Characterization of M-Current in Ventral Tegmental Area Dopamine Neurons

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Koyama, Susumu and Sarah B. Appel. Characterization of M-current in ventral tegmental area dopamine neurons. J Neurophysiol 96: 535–543, 2006. First published January 4, 2006; doi:10.1152/jn.00574.2005. M-current (I_M) is a voltage-gated potassium current (KCNQ type) that affects neuronal excitability and is modulated by some drugs of abuse. Ventral tegmental area (VTA) dopamine (DA) neurons are important for the reinforcing effects of drugs of abuse. Therefore we studied I_M in acutely dissociated rat DA VTA neurons with nystatin-perforated patch recording. The standard deactivation protocol was used to measure I_M during voltage-clamp recording with hyperpolarizing voltage steps to −65 mV (in 10-mV increments) from a holding potential of −25 mV. I_M amplitude was voltage dependent and maximal current amplitude was detected at −45 mV. The deactivation time constant of I_M was voltage dependent and became shorter at more negative voltages. The I_KCNS antagonist XE991 (0.3–30 μM) caused a concentration-dependent reduction in I_M amplitude with an IC50 of 0.71 μM. Tetraethylammonium (TEA, 0.3–10 mM) caused a concentration-dependent inhibition of I_M with an IC50 of 1.56 mM. In current-clamp recordings, all DA VTA neurons were spontaneously active. Analysis of evoked action potential shape indicated that XE991 (1–10 μM) reduced the fast and slow components of the spike afterhyperpolarization (AHP) without affecting the middle component of the AHP. Action potential amplitude, duration, and threshold were not affected by XE991. In addition, 10 μM XE991 significantly shortened the interspike intervals in evoked spike trains. In conclusion, I_M is active near threshold in DA VTA neurons, is blocked by XE991 (10 μM) and TEA (10 mM), may contribute to the shape of the AHP, and may decrease excitability of these neurons.

INTRODUCTION

The ventral tegmental area (VTA) is located in the ventromedial region of the mesencephalon (Paxinos and Watson 1996) and the neurons in the VTA are classified into two major neuronal subgroups: dopaminergic (DA) and GABAergic neurons (Grace and Onn 1989; Johnson and North 1992). DA neurons constitute the majority of the neuronal population (70%) and γ-aminobutyric acid (GABA) neurons represent 30% of the neuronal population in the VTA (Johnson and North 1992) and DA VTA neurons are involved in the reinforcing effects of natural stimuli and drugs of abuse (Appel et al. 2004; Robinson and Berridge 2003; Wise 1987, 2002). The cell bodies of DA VTA neurons send terminal projections that synapse in the nucleus accumbens. Excitation of DA VTA neurons by drugs of abuse such as ethanol or nicotine (Brodie 1991; Brodie et al. 1999b) results in increased dopamine release in the nucleus accumbens (DiChiara and Imperato 1988; Weiss et al. 1993), which is important for the reinforcing effects of these drugs (Corrigall et al. 1994; Pfeffer and Samson 1988). DA VTA neurons have intrinsic pacemaker activity and several voltage-dependent ionic currents contribute to the excitability of these neurons (Brodie et al. 1999b; Grace and Onn 1989; Johnson and North 1992; Neuhoff et al. 2002).

M-current (I_M) is a voltage-dependent, slow delayed rectifier K+ current that is activated at the subthreshold range of membrane potential and contributes to regulation of repetitive firing and excitability (Aiken et al. 1995; Brown and Adams 1980). I_M has been shown to be modulated by drugs of abuse including cannabinoids, opiates, and ethanol (Koyama and Appel 2004; Moore et al. 1990, 1994; Schweitzer 2000). I_M is mediated by current through KCNQ-type potassium channels (Wang et al. 1998). To date, five different KCNQ genes have been cloned and KCNQ2, 3, 4, and 5 channel subunits have been reported to mediate I_M in various types of neurons (Lerche et al. 2000; Passmore et al. 2003; Selyanko et al. 2000; Shah et al. 2002; Shapiro et al. 2000; Segard et al. 2001; Wang et al. 1998). Immunohistochemical studies have shown that the KCNQ2 and KCNQ4 channel proteins are present in VTA neurons (Cooper et al. 2001; Kharkovets et al. 2000). Consistent with the presence of KCNQ channel proteins found in these anatomical studies, I_M has been shown to be present in midbrain dopamine neurons with intracellular voltage-clamp recording in brain slices (Lacey et al. 1990), although it has not been studied in detail in these neurons.

The aims of the present study were 1) to characterize the electrophysiological parameters of I_M in DA VTA neurons with voltage-clamp recording for comparison to I_M in other types of neurons; 2) to determine the sensitivity of I_M in DA VTA neurons to tetraethylammonium (TEA) and 10,10-bis(4-pyridinylmethyl)-9-(10H)-anthracene (XE991), which has been shown to depend on the KCNQ channel subtypes that mediate the current in other types of neurons; and 3) to examine the physiological role of I_M in the electrical behavior of DA VTA neurons in current-clamp experiments by blocking I_M with XE991. The present study was performed in acutely dissociated rat DA VTA neurons, stripped of synaptic terminals (Koyama et al. 2005), and therefore the actions of the drugs tested reflect a direct postsynaptic action on these neurons. Nystatin-perforated patch-clamp recording was used to minimize intracellular dialysis and prevent the rundown of I_M. Rundown occurs rapidly with whole cell patch-clamp recording because intracellular nucleotide phosphates are indispensable for maintenance of I_M (Simmons and Schneider 1998). Some of the results of the present study were reported previously in abstract form (Koyama and Appel 2004).

