Impairment of Hyperpolarization-Activated, Cyclic Nucleotide-Gated Channel Function by the Intravenous General Anesthetic Propofol


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Received June 29, 2005; accepted July 18, 2005

ABSTRACT

Propofol (2,6-diisopropylphenol) is a widely used intravenous general anesthetic, which has been reported to produce bradycardia in patients at concentrations associated with profound sedation and loss of consciousness. Hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels conduct a monovalent cationic current Ih (also known as Iq or If) that contributes to autorhythmicity in both the brain and heart. Here we studied the effects of propofol on recombinant HCN1, HCN2, and HCN4 channels and found that the drug inhibits and slows activation of all three channels at clinically relevant concentrations. In oocyte expression studies, HCN1 channel activation was most sensitive to slowing by propofol (EC50 values of 5.6 ± 1.0 μM for fast component and 31.5 ± 7.5 μM for slow component). HCN1 channels also showed a marked propofol-induced hyperpolarizing shift in the voltage dependence of activation (EC50 of 6.7 ± 1.0 μM) and accelerated deactivation (EC50 of 4.5 ± 0.9 μM). Furthermore, propofol reduced heart rate in an isolated guinea pig heart preparation over the same range of concentrations. These data suggest that propofol modulation of HCN channel gating is an important molecular mechanism that can contribute to the depression of central nervous system function and also lead to bradyarrhythmias in patients receiving propofol during surgical anesthesia.

Propofol (2,6-diisopropylphenol) is a widely used intravenous hypnotic agent that is used to produce both mild to moderate sedation as well as the induction and maintenance of general anesthesia (Reves et al., 2005). An undesirable side effect associated with its use is bradycardia, including asystole, with a reported bradycardia-related death rate of 1.4 per 100,000 patients (Tramèr et al., 1997). Oscillations entrained by the intrinsic electrical activity of neurons in thalamocortical networks are thought to contribute to different levels of consciousness (Steriade and Llinás, 1988; Llinás and Paré, 1991; Bal and McCormick, 1997; Steriade, 2000), and comparable intrinsic activity in cardiac pacemaker cells is thought to establish baseline heart rate (Schram et al., 2002).

One ionic current has been identified in both neurons and cardiac pacemaker cells that appears to regulate this intrinsic electrical activity. Depending on the tissue in which it was characterized, this current has been termed Iq, Iq, or Ih (Pape, 1996; Ludwig et al., 1999a; Kaupp and Seifert, 2001; Robinson and Siegelbaum, 2003). These currents are conducted by hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels, which are voltage-activated cation channels that belong to the voltage-gated K+ channel superfamily (Kaupp and Seifert, 2001; Robinson and Siegelbaum, 2003). Genes coding for four distinct channel isoforms have been cloned (HCN1–4), and HCN channel transcripts and proteins are widely and variably distributed throughout the mammalian central nervous system (Monteggia et al., 2000; Santoro et al., 2000; Notomi and Shigemoto, 2004) and in cardiac sinoatrial node and Purkinje cells (Ludwig et al., 1999b, 2003; Santoro et al., 2000; Notomi and Shigemoto, 2004) and in cardiac sinoatrial node and Purkinje cells (Ludwig et al., 1999b, 2003; Santoro et al., 2000; Notomi and Shigemoto, 2004) and in cardiac sinoatrial node and Purkinje cells (Ludwig et al., 1999b, 2003; Santoro et al., 2000; Notomi and Shigemoto, 2004) and in cardiac sinoatrial node and Purkinje cells (Ludwig et al., 1999b, 2003; Santoro et al., 2000; Notomi and Shigemoto, 2004) and in cardiac sinoatrial node and Purkinje cells (Ludwig et al., 1999b, 2003; Santoro et al., 2000; Notomi and Shigemoto, 2004) and in cardiac sinoatrial node and Purkinje cells (Ludwig et al., 1999b, 2003; Santoro et al., 2000; Notomi and Shigemoto, 2004) and in cardiac sinoatrial node and Purkinje cells (Ludwig et al., 1999b, 2003; Santoro et al., 2000; Notomi and Shigemoto, 2004) and in cardiac sinoatrial node and Purkinje cells (Ludwig et al., 1999b, 2003; Santoro et al., 2000; Notomi and Shigemoto, 2004) and in cardiac sinoatrial node and Purkinje cells (Ludwig et al., 1999b, 2003; Santoro et al., 2000; Notomi and Shigemoto, 2004) and in cardiac sinoatrial node and Purkinje cells (Ludwig et al., 1999b, 2003; Santoro et al., 2000; Notomi and Shigemoto, 2004).
In central neurons, $I_h$ contributes to resting membrane potential, action potential firing, dendritic integration, neuronal automaticity, and temporal summation and determines periodicity and synchronization of oscillations in a number of neuronal networks (Robinson and Siegelbaum, 2003). In cardiac pacemaker cells, $I_f$ contributes to the cardiac pacemaker depolarization (Yanagihara and Irisawa, 1980; DiFrancesco, 1981) that establishes heart rate and rhythm (DiFrancesco, 1993; Irisawa et al., 1993). Both volatile (Tokimasa et al., 1990; Sirois et al., 1998) and intravenous (Wan et al., 2003) anesthetics have been reported to modulate native $I_h$ currents at clinically relevant concentrations.

