Depression by isoflurane of the action potential and underlying voltage-gated ion currents in isolated rat neurohypophysial nerve terminals

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**Abbreviations:** AHP, afterhyperpolarization; AP, action potential; 4-AP, 4-aminopyridine; ANOVA, analysis of variance; BK, big Ca\(^{2+}\)-activated K\(^+\) current; \(I_{Ca}\), Ca\(^{2+}\) current; \(I_K\), K\(^+\) current; \(I_{Na}\), Na\(^+\) current; NHP, neurohypophysial; TEA, tetraethylammonium chloride; IC\(_{50}\), concentration for 50% inhibition; \(V_{1/2}\), voltage of half-maximal activation
Abstract

We characterized the effects of the volatile anesthetic isoflurane on the ion currents that contribute to the action potential (AP) in isolated rat neurohypophysial (NHP) nerve terminals using patch clamp electrophysiology. Mean resting membrane potential and AP amplitude was -62.3±4.1 mV and 69.2±2.9 mV, respectively in NHP terminals. Two components of outward K\(^+\) current (\(I_K\)) were identified in voltage-clamp recordings: a transient \(I_K\) and a sustained \(I_K\) with minimal inactivation. Some terminals displayed a slowly activating \(I_K\), probably Ca\(^{2+}\)-activated K\(^+\) current (BK). Isoflurane reversibly inhibited AP amplitude and increased AP half-width in normal extracellular Ca\(^{2+}\) (2.2 mM). In high extracellular Ca\(^{2+}\) (10 mM), isoflurane also reduced the afterhypolarization (AHP) peak amplitude. A transient tetrodotoxin-sensitive Na\(^+\) current (\(I_{Na}\)) was the principal current mediating the depolarizing phase of the AP. A slowly-inactivating Cd\(^{2+}\)-sensitive current (probably a voltage-gated Ca\(^{2+}\) current; \(I_{Ca}\)) followed the initial \(I_{Na}\). Isoflurane reversibly inhibited both \(I_{Na}\) and \(I_{Ca}\) elicited by a voltage-stimulus based on an averaged AP waveform. The isoflurane IC\(_{50}\) for AP waveform-evoked \(I_{Na}\) was 0.36 mM. Isoflurane (0.84±0.04 mM) inhibited AP waveform-evoked \(I_{Ca}\) by 37.5±0.16% (p<0.05). The isoflurane IC\(_{50}\) for peak \(I_K\) was 0.83 mM and for sustained \(I_K\) was 0.73 mM, with no effect on the voltage dependence of activation. The results indicate that multiple voltage-gated ion channels (Na\(^+\)>K\(^+\)>Ca\(^{2+}\)) in NHP terminals, though not typical CNS terminals, are inhibited by the volatile general anesthetic isoflurane. The net inhibitory effects of volatile anesthetics on nerve terminal action potentials and excitability result from integrated actions on multiple voltage-gated currents.
Introduction

The molecular and cellular mechanisms of general anesthetics remain unclear despite over a century of research. Modulation of synaptic transmission is the principal neurophysiologic effect of general anesthetics, though the molecular targets for these actions have not been clearly established (Campagna et al., 2003). Considerable evidence indicates that presynaptic targets are important in the depression of excitatory transmission by volatile anesthetics in the CNS (Perouansky and Hemmings, 2003). Characterization of the effects of volatile anesthetics on nerve terminal ion currents is an important step in identifying presynaptic mechanisms that underlie anesthetic actions on nerve terminal excitability and hence neurotransmitter release. However, the inaccessibility of most presynaptic terminals to direct electrophysiological analysis has impeded identification and characterization of presynaptic ion channel targets.

Although they are not typical of small CNS terminals, isolated neurohypophysial (NHP) nerve terminals are useful as a model nerve terminal that is large enough in diameter (5-16 µm) to measure presynaptic ion channel function using patch-clamp electrophysiological techniques (Lemos and Nordmann, 1986). These nerve terminals express voltage-gated Na⁺, K⁺ and Ca²⁺ channels, which are involved in the control of excitability and neuropeptide release (Lemos and Nowycky, 1989; Bielefeldt et al., 1992; Lindau et al., 1992; Turner and Stuenkel, 1998; Wang et al., 1999). Previous studies have shown that voltage-gated \( I_{Na} \) in NHP terminals is inhibited by isoflurane (Ouyang et al., 2003). Presynaptic Ca²⁺ channels are apparently more resistant to volatile anesthetics, as evident in studies of transmitter release from nerve terminals (Schlame and Hemmings 1995; Westphalen and Hemmings, 2003; Wu et al., 2003). Volatile anesthetic effects on
presynaptic voltage-gated $I_k$ have not been studied, though heterologously expressed $K^+$ channels appear to be relatively insensitive (Yamakura et al., 2001). We investigated the effects of the widely used volatile general anesthetic ether isoflurane on action potentials (AP) and underlying voltage-gated ion currents in isolated rat NHP nerve terminals. An action potential-clamp technique employing an averaged AP waveform as the command voltage (Llinas et al., 1982) was used to investigate the contribution of outward ($K^+$) and inward ($Na^+$, $Ca^{2+}$) currents to the AP and their sensitivities to isoflurane. This approach demonstrates that volatile anesthetics likely modulate neurotransmitter release by effects on multiple nerve terminal ion channels that interact to depress AP amplitude and increase AP duration.
Methods