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METHODS

Region-specific cell dissociation

Animals used in this study were treated in strict accordance with the American Physiological Society’s Guiding Principles in the Care and Use of Animals and the U.S. National Institutes for Health Guide for the Care and Use of Laboratory Animals; the protocol for all experimental methods was approved by the Institutional Animal Care Committee of the University of Illinois at Chicago. Fisher 344 rats (14–20 days old, male and female) were decapitated and the brain quickly removed. As an inbred strain, Fisher 344 rats showed smaller animal-to-animal variation in ethanol sensitivity in our previous studies; we used Fisher 344 rats here for the comparison with future ethanol studies on $I_{M}$. The brain was placed in ice-cold cutting solution (in mM: 220 sucrose, 2.5 KCl, 2.4 CaCl$_2$, 1.3 MgSO$_4$, 1.24 NaHPO$_4$, 26 NaHCO$_3$, 11 d-glucose), which was constantly bubbled with 95% O$_2$, 5% CO$_2$. Transverse brain slices (400 µm thick) were made on a Vibratome (Series 1000 plus, St. Louis, MO). Brain slices were incubated for 30 min in artificial cerebrospinal fluid (aCSF) (in mM: 126 NaCl, 2.5 KCl, 2.4 CaCl$_2$, 1.3 MgSO$_4$, 1.24 NaHPO$_4$, 26 NaHCO$_3$, 11 d-glucose; osmolality, 300 mOsm), which was constantly bubbled with 95% O$_2$, 5% CO$_2$ at room temperature (23–25°C). The brain slices were then incubated in a HEPES-buffered solution (in mM: 145 NaCl, 2.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 11 d-glucose; pH 7.4 adjusted with NaOH; osmolality, 300 mOsm) containing papain (15–18 U/ml) at 32°C for 20–25 min. After papain treatment, the brain slices were further incubated in aCSF for 20–40 min. VTA neurons were dissociated with a vibrating stylus apparatus for dispersing cells from the brain slices as previously described (Koyama et al. 2005). A brain slice was transferred to a poly-d-lysine–coated 35-mm culture dish (Becton Dickinson, Bedford, MA) containing the HEPES-buffered solution. A grid of nylon threads glued to a U-shaped metal frame was used to hold the brain slice down during cell dissociation. After the VTA was visually identified, a vibrating stylus was placed in the appropriate region with a micromanipulator. The stylus was made of glass capillary tubing (1.5 mm OD) pulled to a fine tip, fire-polished (200–400 µm in diameter), and mounted on a vibrating apparatus, which horizontally vibrated the stylus tip (excursions of 100–200 µm at 20–25 Hz). 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. The dissociation procedure with papain described above has been shown to yield acutely dissociated VTA neurons stripped of synaptic terminals (Koyama et al. 2005) and no spontaneous synaptic potentials or synaptic currents were recorded from these neurons in the present study.

Electrophysiological recording

Electrophysiological measurements were made with an Axopatch-1B patch-clamp amplifier (Axon Instruments, Union City, CA). Microelectrodes were fabricated on a P-87 puller (Sutter Instrument Company, Novato, CA), from LE16 glass capillaries (Dagan, Minneapolis, MN) and heat-polished on a microforge (Narishige, Tokyo, Japan). The tip resistances of the electrodes were 1–2.5 MΩ when filled with pipette solution (in mM: 60 K-acetate, 60 KCl, 1 CaCl$_2$, 2 MgCl$_2$, 40 HEPES; pH 7.2 adjusted with KOH; final [K$^+$], 131 mM; osmolality was adjusted to 290 mOsm with sucrose). Nystatin-perforated patch-clamp recording (Akaife and Harata 1994) was used to minimize intracellular dialysis. Nystatin was dissolved in methanol at a concentration of 10 mg/ml. This nystatin stock solution was diluted with pipette solution to a final concentration of 100–200 µg/ml and the electrodes were backfilled with this solution. After the cell-attached configuration was attained, the access resistance was periodically monitored with hyperpolarizing voltage steps (amplitude 10 mV, duration 100 ms) and capacitive transients were cancelled.

When the access resistance had reached a steady level (10.6 ± 0.4 MΩ, n = 72), the recording was started. In case of the sudden change of the access resistance, the recording was stopped. Current- and voltage-clamp recordings were done in the HEPES-buffered solution constantly bubbled with 100% O$_2$. The liquid junction potential between the pipette solution and the HEPES-buffered solution was estimated to be 5 mV (Neher 1992) and the results have been corrected by this amount. Membrane currents and voltage were filtered at 1 kHz by a –3-dB four-pole filter and acquired at a sampling frequency of 10 kHz. Data acquisition was performed with a DigiData 1322A interface and pClamp software version 9.0 (Axon Instruments). The dissociated VTA neurons were visualized under phase-contrast optics on an inverted microscope (Diaphot 300, Nikon, Tokyo, Japan). All experiments were performed at room temperature (23–25°C).

Drug application

Neurons were continuously bathed in control HEPES-buffered solution and drugs were dissolved at final concentration in the same solution. Drug solutions were applied by a multiple channel manifold (Becton Dickinson, Bedford, MA). Each channel of the manifold was connected to a gravity-fed reservoir with tubing (860 µm, ID). The output of the manifold was connected to an outflow tube (580 µm, ID), 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 following drugs and chemical agents were used in this study. Nystatin and TEA were purchased from Sigma (St. Louis, MO). Papain was purchased from Worthington (Lakewood, NJ), and XE991 dihydrochloride was purchased from Tocris-Cookson (Ellisville, MO).