HCN1 and HCN2 channel conductances have been shown to contribute to the resting membrane potential, action potential firing (excitability), and the stabilization of integrative properties in central neurons (Ludwig et al., 2003; Nolan et al., 2003). In isolated sinoatrial node cells, the HCN1, HCN2, and HCN4 channels generate cardiac pacemaking activity, their relative contributions depending upon species (see Discussion) (Ludwig et al., 2003). Thus, the ability of propofol to modulate $I_h$ in central neurons may contribute to its general anesthetic properties, whereas its effects on $I_h$ in cardiac pacemaker cells may contribute to the bradycardia that has been associated with its use (Tramèr et al., 1997). To clarify the issue of propofol modulation of $I_h$, we studied the effects of propofol on HCN1, HCN2, and HCN4 channels expressed in *Xenopus laevis* oocytes and HEK 293 cells. In addition, the effect of propofol on heart rate was examined using the isolated guinea pig heart preparation.

**Materials and Methods**

**Heterologous Expression in *X. laevis* Oocytes.** cDNA constructs containing murine HCN channels (mHCN1, mHCN2, or mHCN4) (Santoro et al., 1998, 2000) in vectors facilitating in vitro transcription were column-purified, linearized with the appropriate restriction enzyme, purified by precipitation, and then transcribed in vitro using the mMessage mMachine in vitro transcription kit (Ambion, Austin, TX). Transcribed HCN cRNA was purified by precipitation and resuspended in RNase-free water for oocyte injection. *X. laevis* oocytes (Stage V—VI) were obtained from Nasco (Fort Atkin-

**Fig. 1.** A, exemplar traces recorded in HCN1 cRNA-injected *X. laevis* oocytes demonstrating that propofol both reduces current amplitude and slows current activation in a concentration-dependent manner. Top left, scale bars. Bottom left, voltage protocol for all panels. Middle, propofol shifts the voltage dependence of HCN1 activation. B, tail currents from traces in A shown on expanded time scale and normalized for current amplitude. Current scale bar indicates: 0.2 $\mu$A, control; 0.25 $\mu$A, 5 $\mu$M propofol; and 0.13 $\mu$A, 10 $\mu$M propofol. C, cumulative data demonstrating that the greatest degree of current inhibition occurs at physiologic membrane potentials; $n$ = 5–34 oocytes/data point. Symbols for propofol concentration are as follows: $\bigcirc$, 0.1 $\mu$M; $\square$, 0.5 $\mu$M; $\bullet$, 1 $\mu$M; $\bigcirc$, 2 $\mu$M; $\triangle$, 5 $\mu$M; $\bigcirc$, 10 $\mu$M; $\Delta$, 20 $\mu$M; and $\triangle$, 50 $\mu$M. D, normalized peak tail conductance versus prepulse voltage at selected propofol concentrations (0.1, 0.5, 5, and 20 $\mu$M removed for clarity). $\bigcirc$ and $\square$: control data. Boltzmann fits were normalized to the fit-determined value of the amplitude in each case to account for the lack of G/V relationship saturation at the higher propofol concentrations. E, shift in $V_{1/2}$ of activation for HCN1 from control versus all propofol concentrations, calculated from data in D.
son, WI). Oocytes were defolliculated by agitation in Ca2+-free Ringer solution containing collagenase (concentration titrated for activity of each batch; Invitrogen, Carlsbad, CA) for 1 h, washed, and then incubated at 15°C in full Ringer solution before injection with cRNA sufficient to give 1 to 10 μA of current at hyperpolarized potentials for each channel type (typically 0.1–100 ng of cRNA in a volume of 25–50 nl). Oocytes were maintained at 15 to 17°C before two-electrode voltage clamp (TEVC) recording.