Materials

Isoflurane was obtained from Abbott Laboratories (North Chicago, IL). Amphotericin B, tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), and tetrodotoxin were purchased from Sigma (St. Louis, MO). Male Sprague-Dawley rats (260-300 g) were from Charles River Laboratories (Wilmington, MA).

Nerve Terminal Preparation

Experimental protocols were approved by the IACUC of Weill Medical College of Cornell University. NHP terminals were prepared as described previously (Ouyang et al., 2003) with minor modifications. Rats were anesthetized with 80% CO$_2$/20% O$_2$ and decapitated; this technique avoids hypoxemia and exposure to used anesthetic drugs (unpublished observations). The neurohypophysis was separated from pars anterior and pars intermedia of the pituitary, and gently homogenized in a solution containing (in mM): sucrose, 270; HEPES-Tris, 10; K-EGTA, 0.01, pH 7.25 using a 0.5 ml Teflon/glass homogenizer. The NHP homogenate was pipetted into a plastic Petri dish (35 × 10 mm), and allowed to settle for 5-8 min. The Petri dish with dissociated NHP nerve terminals was placed onto the stage of a Nikon ECLIPSE TE300 inverted microscope equipped with Hoffmann interference contrast optics (Melville, NY, USA) and superfused (2–3 ml/min) with modified Locke’s solution consisting of (in mM) NaCl, 145; KCl, 5; CaCl$_2$, 2.2; MgCl$_2$, 1; HEPES, 10; and D-glucose, 2; pH 7.30 with NaOH at room temperature (24-26°C). Terminals with diameters of 5-16 µm were identified by their bright refraction, smooth spherical shape, and absence of a nucleus. Isolated NHP terminals were readily
distinguished from the larger and nucleated pars intermedia cells. Large terminals (diameter of 11-16 µm) were selected for study in order to minimize variability in individual current densities, channel kinetics, and AP shape.

**Electrophysiological Recordings**

Recording pipettes were made from borosilicate glass capillaries (Drummond Scientific, Broomall, PA) using a P-97 micropipette puller (Sutter Instruments, Novato, CA) and polished (MF-200 microforge, World Precision Instrument, Sarasota, FL). Pipette tips were fire coated with SYLGARD (Dow Corning Corporation, Midland, MI) to lower background noise and reduce pipette capacitance.

In order to avoid rundown of ion currents and sweep-to-sweep AP amplitude decline observed with whole terminal patch-clamp, a perforated patch-clamp technique was used (Wang et al., 1999). Pipette resistance in the bath was 3-7 MΩ, and seal resistance was 1-5 GΩ. Pipettes were filled with a pipette solution containing (in mM): NaCl 10; K-glutamate, 135; CaCl₂, 2; MgCl₂, 1; HEPES, 10; D-glucose, 2; pH 7.25 with KOH plus amphotericin B (240-300 µg/ml); addition of ATP or GTP to the recording pipette did not alter control recordings or anesthetic effects (data not shown). Terminals were attached with GΩ seal under voltage-clamp mode to record K⁺ current, and then switched to current-clamp mode for AP recording elicited by 0.5 ms stimulus pulses of 0.2-0.8 nA. In current clamp recording, an additional channel measured membrane current (as shown in Figs. 3C and 4D) during membrane potential changes. This is not a typical voltage-clamp method (no holding potential), but it provides useful information on the effects of isoflurane on ion currents underlying the AP. For recording voltage-gated Na⁺ and Ca²⁺ currents in AP waveform-clamp mode, the amphotericin B pipette solution was changed
to (mM): NaCl 10; Cs-glutamate 135; CaCl₂ 2; MgCl₂ 1; HEPES, 10; D-glucose 2; pH 7.25 with CsOH. An average AP waveform (see below) was used as the command voltage. Capacitance and series resistance (60-85%) were compensated on-line, and membrane potentials were corrected on-line for the liquid junction potential (16 mV in normal Locke’s solution and 17 mV in high Ca²⁺ Locke’s solution). To maintain the osmolarity, high Ca²⁺ Locke’s solution contained (in mM): NaCl, 135; KCl, 5; CaCl₂, 10; MgCl₂, 1; HEPES, 10; and D-glucose, 4; pH 7.30 with NaOH.