Data analysis and curve fitting

Action potentials were analyzed off-line with pClamp 9.0 software (Axon Instruments). All average values are expressed as mean ± SE. Statistical comparison was done by Student’s t-test to assess significant differences. Graphing and curve fitting of data were performed with Origin 7 software (OriginLab, Northampton, MA). The inward relaxation current, which was attributed to deactivation of $I_{M}$, was fitted by a single exponential function of the form

$$I(t) = A \exp(-t/\tau)$$

where $A$ is amplitude obtained from the beginning of the fit and $\tau$ is the decay time constant.

Concentration–response curves for XE991 and TEA were constructed by plotting percentage inhibition of $I_{M}$ as a function of drug concentration plotted on a log scale. Smooth curves were fit to these data with the Hill equation of the form

$$y = y_{\text{max}}x^k/(x^k + x^\delta)$$

where $x$ is the concentration, $y$ is the percentage inhibition, and $y_{\text{max}}$ is the maximal value of $y$ (at saturation); in the fitting procedure $y_{\text{max}}$ was constrained not to exceed 100%. The term $k$ is the IC$_{50}$ (the concentration giving half-maximal inhibition) and $\delta$ (Hill slope) is the power term related to the slope of the curve.

RESULTS

$M$-current ($I_{M}$) in DA VTA neurons

In the present study, we recorded 72 spontaneously active dopamine neurons dissociated from the VTA with nystatin-
perforated patch current-clamp recording. To identify VTA neurons as dopaminergic, we analyzed cell capacitance, spontaneous firing frequency, and action potential shape parameters in all of these neurons as shown in Table 1 and Fig. 1A. In addition, in some of the same experiments, we measured input resistance and estimated resting membrane potential in 20 typical neurons out of the 72 DA VTA neurons (Table 1).

Nystatin-perforated patch-clamp recording was used in this study because this technique does not dialyze intracellular contents and therefore prevents rundown of \( I_M \). After obtaining a stable perforated patch recording, dissociated VTA neurons were identified as dopaminergic in current-clamp recording mode according to the parameters described above; then \( I_M \) was measured in the same DA VTA neuron by voltage-clamp recording mode. Figure 1A (left) illustrates a current-clamp recording of a spontaneously active DA VTA neuron. The action potential of this neuron is shown on a faster timescale in Fig. 1A (right); the duration at half-amplitude of this spontaneous action potential was 1.9 ms. \( I_M \) in this neuron was examined with a standard \( I_M \) deactivation voltage protocol (Brown and Adams 1980). Hyperpolarizing voltage steps (1-s duration) were given from a holding potential of −25 to −65 mV (in 10-mV increments) as shown in Fig. 1B (the currents recorded in response to this protocol are shown below in Fig. 1B, left). \( I_M \) was measured as the inward relaxation current caused by deactivation of \( I_M \) during the voltage step as shown in Fig. 1B (right). Figure 1C shows the current–voltage relationship of \( I_M \) for 13 DA VTA neurons. The mean \( I_M \) amplitude was voltage dependent and the maximal \( I_M \) amplitude (64.9 ± 7.4 pA) was measured at −45 mV. The deactivation time constant of \( I_M \) was determined by fitting the current measured at each voltage with a single exponential function. Figure 1D shows the mean deactivation time constant of \( I_M \) as a function of voltage (n = 13). The mean \( I_M \) deactivation time constant was 105.6 ± 10.4 ms (at −65 mV), 138.4 ± 13.2 ms (at −55 mV), 193.5 ± 24.5 ms (at −45 mV), and 241.8 ± 30.1 ms (at −35 mV), indicating that it was voltage dependent. Note that the deactivation time constant was a linear function of voltage (correlation coefficient \( r = 0.99 \)). Deactivation time constant was determined by fitting the current measured at each voltage by a single exponential function.

### Table 1. Membrane properties of spontaneously firing DA VTA neurons measured with nystatin-perforated patch recording

<table>
<thead>
<tr>
<th>Property</th>
<th>Mean ± SE</th>
<th>n = 72</th>
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<tbody>
<tr>
<td>Cell capacitance, pF</td>
<td>33.0 ± 0.9</td>
<td>72</td>
</tr>
<tr>
<td>AP parameters (n = 72)</td>
<td></td>
<td></td>
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<tr>
<td>Amplitude, mV</td>
<td>74.7 ± 1.1</td>
<td></td>
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<tr>
<td>Half-width, ms</td>
<td>2.0 ± 0.1</td>
<td></td>
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<tr>
<td>Threshold, mV</td>
<td>−38.6 ± 0.4</td>
<td></td>
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<tr>
<td>AHP amplitude, mV</td>
<td>26.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>902.4 ± 43.5</td>
<td>20</td>
</tr>
<tr>
<td>Estimated RMP, mV</td>
<td>48.5 ± 0.7</td>
<td>20</td>
</tr>
<tr>
<td>Firing frequency, Hz</td>
<td>2.6 ± 0.2</td>
<td>72</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers of neurons (n) are in parentheses. Cell capacitance was measured after the capacitive transients were cancelled. Action potential (AP) parameters were measured from spontaneous APs recorded in current-clamp mode at resting membrane potential (no current injection). AP amplitude was measured from threshold to AP peak. AP half-width was measured as the duration of the spike halfway between threshold and the AP peak. Afterhyperpolarization (AHP) amplitude was measured from threshold to the peak of the AHP. In 20 of these 72 cells, input resistance and estimated resting membrane potential (RMP) were also measured. Input resistance was determined by hyperpolarizing current pulses that induced a voltage shift of 10–15 mV negative to the resting potential, a voltage range in which \( I_M \) (voltage sag on current-clamp recording) was not present (Koyama et al. 2005). Estimated RMP was determined by the membrane potential measured at 60 ms before the peak of the spontaneous AP (Brodie et al. 1999a).
DA VTA neurons have a prominent A-type transient $K^+$ current that was inactivated at the holding potential of $-25$ mV and therefore did not obscure the measurement of $I_M$ with the voltage protocol shown in Fig. 1. A-current activation can be seen after the offset of the larger hyperpolarizing voltage steps in Fig. 1B (left).