**TEVC Oocyte Recording.** HCN currents were recorded 18 to 48 h after injection of HCN subunit cRNA into X. laevis oocytes. Currents were recorded using a OC-725C TEVC amplifier (Warner Instruments, Hamden, CT) and pClamp8.2 software (Axon Instruments, Union City, CA) or Pulse 8.5 (HEKA, Lambrecht/Pfalz, Germany) from oocytes bathed in a small-volume oocyte bath with 2 mM MgCl2 substituting for 2 mM MgCl2, when indicated, to minimize endogenous HCN activation when filled with intracellular solution. The extracellular solution contained 2 mM NaCl, 96 mM KCl, 2 mM MgCl2, 2 mM ATP (sodium salt), 0.1 mM GTP (sodium), 5 mM HEPES, pH 7.2 (KOH) at an osmolarity of 295 to 305 mOsm. Patch pipettes had a resistance of 4 to 8 MΩ when filled with intracellular solution. The extracellular solution contained 110 mM NaCl, 0.5 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES, and 5 mM KCl, pH 7.4 (NaOH), at an osmolarity of 290 to 310 mOsm. For some recordings, an extracellular solution containing 130 mM NaCl was substituted and had no effect on the data.

Cells were voltage-clamped at −40 mV, and 10-mV voltage steps lasting 10 s were applied from −60 to −130 mV. To evaluate the effects of propofol on the HCN2 channel, propofol in dimethyl sulfoxide (DMSO) was added to extracellular solution to yield a final concentration of 5 μM. After a baseline recording, the bath solution containing DMSO (at the same final concentration as for propofol) was switched to the solution containing propofol for a duration of 2 min. After 2 min, the voltage step protocol was repeated in the presence of propofol. The protocol was repeated again after 5- to 10-min washes. Data were digitized and acquired with an EPC10...
amplifier (HEKA) and Pulse software (version 8.65) with no series resistance compensation. Data were filtered at 1.25 kHz with a low-pass Bessel filter and sampled at 2.5 kHz.

Isolated Heart Preparation. Cardiac measurements were made using the isolated guinea pig heart preparation (Levi, 1972). Hearts were perfused with warmed Krebs-Henseleit solution (37°C) at a constant flow of 10 ml/min. The perfusate contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4·7H2O, 24 mM NaHCO3, 1.1 mM KH2PO4, 10 mM glucose, and 2.5 mM CaCl2·2H2O. Propofol (100 mM in DMSO) was diluted with Krebs-Henseleit solution to a stock perfusate solution of 2 mM.

Data Analysis. Analysis of evoked currents was performed as described previously (Santoro et al., 1998, 2000). Analysis was performed with pClamp8.2 software and Origin 6.1 (OriginLab Corp., Northampton, MA), Pulsefit (HEKA, Lambrecht/Pfalz, Germany), Prism 4 (GraphPad Software, Inc., San Diego, CA), and Igor Pro (Wavemetrics, Lake Oswego, OR). The voltage dependence of activation was determined from the amplitude of tail currents observed after each hyperpolarizing voltage step on return to −40 mV. Normalized tail current values were plotted as a function of the test voltage and fit with the Boltzmann function: I(V) = A1 + (A2 - A1)/(1 + exp(-(V1/2 - V)/s)), where A1 and A2, respectively, are maximum response and offset amplitudes, V1/2 is the midpoint of activation voltage, and s is the slope factor. Traces were fitted to a biexponential function in Pulsefit. An initial “lag” not well described by a biexponential function was excluded from each fit, as described previously (Santoro et al., 1998, 2000).

Concentration-effect curves were fit using a Hill equation in the form, r = Rmax*(agonist)m/(agonist)m + EC50n, where r is the peak response, Rmax is the maximum response, [agonist] is the agonist concentration, EC50 is the agonist concentration eliciting a half-maximal response, and nH is the Hill coefficient. Data are represented as mean ± S.E.M. Statistical comparisons were determined using one-way analysis of variance (p < 0.05).

Results

Propofol Decreases Current Amplitude and Slows the Activation of HCN1 Currents. External application of clinically relevant concentrations of propofol to X. laevis oocytes expressing HCN1 channels gave rise to current inhibition that was most pronounced near physiological membrane potentials (Fig. 1, A–C). Under control conditions, the half-maximal voltage required for activation (V1/2) for HCN1 channels was −70.9 ± 0.8 mV (n = 34; Fig. 1D). In the presence of propofol, the voltage-activation curves demonstrated a concentration-dependent shift to the left; this effect had an EC50 of 6.7 ± 1.0 μM and produced a shift of −22.9 ± 1.2 mV with 10 μM propofol (Fig. 1, D and E), suggesting that propofol inhibition of HCN1 currents was largely due to impaired activation at a given voltage.