Current and voltage data were collected using an Axon 200B amplifier, digitized via a Digidata 1321A interface, and transferred to a Pentium III PC for analysis by pClamp 8.2 software (Axon Instruments, Burlingame, CA). Whole-terminal currents were sampled at 10 kHz and filtered at 1-3 kHz. Locke’s solution perfused the chamber at 0.10-0.15 ml/min. Isoflurane was diluted into Locke’s solution from stock solutions (10-12 mM isoflurane in Locke’s solution, prepared 12-24 h prior to experiments) into airtight glass syringes, and applied locally to attached terminals at 0.05 ml/min through a 0.15 mm diameter perfusion pipet (30-40 μm away from patched terminals) using an ALA-VM8 pressurized perfusion system (ALA Scientific, Westbury, NY). Concentrations of isoflurane in the recording chamber were determined by local sampling of the perfusate at the site of the recording pipette tip and analysis by gas chromatography (Ratnakumari and Hemmings 1998). With this method of application the average isoflurane loss is 15% of the syringe concentration with local application.

**Data Analysis**

Voltage-dependent activation curves were fitted to a Boltzmann equation of the form 

\[ \frac{G}{G_{\text{max}}} = \frac{1}{1 + e^{(V_{1/2} - V)/k}} \]

where \( \frac{G}{G_{\text{max}}} \) is the normalized fractional conductance,
$G_{\text{max}}$ is the maximum conductance, $V_{1/2}$ is the voltage for half-maximal activation, and $k$ is the slope factor. Na$^+$ conductance ($G_{\text{Na}}$) was calculated using the equation: $G_{\text{Na}} = I_{\text{Na}}/(V_t-V_r)$, where $I_{\text{Na}}$ is the peak Na$^+$ current, $V_t$ is the test potential and $V_r$ is the calculated reversal potential ($E_{\text{Na}} = +68 \text{ mV}; E_{\text{K}}=-85 \text{ mV}$).

Data were analyzed using pCLAMP 8.2 (Axon Instruments), Prism 3.02 (Graph-Pad Software Inc., San Diego, CA, USA) and SigmaPlot 6.0 (SPSS Science Software Inc., Chicago, IL, USA). AP data were analyzed using Synaptosoft 5.4.17 (Decatur, GA, USA). Data are expressed as mean±SD. Concentration-effect data were fit to a sigmoid function to calculate IC$_{50}$ values. Statistical significance was assessed by ANOVA, paired t-test or unpaired t-test as appropriate; p<0.05 was considered statistically significant.
Results

Properties of nerve terminal action potentials and K⁺ current

No spontaneous APs were observed in isolated NHP terminals in current-clamp recordings. Nerve terminal resting membrane potential was -62.3±4.1 mV (diameter=13.6±2.5 µm; n=40). APs were elicited by single short (0.5 ms) current injections (0.3-0.8 nA), and were substantially inhibited by 0.5 µM tetrodotoxin in the superfusion solution (Fig. 1A). AP amplitude was 69.2±2.9 mV, AP half-width was 3.9±0.6 ms, and AHP peak amplitude was -3.6±2.8 mV in normal Locke’s solution (2.2 mM CaCl₂; n=40). With high Ca²⁺ (10 mM) Locke’s solution, AP amplitude was enhanced (from 66.6±5.4 mV to 80.4±10.8 mV; n=5, p<0.05), as was AHP amplitude (from -6.1±2.1 mV to -15.0±4.2 mV; p<0.01, n=5). High Ca²⁺ did not significantly affect AP half-width (4.6±1.4 ms vs. 3.5±1.6 ms for normal Ca²⁺; p>0.05, n=5) or resting membrane potential (-60.8±4.0 mV vs. -58.9±4.8 mV for normal Ca²⁺; p>0.05, n=5; see also similar data in Table 1).