**XE991 inhibition of $I_M$**

XE991 has been shown to be a potent and selective inhibitor of $I_M$ in native neurons and currents from expressed KCNQ channels (Wang et al. 1998). Figure 2A$_1$ (top) shows that a maximal concentration of XE991 (30 $\mu$M) greatly reduced $I_M$ in a typical DA VTA neuron. This effect was accompanied by a reduction of the baseline outward current. Figure 2A$_1$ (bottom) shows the XE991-sensitive current obtained by digital subtraction of the XE991-resistant component (top right) from the control current (top left). The deactivation time constant of XE991-sensitive current was 190.0 ms and that of the control $I_M$ was 186.1 ms. In these experiments, $I_M$ was monitored with a 1-s-long hyperpolarizing voltage step from a holding potential of $-25$ to $-40$ mV (Fig. 2B$_1$, inset). To determine the time course of the XE991 inhibition, $I_M$ was monitored with this voltage step every 20 s. Figure 2A$_2$ shows the inhibition by 30 $\mu$M XE991 of the mean $I_M$ measured in seven DA VTA neurons versus time. The mean maximal reduction of $I_M$ amplitude with 30 $\mu$M XE991 was 90.2 $\pm$ 2.3% and this reduction was partially reversible after the washout of XE991. The concentration dependency of XE991 inhibition is shown in Fig. 2, B$_1$ and B$_2$. Note that XE991 reduced both $I_M$ and the baseline outward current in a concentration-dependent manner in this typical DA VTA neuron (Fig. 2B$_1$). Figure 2B$_2$ shows the pooled concentration–response curve that plots mean percentage inhibition of $I_M$ amplitude versus log concentration of XE991 from seven DA VTA neurons. The mean inhibition of $I_M$ by XE991 was $27.2 \pm 3.8\%$ (at 0.3 $\mu$M), $51.4 \pm 2.9\%$ (at 1 $\mu$M), $81.4 \pm 1.7\%$ (at 3 $\mu$M), and nearly complete block of $I_M$ was seen with the two highest concentrations tested: $86.6 \pm 2.5\%$ (at 10 $\mu$M) and $90.2 \pm 2.3\%$ (at 30 $\mu$M). A smooth curve was fit to the mean data in Fig. 2B$_2$ with the Hill equation (see METHODS). The IC$_{50}$ for XE991 was 0.71 $\mu$M and the power term $n$ (Hill slope), which is related to the steepness of curve, was 1.14. The goodness of fit $R^2$ was 0.988.

**TEA inhibition of $I_M$**

Previous studies reported that different KCNQ channel proteins have different sensitivities to TEA (Hadley et al. 2000; Shapiro et al. 2000; Wang et al. 1998), and therefore it was of interest to examine the TEA sensitivity of $I_M$ in DA VTA neurons. Figure 3A shows that TEA caused a concentration-dependent reduction in $I_M$ in this typical DA VTA neuron. TEA also caused a concentration-dependent reduction in the baseline outward current. The voltage protocol used to measure $I_M$ is shown in the inset. Figure 3B shows the pooled concentration–response curve that plots mean percentage inhibition of $I_M$ amplitude versus log concentration of TEA from five DA VTA neurons. The mean inhibition of $I_M$ by TEA was $13.5 \pm 3.2\%$ (at 0.3 mM), $32.3 \pm 3.9\%$ (at 1 mM), $74.0 \pm 5.3\%$ (at 3 mM), and nearly complete block of $I_M$ ($97.6 \pm 0.5\%$) was seen with 10 mM TEA. A smooth curve was fit to the mean data in Fig. 3B with the Hill equation (see METHODS). The IC$_{50}$ for TEA was 1.51 mM and the power term $n$ (Hill slope) was 1.49. The goodness of fit $R^2$ was 0.990. The sensitivity to TEA has been used as an indication of KCNQ subunit composition, and thus for comparison, we also used the Hill equation to fit the log

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**FIG. 2.** Inhibition of $I_M$ by XE991 in DA VTA neurons. A$_1$, top: $I_M$ before and after treatment with 30 $\mu$M XE991 in a DA VTA neuron; bottom: XE991-sensitive current determined by digital subtraction of the XE991-resistant component from the control current shown on an expanded timescale. A$_2$: time course of 30 $\mu$M XE991 block of $I_M$ (mean data from 7 DA VTA neurons). A 1-s-long hyperpolarizing voltage step from a holding potential of $-25$ to $-40$ mV was given every 20 s to monitor $I_M$. All $I_M$ amplitudes were normalized to the mean amplitude of the five currents measured before XE991 application in each cell. B$_1$: XE991 inhibition of $I_M$ was concentration dependent. $I_M$ values are superimposed. Note the shift in the baseline outward current in XE991 (at holding potential of $-25$ mV). B$_2$: concentration–response curve showing mean percentage inhibition of $I_M$ amplitude as a function of log XE991 concentration for 7 DA VTA neurons. Nearly maximal block of $I_M$ was seen with 10 $\mu$M ($86.6 \pm 2.5\%$) and 30 $\mu$M ($90.2 \pm 2.3\%$) XE991. The smooth curve was fit to the Hill equation. The IC$_{50}$ value for XE991 inhibition was 0.71 $\mu$M.
concentration–response curves and to determine the IC$_{50}$ and Hill slope for each of the five DA VTA neurons individually.

