286 ms (Neuhoff et al. 2002), which are larger than the activation time constant of I_A presented in the present study. Therefore I_A is thought to have a more critical influence on repolarization in DA VTA neurons. Similarly, the effect of I_A to delay repolarization has been reported in cerebellar granule neurons (Shibata et al. 2000). On the other hand, GABA VTA neurons exhibited short repolarization latency in current-clamp recording and small I_A current density in voltage-clamp configuration. In GABA VTA neurons, since I_A had small magnitude with the inactivation time constant of about 15 ms at subthreshold membrane potentials, I_A may not contribute significantly to the delay of repolarization as a hyperpolarizing driving force.

When depolarizing current was injected at various times after spontaneous AP generation, a train of spikes were evoked and the delay of first spike was detected in DA VTA neurons. This delay of spike generation became longer when depolarizing current was initiated at membrane potentials negative to -55 mV. In the similar voltage rage, I_A of DA VTA neurons could be available for activation under physiological conditions. The long delay to the first spike was induced by the duration of 30–120 ms measured from spontaneous AP to the initiation of depolarizing current injection. Since the time constant of I_A to recover from inactivation was 50 ms, I_A could be activated during the time window between 30 to 120 ms. Therefore the component for the time- and voltage-dependent delay of spike generation induced in DA VTA neurons is probably I_A.

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