Two components of outward K⁺ current (I_K) were present in voltage-clamp recordings: a transient I_K and a sustained I_K that exhibited minimal inactivation (Fig. 1B). A Ca²⁺-activated K⁺ current, probably mediated by the BK channel (Wang et al., 1992), was also observed (see below). Average peak I_K amplitude was 1460±210 pA and sustained I_K (measured at the end of the depolarizing pulse) was 920±160 pA (n=27). Peak and sustained I_K were essentially completely blocked by a combination of the selective transient K⁺ channel (K_A) blocker 4-AP (5 mM) and the non-selective K⁺ channel blocker TEA (5 mM; Fig. 1B). TEA (5 mM) alone blocked most of the sustained and part of the transient I_K (data not shown); 4-AP (5 mM) alone completely blocked the transient I_K and
partially blocked the sustained $I_K$ (Fig. 1B). Action potentials were inhibited by TEA plus 4-AP and lengthened (~60 ms) with delayed repolarization by 5 mM 4-AP alone (Fig. 1A).

**Effects of isoflurane on the action potential**

Using current-clamp recording, isoflurane concentration-dependently inhibited AP amplitude and increased half-width in either normal or high extracellular Ca$^{2+}$ solution (Fig. 2A, Table 1). Isoflurane significantly reduced the nerve terminal AP amplitude by 18% at 0.41±0.05 mM (n=16) and by 34% at 0.82±0.06 mM (n=14), equivalent to 1.2 and 2.3 times, respectively, the effective aqueous isoflurane EC$_{50}$ for general anesthesia in rats of 0.35 mM (Taheri et al., 1991); this aqueous concentration for volatile anesthetic potency is temperature insensitive (Franks & Lieb, 1996). This effect of isoflurane was reversible with washout by superfusion of control solution for 1 min. Isoflurane also reversibly depolarized resting membrane potential (Fig. 3A, Table 1). To assess the contribution of voltage-gated ion channel inactivation as a result of the slight depolarization to the depressant effects of isoflurane, experiments were also performed with the membrane potential held at the control level in the presence of isoflurane. Comparable depression of AP amplitude was observed when the resting potential was held at control (-65 mV) or slightly depolarized (-55 mV) potentials (67% vs. 63% of control respectively; n=4, Fig. 3). The AP amplitude was significantly enhanced by high Ca$^{2+}$ (10 mM). In the presence of high external Ca$^{2+}$, isoflurane (0.82±0.07 mM) reversibly inhibited AP amplitude (p<0.001, n=4) and AHP peak amplitude (p<0.001, n=4), and increased AP half-width (Table 1, Fig. 4A,B; p<0.01, n=4).

**Effects of isoflurane on action potential waveform-evoked ion currents**
The action potentials recorded from 7 NHP terminals were averaged using pClamp 8.2. This averaged AP waveform was used as a voltage stimulus to study the effects of isoflurane on the underlying ion currents (action potential-clamp). A transient tetrodotoxin-sensitive $I_{Na}$ was the principal current mediating the depolarizing phase of the AP. During the repolarizing phase, a slowly-inactivating voltage-gated Ca\(^{2+}\) current (blocked by 400 $\mu$M external Cd\(^{2+}\); data not shown) followed the initial transient $I_{Na}$ in 20-30\% of terminals, and was enhanced in high Ca\(^{2+}\) solution (Fig. 5). Isoflurane reversibly inhibited both currents (Fig. 5C; Fig. 6A). The isoflurane IC\(_{50}\) for AP waveform-evoked $I_{Na}$ was 0.36 mM (Fig. 6B). Isoflurane (0.84±0.04 mM) inhibited the AP waveform-evoked $I_{Ca}$ by 37.5±0.16\% (Fig. 5C; p<0.05, n=3).

**Effects of isoflurane on potassium current**

The effects of isoflurane on the AP and $I_{K}$ were observed in the same terminals by switching between current clamp and voltage clamp recording modes, respectively. Isoflurane concentration-dependently and reversibly inhibited peak $I_{K}$ (IC\(_{50}\) = 0.83 mM) and sustained $I_{K}$ (IC\(_{50}\) = 0.73 mM; Fig. 7). The voltage dependence for inhibition of $I_{K}$ by isoflurane was determined by plotting normalized $I_{K}$ amplitude vs. test potential (Fig. 8A,B). Inhibition of peak $I_{K}$ by isoflurane was not voltage-dependent, but inhibition of sustained $I_{K}$ was greater at more positive test potentials. Isoflurane produced a small hyperpolarizing shift in the voltage dependence of sustained $I_{K}$ activation (Fig. 8C,D; Table 2). In the presence of high Ca\(^{2+}\), isoflurane (0.85±0.04 mM) inhibited peak $I_{K}$ by 37.5±0.09\% (p<0.01, n=4) and sustained $I_{K}$ by 66.7±0.12\% (p<0.001, n=4), and totally inhibited the enhanced Ca\(^{2+}\)-activated K\(^{+}\) current (Fig. 4D).
Discussion