The mean of the IC$_{50}$ values determined for each individual experiment was $1.56 \pm 0.27$ mM and the mean Hill slope was $1.58 \pm 0.08$. These were very similar to the IC$_{50}$ and Hill slope determined from the pooled log concentration–response curve shown in Fig. 3B described above.

$I_M$ contribution to the shape of the evoked action potential

The specific antagonist XE991 was used to block $I_M$ to assess the contribution of $I_M$ to the shape of the evoked action potential in DA VTA neurons. These experiments were performed with nystatin-perforated patch recording in current-clamp mode. Continuous DC hyperpolarizing current was injected to prevent spontaneous action potential firing and to maintain the baseline membrane potential constant throughout the experiment. A small depolarizing current pulse (+10 pA, inset) was used to evoke one or two action potentials before and after XE991 treatment. Figure 4A$_1$ shows action potentials evoked in a typical DA VTA neuron before (left) and in the

![Figure 4A](https://example.com/figure4a.png)

**FIG. 4.** $I_M$ contribution to evoked action potential shape in DA VTA neurons. A$_1$: current-clamp recording of voltage responses of a DA VTA neuron to depolarizing (+10 pA) and hyperpolarizing (−70 pA) square-wave current pulses (insets), before (left) and after treatment with 10 μM XE991 (right). Continuous DC hyperpolarizing current was injected to prevent spontaneous firing and to maintain the baseline membrane potential at −62 mV. Sag on the voltage responses to the hyperpolarizing pulses is attributed to the time-dependent inward rectifier current $I_h$, which is characteristic for identification of DA VTA neurons. Measurements were made on the first action potential evoked in each case (arrows). A$_2$: comparison of evoked action potentials recorded from the DA VTA neuron shown in A$_1$, before (black line) and after 10 μM XE991 treatment (gray line); action potentials were superimposed and timescale is 20 ms long. Note that XE991 reduced the fast component of the afterhyperpolarization (AHP). A$_3$: comparison of evoked action potentials recorded from the same DA VTA neuron before (black line) and after 10 μM XE991 treatment (gray line) shown on a longer timescale (340 ms long). Note that XE991 reduced the slow and fast components of AHP without affecting the middle component of AHP. B: diagram illustrating measurement of the action potential shape parameters listed in Table 2. Note that half-width refers to the duration of the spike at half-amplitude.

presence of 10 μM XE991 (right). A large hyperpolarizing current pulse (−70 pA) activated a prominent time-dependent inward rectification (voltage sag) arising from the presence of

![Figure 3](https://example.com/figure3.png)

**FIG. 3.** Inhibition of $I_M$ by tetraethylammonium ion (TEA) in DA VTA neurons. A: TEA inhibition of $I_M$ was concentration dependent. $I_M$ values are superimposed. Note the shift in the baseline outward current in TEA (at holding potential of −25 mV). B: concentration–response curve showing mean percentage inhibition of $I_M$ amplitude as a function of log TEA concentration for 5 DA VTA neurons. Nearly complete blockage of $I_M$ was seen with 10 mM TEA (97.6 ± 0.5%). Smooth curve was fit with the Hill equation. IC$_{50}$ value for TEA inhibition determined from this pooled log concentration–response curve was 1.51 mM.
Table 2. Effect of XE991 on the shape of the evoked action potential (AP) in DA VTA neurons

<table>
<thead>
<tr>
<th></th>
<th>Control (7)</th>
<th>XE991, 1 μM (7)</th>
<th>Control (6)</th>
<th>XE991, 3 μM (6)</th>
<th>Control (7)</th>
<th>XE991, 10 μM (7)</th>
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<tbody>
<tr>
<td>Amplitude, mV</td>
<td>86.1 ± 2.0</td>
<td>87.0 ± 2.0</td>
<td>87.8 ± 2.1</td>
<td>88.7 ± 2.2</td>
<td>87.6 ± 1.2</td>
<td>87.2 ± 1.5</td>
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<tr>
<td>Half-width, ms</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Threshold, mV</td>
<td>−42.7 ± 0.9</td>
<td>−42.8 ± 0.8</td>
<td>−42.7 ± 1.0</td>
<td>−42.4 ± 1.1</td>
<td>−42.8 ± 1.2</td>
<td>−42.9 ± 1.4</td>
</tr>
<tr>
<td>AHP-1, mV</td>
<td>25.6 ± 1.5</td>
<td>24.0 ± 1.3*</td>
<td>27.3 ± 1.9</td>
<td>25.1 ± 1.7**</td>
<td>27.5 ± 1.1</td>
<td>22.5 ± 1.5**</td>
</tr>
<tr>
<td>AHP-2, mV</td>
<td>26.6 ± 2.8</td>
<td>26.6 ± 3.1*</td>
<td>27.8 ± 3.8</td>
<td>28.6 ± 2.4</td>
<td>25.5 ± 4.1</td>
<td>24.4 ± 4.4</td>
</tr>
<tr>
<td>AHP-3, mV</td>
<td>16.6 ± 1.0</td>
<td>15.7 ± 1.0*</td>
<td>16.5 ± 1.5</td>
<td>14.8 ± 1.4*</td>
<td>15.0 ± 1.2</td>
<td>11.2 ± 2.3**</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers of neurons (n) are in parentheses. AHP, spike afterhyperpolarization. All AP shape parameters were analyzed on the first AP evoked by a 10-pA depolarizing current injection (see Fig. 4, A1 and B). APs were evoked from the same membrane potential before and after XE991 treatment. DC current injection was used to keep the membrane potential constant. Statistical significance was assessed with the paired Student’s t-test. The asterisks indicate significant differences between values in control and in the presence of XE991 (*P < 0.05; **P < 0.01).