Analysis of HCN1-gating kinetics revealed further effects of propofol (Fig. 2). Fitting with a biexponential function of HCN1 current activation in the absence of propofol at −100 mV (Fig. 2A) gave values of 143 ± 8 ms (τfast) and 904 ± 52 ms (τslow) (n = 34). Propofol increased τfast and τslow in a concentration-dependent manner (Fig. 2, A–C), with respective EC50 values of 5.6 ± 1.0 μM, slope = 1.4 ± 0.2 (Fig. 2B), and 31.5 ± 7.5 μM, slope = 1.6 ± 0.2 (Fig. 2C). At clinically relevant concentrations (≤10 μM), propofol did not alter the relative current amplitude of the fast component (Fig. 2D). Propofol also affected HCN1 deactivation kinetics, as is evident in Fig. 1B. Fitting of HCN1 deactivation at −40 mV with a single exponential function revealed a dose-dependent acceleration of deactivation in response to propofol, with an EC50 value of 4.5 ± 0.9 μM, slope = 1.1 ± 0.2 μM (Fig. 2E).
Effects of Propofol on HCN2 Channels in *X. laevis* Oocytes. Assessment of the effects of 5 μM propofol on the voltage dependence of activation of HCN2 channels expressed in *X. laevis* oocytes revealed a lower sensitivity than HCN1 channels. Thus, neither significant inhibition of HCN2 channels (Fig. 3, A–C) nor a shift in the voltage dependence of HCN2 activation (Fig. 3, C and D) was observed with application of 5 μM propofol. Even 50 μM propofol had relatively small effects on the peak HCN2 current amplitude, producing only 12.7 ± 0.07% inhibition at −120 mV (data not shown).

However, application of propofol at concentrations above 5 μM produced slowing of HCN2 channel activation (Fig. 4A). Fitting of HCN2 channel activation kinetics in the absence of propofol at −120 mV with a double exponential function (Fig. 4B) gave values for $\tau_{fast}$ of 327 ± 25 and a $\tau_{slow}$ of 2504 ± 237 ms ($n = 23$). Application of 10 μM propofol produced a ~2-fold slowing in $\tau_{fast}$ and a ~20% increase in $\tau_{slow}$ (Fig. 4, A–D). The EC$_{50}$ values for the propofol effects on activation time constants were 23.9 ± 30.7 μM ($\tau_{fast}$) and 30.0 ± 20.2 μM ($\tau_{slow}$) (Fig. 4, C and D), with no significant change in the relative amplitude of the fast component of activation, which was ~0.8 from 0 to 50 μM propofol (Fig. 4E).

Effects of Propofol on HCN2 Activation Kinetics in HEK Cells. In the oocyte expression system, the effects of propofol on HCN1 and HCN2 channels were essentially irreversible; i.e., washout took long enough to preclude accurate assessment of reversibility. This is a relatively common feature of oocyte-based experiments in which small hydrophobic molecules are bath-superfused and may be due to absorption by the yolk and other intracellular bodies. Therefore, we repeated the analysis of propofol effects on HCN2 expressed in HEK cells and found that the effects on kinetics of activation were reversible using this system and also that propofol was more effective in HEK cells than in oocytes (Fig. 5).

Kinetic analysis (Fig. 5A) indicated that 5 μM propofol significantly slowed HCN2 activation, producing a ~2-fold increase in $\tau_{fast}$ and a ~4-fold increase in $\tau_{slow}$ at −130 mV in the HEK cell system; these effects were fully reversible upon washout of propofol (Fig. 5, B–D). Thus, HCN2 channels expressed in HEK cells were sensitive to a clinically relevant concentration of propofol. As with reversibility, the lower sensitivity of HCN2 channels in oocytes probably relates to the tendency for hydrophobic small molecules to be sequestered intracellularly in this system.