The ether anesthetic isoflurane inhibited several prominent ion currents involved in determining excitability and shaping the action potential in isolated neurohypophysial nerve terminals. Action potential generation in isolated NHP terminals depends largely on fast inward Na$^+$ current during the depolarizing phase and outward A-type K$^+$ current in the repolarizing phase; Ca$^{2+}$ current and Ca$^{2+}$-activated K$^+$ current also contribute to the AP (Faber and Sah, 2003). The basic properties of the current pulse-induced APs reported here are similar to those reported using intraterminal recordings from NHP terminals in situ activated by electrical stimulation of the neural stalk (Bourque, 1990), which included a resting potential of -60 mV and AP amplitude of 72 mV. Isoflurane reversibly inhibited the amplitude and increased the duration of the AP and inhibited peak and sustained voltage-gated K$^+$ currents, as well as Na$^+$ and Ca$^{2+}$ currents activated by an action potential waveform voltage protocol. The effects of isoflurane on $I_{Na}$ occurred at clinically relevant concentrations ($IC_{50}=0.36 \text{ mM} \approx 1.0 \text{ MAC}$), while effects on $I_K$ (peak $IC_{50}=0.83 \text{ mM}$; sustained $IC_{50}=0.73 \text{ mM}$) and $I_{Ca}$ were less potent compared to isoflurane potency ($EC_{50}$) for general anesthesia in rats (aqueous MAC=0.35 mM Taheri et al., 1991). Thus, isoflurane inhibits a number of presynaptic voltage-gated ion currents, most potently $I_{Na}$. These currents are all mediated by members of the voltage-gated ion channel superfamily (Nelson et al., 1999), which suggests a role for a common feature of these channels in their sensitivities to isoflurane. Whether the reduced action potential amplitude by isoflurane results in reduced transmitter release in NHP terminals as volatile anesthetics do in glutamatergic and GABAergic CNS terminals (Perouansky and Hemmings, 2003) will require further investigation.
Effects of isoflurane on the action potential

Isoflurane inhibited AP and AHP amplitude and prolonged AP duration (increased half-width) in both normal and high external Ca$^{2+}$ superfusion solution at concentrations in the clinical range. The reduction in AP amplitude results from reduced $I_{Na}$, the driving current for the depolarizing phase of the AP. A role for presynaptic anesthetic effects is also supported by a study of the large calyceal synapse in rat brainstem (Wu et al., 2004) in which isoflurane inhibited AP-evoked excitatory postsynaptic current (EPSC) (IC$_{50}$=0.49 mM); this effect was attributed largely to inhibition of AP amplitude based on AP waveform simulation studies. The degree of AP depression by 0.7 mM isoflurane in the calyceal synapse (5.5%) was less than that produced by 0.41±0.05 mM (n=16) isoflurane (18%) in the NHP terminal, which may reflect the larger calyceal AP amplitude (106 mV; Wu et al., 2004), distinct ion channel complements and/or other factors that determine nerve terminal properties and anesthetic sensitivity. The increase in AP half-width by isoflurane most likely results from inhibition of $I_{K}$ (Suzuki et al., 2002). Increased AP duration due to inactivation of $I_{K}$ resulting from high frequency-stimuli contributes to frequency-dependent facilitation of [Ca$^{2+}$]$_i$ changes (Jackson et al., 1991), which may actually mitigate the reduced release probability due to depression of $I_{Na}$ and $I_{Ca}$. However, increased AP duration may also reduce the ability of the nerve terminal to respond to high frequency stimuli, enhancing the depression in synaptic transmission produced by depression of AP amplitude. The net effects of isoflurane on neuropeptide release from NHP terminals requires further investigation.

Isoflurane slightly depolarized the resting membrane potential in NHP terminals. Depolarization by isoflurane has also been reported in smooth muscle cells (Wilde, 1996).
Since resting membrane potential depends primarily on permeability to $K^+$ (Hille, 2001), depolarization of NHP terminals by isoflurane is likely due to $K^+$ channel blockade. The depolarizing effect of isoflurane was insufficient alone to explain the depression in AP amplitude, as indicated by experiments in which the resting membrane potential was maintained at control values. The observations that isoflurane does not depolarize the calyceal (Wu et al., 2004) or NHP nerve terminal suggest that these terminals lack isoflurane-sensitive $K^+$ channels. Evidence from several nerve terminal preparations (Schlame & Hemmings, 1995; OuYang et al., 2003; Wu et al., 2004) suggests that inhibition of presynaptic bouton APs is sufficient to inhibit transmitter release.