\( I_{M} \) indicating that this neuron was a dopamine neuron (Figs. 4A1 and 5A1). Blockade of \( I_{M} \) with 10 μM XE991 shortened the latency to the first evoked action potential (arrows) and reduced the interspike interval (ISI) (Fig. 4A1). Figure 4A2 and A3 show the first evoked action potentials before and after treatment with 10 μM XE991 from the same neuron, on a short (20-ms) and long (340-ms) timescale, respectively. XE991 reduced the fast and slow components of the afterhyperpolarization (AHP) without affecting the middle component of AHP. Figure 4B illustrates measurement of the evoked action potential shape parameters shown in Fig. 4A1 and Table 2. AHP-1, AHP-2, and AHP-3 correspond to the fast, middle, and slow components of AHP mentioned above. Table 2 shows the effects of 1, 3, and 10 μM XE991 on evoked action potential shape parameters in DA VTA neurons. XE991 significantly reduced the amplitude of the fast (AHP-1) and slow (AHP-3) components, without affecting the amplitude of the middle component (AHP-2). The size of these effects appeared to be concentration dependent. XE991 (1–10 μM) did not affect evoked action potential amplitude, half-width (duration at half-amplitude), or threshold. In addition, 10 μM XE991 did not significantly change \( I_{M} \), as measured by the amplitude of the voltage sag induced by a hyperpolarizing current step of −70 pA (39.7 ± 5.4 mV in control, 37.8 ± 5.0 mV with XE991; paired Student’s t-test, \( P > 0.4, n = 7 \)). In five DA VTA neurons, 30 μM XE991 caused a small prolongation of action potential half-width (1.4 ± 0.2 ms in control, 1.6 ± 0.3 ms in XE991; paired Student’s t-test significant, \( P < 0.05 \)); this effect was not seen at lower concentrations. As seen with 1–10 μM XE991, 30 μM XE991 also caused a significant reduction in AHP-1 (26.9 ± 2.7 mV in control, 15.8 ± 1.8 mV in XE991; paired Student’s t-test, \( P < 0.01 \)) and AHP-3 (12.8 ± 1.8 mV in control, 8.5 ± 2.0 mV in XE991; paired Student’s t-test, \( P < 0.05 \)) with no effect on AHP-2.

**I\(_{M}\)** contribution to the interspike interval

To examine the contribution of \( I_{M} \) to the ISI in DA VTA neurons, a larger depolarizing current pulse than that in Fig. 4 was used to evoke a train of several action potentials in control and in the presence of XE991. Figure 5A shows action potentials evoked by a +30 pA depolarizing current pulse before (left) and in the presence of 10 μM XE991 (right) in the same DA VTA neuron as in Fig. 4A. Measurements were made of the first ISI evoked in each case (arrows). Figure 5A2 shows evoked action potentials recorded from the same DA VTA neuron before (black line) and after 10 μM XE991 treatment (gray line), superimposed for comparison of the first ISIs. Note that XE991 reduces the slow AHP, which leads to earlier occurrence of the second action potential, i.e., a shortening of the ISI. Figure 5B summarizes the mean effect of XE991 on the

\( I_{M} \) contribution to the interspike interval (ISI) in DA VTA neurons. \( A_{1} \): current-clamp recording of voltage responses of a DA VTA neuron to depolarizing (+30 pA) and hyperpolarizing (~70 pA) square-wave current pulses (insets), before (left) and after treatment with 10 μM XE991 (right). Continuous DC hyperpolarizing current was injected to prevent spontaneous firing and to maintain the baseline membrane potential constant throughout the experiment. Measurements were made of the first ISI evoked in each case (arrows). \( A_{2} \): evoked action potentials recorded from the DA VTA neuron shown in \( A_{1} \) before (black line) and after 10 μM XE991 treatment (gray line), superimposed for comparison of the first ISIs; timescale is 250 ms long. Note that reduction in the slow AHP by XE991 leads to earlier occurrence of the second action potential. \( B \): mean of first ISIs before and after 10 μM XE991 treatment recorded from 7 DA VTA neurons. Asterisks indicate a significant difference on a paired Student’s t-test (\( P < 0.01 \)).
first ISI in seven DA VTA neurons. XE991 (10 µM) significantly reduced the first ISI (278.0 ± 44.2 ms in control, 221.6 ± 44.6 ms in XE991; paired Student’s t-test, P < 0.01). As can be seen in Fig. 5A, XE991 also increased the number of action potentials evoked by the +30 pA depolarizing current pulse. In seven DA VTA neurons, the mean number of action potentials evoked by this current pulse was 4.14 ± 0.55 in control and 5.57 ± 0.81 in 10 µM XE991, which was a significant increase (paired Student’s t-test, P < 0.01).

**DISCUSSION**

The electrophysiological properties of rat DA VTA neurons, measured in this study with nystatin-perforated patch-clamp recording, were consistent with values previously reported for DA VTA neurons with other recording techniques (Brodie et al. 1999b; Grace and Ond 1989; Johnson and North 1992; Neuhoff et al. 2002). Furthermore, with nystatin-perforated patch voltage-clamp recording, it was possible to study I_M with the standard I_M deactivation protocol, without the rundown of this current seen in whole cell recordings. The presence of I_M in DA VTA neurons found in our study agrees with the earlier observation of I_M in mesencephalic DA neurons recorded with sharp intracellular electrodes from brain slices, with a similar voltage protocol (Lacey et al. 1990).

XE991 has been shown to be a potent and selective inhibitor of I_M in native neurons and expressed KCNQ channels (Wang et al. 1998). In the present study, XE991 (10–30 µM) caused about 90% block of the current measured with the standard I_M deactivation voltage protocol, confirming the identity of this current as I_M in DA VTA neurons. The IC_{50} for XE991 in DA VTA neurons was 0.71 µM, which is closest to the IC_{50} value for XE991 inhibition of expressed KCNQ2 channels (0.71 µM), but is also similar to expressed KCNQ2/3 heteromeric channels (0.6 µM) and native I_M (0.98 µM) in sympathetic ganglion neurons (Wang et al. 1998). IC_{50} values reported for XE991 inhibition of expressed KCNQ4 and KCNQ5 channels are higher: 5.5 µM (Søgaard et al. 2001) and 65 µM (Schroeder et al. 2000), respectively.

Currents mediated by the expressed KCNQ1–5 channel subunits differ considerably in their sensitivity to inhibition by TEA (Hadley et al. 2000; Lerche et al. 2000) and therefore TEA sensitivity has been used as an indication of possible subunit composition of native I_M currents (Hadley et al. 2003; Wang et al. 1998). Homomeric KCNQ2 channels have an IC_{50} value for TEA inhibition in the range of 0.16–0.3 mM (Hadley et al. 2000; Shapiro et al. 2000; Wang et al. 1998). The IC_{50} value of TEA for homomeric KCNQ4 channels is 3.0 mM (Hadley et al. 2000). Interestingly, the IC_{50} value for TEA inhibition of I_M in DA VTA neurons found in the present study was 1.56 mM, which lies between the IC_{50} values for TEA of KCNQ2 channels and KCNQ4 channels. Similarly, the Hill slope calculated for the TEA inhibition curves for DA VTA neurons in the present study was 1.58, which is also intermediate between the Hill coefficients reported for KCNQ2 (0.9) and KCNQ4 (2.0) channels (Hadley et al. 2000). Both KCNQ2 and KCNQ4 subunits have been shown to be present in the VTA in immunohistochemical studies (Cooper et al. 2001; Kharkovets et al. 2000). A study in oocytes concluded that coexpression of KCNQ2 and KCNQ4 subunits did not lead to formation of functional heteromeric channels (Kubisch et al. 1999). The IC_{50} for TEA for homomeric KCNQ3 is >30 mM (Hadley et al. 2000). KCNQ1 channels have been detected in heart, kidney, and cochlea, but not in brain (Robbins 2001; Wang et al. 1996; Wangemann 2002). Taken together, these data raise the possibility that both homomeric KCNQ2 and homomeric KCNQ4 channels contribute to I_M in DA VTA neurons.

Although KCNQ3 subunits have not been demonstrated in the VTA, the IC_{50} for TEA inhibition of heteromeric KCNQ2/3 channels is 3.5–3.8 mM (Hadley et al. 2000; Wang et al. 1998), which is close to the IC_{50} for homomeric KCNQ4 channels (3.0 mM). KCNQ3 has also been shown to form heteromeric channels with KCNQ4 when these subunits are coexpressed (Kubisch et al. 1999). Therefore the contribution of KCNQ2/3 and KCNQ3/4 heteromeric channels to I_M in DA VTA neurons cannot be conclusively excluded at this time. The present study shows that 10 mM TEA causes essentially complete inhibition (97.6%) of I_M in DA VTA neurons. By contrast, homomeric KCNQ3, homomeric KCNQ5, and heteromeric KCNQ3/5 channels are very insensitive to TEA with estimated IC_{50} values of >30, 71, and 200 mM, respectively (Hadley et al. 2000; Lerche et al. 2000; Schroeder et al. 2000; Shapiro et al. 2000), making it very unlikely that these channels contribute to I_M in DA VTA neurons.

Hill slopes >1 are generally believed to indicate a cooperativity of binding. The difference between the interaction of TEA with the KCNQ channel compared with XE991 may explain why TEA reduced I_M by 98% and XE991 reduced I_M by 90%. The 8% difference may indicate an additional action of TEA on a KCNQ channel that is related to the cooperativity of binding of TEA (Hill slope 1.58) compared with the lack of cooperativity induced by the lower Hill slope (1.14) seen with XE991. Additional studies will be necessary to investigate this difference in detail.