**Effects of isoflurane on nerve terminal $Na^+$ and $Ca^{2+}$ currents**

Voltage-gated $Na^+$ channel activation is required for the generation and propagation of the AP and nerve terminal depolarization, which leads to activation of voltage-gated $Ca^{2+}$ channels, $Ca^{2+}$ entry and $Ca^{2+}$-dependent neurotransmitter release from nerve terminals (Tibbs et al., 1989; Nicholls, 1993; Hille, 2001). Using an AP waveform voltage clamp technique, transient voltage-gated $Na^+$ and $Ca^{2+}$ currents activated during the depolarizing and repolarizing phases of the AP, respectively, were inhibited by isoflurane. The $IC_{50}$ of isoflurane for $I_{Na}$ (0.36 mM at a resting potential of -65 mV) is lower than $IC_{50}$ values determined using voltage clamp protocols in the same preparation at more negative holding potentials ($IC_{50}$=0.45 mM at -70 mV; $IC_{50}$=0.56 mM at -90 mV; Ouyang et al., 2003). Enhanced sensitivity of $Na^+$ channels to isoflurane at more depolarized potentials results from the greater inactivation of $Na^+$ channels at more depolarized potentials due to a hyperpolarizing shift in the voltage-dependence of $Na^+$ channel inactivation produced by volatile anesthetics (Rehberg et al., 1996; Ouyang et al.,
2003), but may also reflect distinct modes of activation by AP-waveform vs. voltage pulse. The specific Na\(^+\) channel isoforms present in NHP terminals have not been identified. Magnocellular neurons in the supraoptic nucleus, which send axons to the neurohypophysis, express both Nav1.2 and Nav1.6 α subunits (Tanaka et al., 1999), which may also be present in NHP nerve terminals. Heterologously expressed Nav1.2, Nav 1.4 and Nav 1.6 channels are inhibited by isoflurane and other volatile anesthetics at clinically relevant concentrations when held at resting membrane potentials (Rehberg et al., 1996; Shiraishi & Harris, 2004). The Ca\(^{2+}\) current observed in high Ca\(^{2+}\) solution is less sensitive to isoflurane than is \(I_{\text{Na}}\). This is consistent with the relative resistance of presynaptic Ca\(^{2+}\) channels to isoflurane detected in neurochemical assays of neurotransmitter release (Schlame and Hemmings 1995; Westphalen and Hemmings, 2003) and exocytosis (Wu et al., 2004; Hemmings et al., 2004).

**Effects of isoflurane on K\(^+\) current**

Potassium channels are important in regulating neuronal excitability and in shaping the AP. Since these properties determine neurotransmitter release, K\(^+\) channels are potential targets for the presynaptic effects of anesthetics. Isoflurane inhibited both peak and sustained \(I_K\), though the potencies were less than that for inhibition of \(I_{\text{Na}}\). These effects are consistent with the observation by Yamakura et al. (2001) that 0.6 mM isoflurane slightly inhibited a voltage-gated K\(^+\) channel (ERG1) in *Xenopus* oocytes. We observed a small hyperpolarizing shift in the activation curve of \(I_K\) (sustained > peak), as has been observed in ventricular myocytes (Suzuki et al., 2003). Isoflurane depressed K\(^+\) current in the AP repolarization phase and depressed the AHP peak, which is probably mediated by BK channels. In addition, depression of the AHP by isoflurane may be
secondary to the partial blockade of Ca$^{2+}$ influx during the AP. Inhibition by isoflurane of K$^+$ current may retard repolarization of the membrane potential and thereby contribute to its depressant effects in nerve terminal firing by slowing AP frequency. But inhibition of AHP by isoflurane may decrease the interburst interval, which tends to facilitate terminal firing and may also hamper recovery of voltage-gated Na$^+$ channels. In neocortical interneurons, which have fast firing properties as do NHP terminals (Erisir et al., 1999), several K$^+$ currents participate in the function of brief action potentials to reduce the amount of Na$^+$ channel inactivation and generate a large AHP to facilitate Na$^+$-channel recovery from inactivation. The net effect of isoflurane on NHP terminal firing will depend on the interaction of multiple intrinsic mechanisms given the multiple presynaptic target sites.

The reported effects of general anesthetics on voltage-gated K$^+$ channels are variable (Sonner et al., 2003). Inhibition of voltage-gated K$^+$ currents by general anesthetics has been reported (Kulkarni et al., 1996; Friederich and Urban, 1999). Isoflurane hyperpolarizes rat vascular smooth muscle, which is attributed in part to enhanced opening of Ca$^{2+}$-activated and ATP-sensitive K$^+$ channels, but not voltage-gated or inward rectifier K$^+$ channels (Kokita et al., 1999). Suzuki et al. (2002) reported biphasic effects of isoflurane on the cardiac action potential in guinea pig ventricular myocytes, with prolongation of AP duration at low concentrations attributed to inhibition of delayed rectifier K$^+$ current, and shortened AP duration at high concentrations attributed to inhibition of $I_{Ca}$. Thus, anesthetic actions on the large and diverse K$^+$ channel family appear to be subtype dependent. The specific K$^+$ channel subtypes involved in mediating
NHP $K^+$ currents have not been identified. The net inhibitory effects of isoflurane on $K^+$ currents may involve effects, possibly opposing, on multiple $K^+$ channel subtypes.

In summary, our findings in isolated NHP nerve terminals indicate that voltage-gated $Na^+$, $K^+$, and $Ca^{2+}$ channels that regulate and define the presynaptic action potential are targets of the volatile general anesthetic isoflurane. Net inhibitory effects on nerve terminal excitability result from integration of these actions on multiple inward and outward currents, with $I_{Na}$ being most sensitive to isoflurane. These findings add to a growing body of evidence implicating depression of presynaptic action potential and transmitter release in the actions of volatile anesthetics (MacIver et al., 1996, Mikulek et al., 1998; Perouansky & Hemmings, 2003).

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**Footnote**

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Legends for Figures

Fig. 1 Properties of action potentials and K⁺ currents observed in a single neurohypophysial nerve terminal. A: Current clamp recording of action potentials. Tetrodotoxin (TTX) substantially inhibited the action potential (AP), demonstrating that Na⁺ current is critical for generation of the AP. 4-AP (5 mM) prolonged AP duration, indicating that an A-type K⁺ current mediates AP repolarization. Addition of TEA and 4-AP together (5 mM each) inhibited the AP, indicating that block of multiple K⁺ currents inhibits AP generation. TEA: tetraethylammonium; 4-AP: 4-aminopyridine. B: Voltage clamp recording of voltage-activated K⁺ current. Depolarization-evoked $I_K$ consisted of a small transient component blocked by 4-AP (5 mM) and a sustained component blocked by 4-AP and TEA (5 mM each). Terminal diameter=13.5 µm.

Fig. 2 Inhibition of action potential and $I_K$ by isoflurane in a single neurohypophysial terminal. A: Representative traces showing reversible inhibition of the AP by 0.76 mM isoflurane. Note reduced amplitude and greater half-width produced by isoflurane. The membrane potential was held at control level during application of isoflurane. B: Membrane current changes produced by the application of isoflurane. The initial positive peak is the injected current (0.5 ms, 0.5 nA) followed by fast inward (probably Na⁺ current) and outward (probably A-type current) currents. Isoflurane inhibited both the inward and outward currents. A and B are plotted with the same time scale. C: The same data as B with an expanded timescale to better display the reduction in both inward and outward currents with application of isoflurane. D: Representative traces showing...
inhibition by 0.76 mM isoflurane of voltage-gated $I_K$. Both transient and sustained $I_K$ were inhibited reversibly. Terminal diameter=14.2 μm.

Fig. 3 Inhibition of action potential by isoflurane at different membrane potentials in a single neurohypophysial terminal. A: Representative traces showing inhibition by 0.81 mM isoflurane of the AP at resting membrane potential (-65 mV) and depolarized potential (-55 mV). Note the reduced amplitude and increased half-width in both. B: Membrane current changes produced by the application of isoflurane at resting membrane potential and a more depolarized potential. C: The same recording as B with an expanded timescale to better display the similar potent inhibition of fast inward and outward currents by isoflurane. Terminal diameter=15.3 μm.