I_M amplitude in DA VTA neurons was found to be voltage dependent in the present study, similar to native I_M measured in sympathetic ganglion and hippocampal neurons (Brown and Adams 1980; Schweitzer 2000; Shah et al. 2002 Wang et al. 1998). The maximal I_M amplitude in the DA VTA neurons was obtained at −45 mV with the deactivation protocol. The deactivation time constant was voltage dependent in DA VTA neurons, becoming shorter at more hyperpolarized membrane potentials, as has been observed for native I_M currents in other neuronal cell types (Brown and Adams 1980; Passmore et al. 2003; Wang et al. 1998). The time course of I_M deactivation in DA VTA neurons was well fitted with a single exponential and the value of the deactivation time constant was 138 ms at −55 mV. This is in contrast to deactivation of native I_M currents in other types of neurons, which have a biphasic (double-exponential) time course (Passmore et al. 2003; Shah et al. 2002; Wang et al. 1998). The deactivation time constant in DA VTA neurons seems to be closest to the fast component of the deactivation time constant in sympathetic neurons, which was reported to be 145 ms at −50 mV (Wang et al. 1998). The slow deactivation time constant observed in native I_M in other types of neurons is typically >500 ms. The absence of this slow component of deactivation in our experiments could be attributable to a difference in the types of KCNQ channels underlying I_M in DA VTA neurons, as compared with I_M in these other types of neurons. It is possible, however, that we did not detect a slow component in our studies as a result of method-
ological differences; we determined the deactivation time constant from the exponential fit to $I_M$ (inward relaxation) measured with the deactivation protocol (Fig. 1B), whereas some of the other studies had larger $I_M$ and determined the deactivation time constant from tail currents following an activation protocol.

The $I_{Kd}/KCNQ$ antagonist XE991 (10 μM) blocked $I_M$ in DA VTA neurons by about 90%. XE991 was then used in nystatin-perforated patch current-clamp experiments, to study the contribution of $I_M$ to the action potential shape and interspike trajectory in DA VTA neurons. XE991 (1–10 μM) did not affect the threshold, amplitude, or duration (at half-amplitude) of the evoked action potential. XE991 shortened the latency to the first evoked action potential and shortened the ISI. Furthermore, XE991 reduced the amplitude of the fast AHP and slow AHP without affecting the amplitude of the middle component of the AHP, which followed the evoked action potential. XE991 reduction of the slow AHP causes a steeper voltage trajectory, leading to earlier occurrence of the next action potential and a shortening of the ISI.

Although a study has reported that a relatively high concentration of linopirdine, an $I_K$ inhibitor structurally related to XE991, also blocks some types of $K^+$ currents in addition to $I_M$ (Schnee and Brown 1998), it seems unlikely that the relatively low concentrations of XE991 (1–10 μM) used in the present study modulated relevant currents other than $I_M$ and affected spike frequency with the change of AP shape and AHP amplitude. In the present study, $I_h$ (as measured by voltage-sag amplitude) was not significantly affected by 10 μM XE991 (Figs. 4 and 5), indicating that XE991 did not affect $I_h$ at this concentration. Consistent with our result, it has been reported that 30 μM linopirdine does not change $I_h$ in hippocampal neurons (Schnee and Brown 1998). It has been reported that the IC₅₀ values of XE991 for types of $K^+$ channels (eag1, erg1, erg3, Kv1.2) other than KCNQ channels are in the range of 49 to >100 μM (Wang et al. 1998). Kv4.3 channels mediate $I_A$ in midbrain dopamine neurons (Liss et al. 2001) but the IC₅₀ of XE991 for Kv4.3 channels is 43 μM (Wang et al. 1998). Therefore it is unlikely that 1–10 μM XE991 affects types of $K^+$ channels in DA VTA neurons other than KCNQ channels and changes the shape of AP in DA VTA neurons.

Our previous study has shown that 200 nM apamin selectively blocks only the middle component of AHP (100 ms after AP peak) without affecting the fast and slow components of AHP in DA VTA neurons (Brodie et al. 1999a). Thus $I_M$ and SK currents contribute to the AHP differently in DA VTA neurons and thus it is unlikely that SK current is the target of 1–10 μM XE991. This conclusion is supported by the finding that 10 μM XE991 does not affect $I_{AHP}$, which is also mediated by the SK current, in sympathetic neurons (Wang et al. 1998).

Based on our voltage-clamp experiments on $I_M$ in DA VTA neurons and the XE991-induced changes in the spike shape and ISI, the role of $I_M$ in DA VTA neurons can be understood as follows. From the voltage dependency of $I_M$ deactivation, the deactivation time constant is predicted to be smaller than 100 ms at ~70 mV, which corresponds to the membrane potential at the peak of the fast AHP of the evoked action potential in DA VTA neurons. At this negative membrane potential, $I_M$ would contribute only to the fast phase near the peak of the AHP. In the subsequent phase, $I_M$ is likely to be fully deactivated and therefore would not contribute to the middle AHP.

As the membrane potential depolarizes, $I_M$ would be reactivated during the slow AHP, decreasing membrane resistance and delaying the generation of the subsequent action potential. Because the activation time constants of KCNQ2, KCNQ3, and KCNQ4 channels have been reported to be more than 40 ms at +40 mV (Lerche et al. 2000; Selyanko et al. 2000; Søgaard et al. 2001), $I_M$ would not be further activated during the very short time from threshold through the depolarizing phase of the action potential. This could explain why inhibition of $I_M$ with 1–10 μM XE991 did not affect action potential amplitude or duration in DA VTA neurons. The duration of the evoked action potential was prolonged by 30 μM XE991, which suggests a nonspecific effect of XE991 at this high concentration.

In conclusion, the present study indicates that $I_M$ is active near the resting membrane potential to decrease excitability and during the slow AHP to decrease the ISI in DA VTA neurons. In addition to the physiological role of $I_M$, KCNQ channels are a potential target for modulation by drugs. Ethanol has been shown to reduce $I_M$ in hippocampal neurons (Moore et al. 1990) and, in our preliminary studies, in DA VTA neurons (Koyama and Appel 2004). It is possible that this action could contribute to the excitatory effect of ethanol on DA VTA neurons and therefore to its reinforcing effects in the mesolimbic pathway.

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REFERENCES


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