Fig. 4 Effects of high Ca$^{2+}$ on the action potential (A-C) and $I_K$ (D) in a single neurohypophysial terminal. A and B are the same recording with an expanded timescale in A to better compare with the membrane current changes shown in C (same timescale as A). The membrane potential was held at control level during application of isoflurane. High Ca$^{2+}$ enhanced the fast inward and outward currents, which are responsible for the depolarizing and repolarizing phases of the AP, respectively. The AHP peak and area were also increased. D. High external Ca$^{2+}$ induced a substantial increase in sustained $I_K$ (probably mediated by a BK channel) and a smaller increase in the initial A-type current. Isoflurane (0.86 mM) inhibited $I_{Na}$, $I_K$ (C,D), and AP amplitude (A,B). Terminal diameter=15.5 μm.
Fig. 5 Currents recorded by action potential waveform clamp in high and low Ca^{2+}. Neurohypophysial terminals were superfused with Locke’s solution containing 10 mM Ca^{2+} (A, C, D) or 2.2 mM Ca^{2+} (B). A, B: Current traces showing isolation of Ca^{2+} current by application of 500 nM tetrodotoxin. The isolated Ca^{2+} current was evident in high Ca^{2+} during AP repolarization (A), and is much less evident in normal Ca^{2+} solution (B). C: Isoflurane (0.86 mM) partially inhibited the Ca^{2+} current recorded in high external Ca^{2+} in presence of tetrodotoxin (500 nM). D: The action potential control stimulus. Terminal diameter=13.6 µm in panel A, C, and 13.2 µm in panel B.

Fig. 6 Inhibition of action potential waveform-evoked Na^{+} current by isoflurane. A representative current trace is shown in A (top) above the AP waveform stimulus shown on the same time scale. A concentration-effect curve is shown in B: Inhibition by isoflurane concentrations of 0.1 mM and greater was statistically significant versus control (p<0.05, ANOVA; n=3-6).

Fig. 7 Inhibition of I_{K} by isoflurane. Representative recordings (A) and concentration-effect curves (B) for inhibition of peak and sustained I_{K} by isoflurane are shown (n=4-15). There is no statistical difference between the curves for inhibition of peak and sustained I_{K} by isoflurane. Inhibition by isoflurane concentrations of 0.1 mM and greater was statistically significant versus control (p<0.05, ANOVA, n=4-15).
Fig. 8 **A,B**: Current-voltage relationship of peak and sustained $I_K$ in the absence or presence of isoflurane. Voltage-dependence of inhibition by isoflurane (0.83±0.06 mM, n=15) was observed for the sustained (B) but not peak (A) $I_K$. **C,D**: Voltage-dependence of activation of peak and sustained $I_K$ in the absence or presence of isoflurane (0.42±0.05 mM and 0.81±0.05 mM, n=10). Isoflurane did not significantly affect the activation curves, fitted to a Boltzmann function. Data expressed as mean±SD; *, p<0.05; **, p<0.01; ***, p<0.001 versus control by ANOVA.
### Table 1. Effects of isoflurane on action potential properties

<table>
<thead>
<tr>
<th></th>
<th>2.2 mM Ca$^{2+}$</th>
<th>10 mM Ca$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP amplitude (mV)</td>
<td>AP half-width (ms)</td>
</tr>
<tr>
<td>Control</td>
<td>64.5 ± 7.4</td>
<td>4.1 ± 0.8</td>
</tr>
<tr>
<td>Isoflurane 0.41±0.05 mM</td>
<td>52.7 ± 7.1**</td>
<td>5.4 ± 0.9**</td>
</tr>
<tr>
<td>Isoflurane 0.82±0.06 mM</td>
<td>42.8 ± 7.2***</td>
<td>7.3 ± 3.1***</td>
</tr>
</tbody>
</table>

AP, action potential; RP, resting membrane potential; AHP, afterhyperpolarization. *, p<0.05; **, p<0.01; ***, p<0.001 vs. control using one way ANOVA with Dunnett’s post hoc test (n=4-16).
Table 2. Effects of isoflurane on K⁺ current activation

<table>
<thead>
<tr>
<th></th>
<th>Peak current</th>
<th>Sustained current</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{1/2}$ (mV)</td>
<td>$k$</td>
</tr>
<tr>
<td>Control</td>
<td>-6.3 ± 0.2</td>
<td>21.6 ± 0.2</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>-6.0 ± 0.1</td>
<td>21.6 ± 0.1</td>
</tr>
<tr>
<td>0.42±0.05 mM</td>
<td>-8.8 ± 0.1*</td>
<td>22.2 ± 0.1</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>-8.8 ± 0.1*</td>
<td>22.2 ± 0.1</td>
</tr>
</tbody>
</table>

$V_{1/2}$, voltage of half-maximal activation of peak or sustained K⁺ current; $k$, slope factor (mean ± SD, n=10-11). *, p<0.05; **, p<0.01; vs. control using one way ANOVA with Dunnett’s post hoc test.