Effects of Propofol on HCN4 Channels in *X. laevis* Oocytes. We next asked whether HCN4, a prevalent cardiac HCN isoform, was sensitive to propofol. Representative records from HCN4 recorded in the absence or presence of 20 μM propofol in Ba$^{2+}$-substituted bath medium to minimize endogenous currents indicate that propofol both slows activation and reduces the fully activated current (Fig. 6A). Fitting of HCN4 channel activation kinetics in the absence of propofol at −140 mV with a double exponential function (Fig. 6A) gave values for $\tau_{fast}$ of 431 ± 6 and a $\tau_{slow}$ of 1827 ± 127
ms (n = 3). Application of 20 μM propofol produced a 15% increase in \( \tau_{fast} \) (494 ± 30 ms) and a 2.6-fold slowing of \( \tau_{slow} \) (4743 ± 707 ms) (Fig. 6, C and D). Slowing of the current was accompanied by a decrease in the relative amplitude of the slow component of activation (Fig. 6E) and in the maximally activated current (Fig. 6B). To directly compare the effect of propofol on HCN1, HCN2, and HCN4, we also recorded currents from HCN1 and HCN2 in the Ba\(^{2+}\)-substituted bath solution. A comparison of data in Fig. 6, B to E, with data presented in Figs. 2 through 4, shows that Mg\(^{2+}\) replacement by Ba\(^{2+}\) did not qualitatively or quantitatively alter the propofol-mediated changes in the gating of either HCN1 or HCN2.

**Propofol Increases the RR Interval but Not the QT\(_{C}\) telescopic:**

The combined results suggested that propofol might produce bradycardia via impaired HCN channel function in the sinoatrial node. Therefore, we tested the effect of propofol on heart rate (Fig. 7). Propofol significantly decreased heart rate at 3 μM, but significant effects on QT\(_{C}\) were only observed at 100 μM; at this supraclinical concentration, a decrease in QT\(_{C}\) (Fig. 7, A–C) and sinus arrest were observed. The lack of an effect on QT\(_{C}\) in the clinical concentration range suggests that the bradycardic effect was not due to inhibition of delayed rectifier K\(^+\) channels. The abrupt rise in QT interval above 10 μM propofol (Fig. 7D), albeit associated with a decrease in QT\(_{C}\), because of dominant effects on RR interval, may reflect inhibition of the cardiac \( I_{Ks} \) current by propofol at these supraclinical concentrations; previous studies showed an IC\(_{50}\) of 250 μM propofol for \( I_{Ks} \) in oocytes (Heath and Terrar, 1997).

**Discussion**

The major finding of the present study is that HCN1, HCN2, and HCN4 channels are sensitive molecular targets for the intravenous general anesthetic propofol. This conclusion is supported by our data demonstrating that propofol inhibits the \( I_{h} \) current generated by HCN1, HCN2, and HCN4 channels, all of which are present in brain and heart, in a concentration- and voltage-dependent fashion. In addition, propofol increases the fast and slow activation time constants for their respective currents, with the HCN1 current considered the most sensitive of the three in the oocyte system. Consistent with its effects on heterologously expressed channels in both oocytes and HEK 293 cells, propofol increased the RR interval (slowed heart rate) in the isolated guinea pig heart.

Propofol reportedly inhibits \( I_{h} \) in central neurons (Funahashi et al., 2001, 2004), with an IC\(_{50}\) for suppressing \( I_{h} \) of 235 μM in CA1 pyramidal neurons and 35 μM in area postrema neurons. It is worth noting that the clinically relevant concentration (Eger et al., 2001) of propofol is on the order of 0.4 to 2 μM after accounting for protein binding (Franks and Lieb, 1994; Reves et al., 2005). We found cloned HCN1, HCN2, and HCN4 channels to be more sensitive than was previously reported for native \( I_{h} \). A possible explanation for the discrepancy is the use of different voltage protocols in each case. In the studies of \( I_{h} \) in CA1 pyramidal neurons and area postrema neurons, a 1-s voltage step was used to generate the currents; thus, the resultant currents are unlikely to be fully activated. In contrast, we used 5-s (HCN1) and 10- to 30-s...
HCN2 and HCN4) voltage steps to activate these slowly gating channels. We demonstrate that propofol at clinically relevant concentrations (100 μM) suppresses and slows the activation of Ih generated by HCN1 and HCN2 channel isoforms and significantly slows HCN4 channel activation in oocytes with 20 μM propofol.

Propofol has negative chronotropic effects in the isolated guinea pig heart (Stowe et al., 1992; Alphin et al., 1995). In the first detailed study on the effects of propofol on cardiac function (Stowe et al., 1992), propofol produced a biphasic response on heart rate. Over a low micromolar concentration range (0.5–50 μM), propofol decreased the heart rate, with an EC50 of 10.7 ± 0.2 μM (determined post hoc), whereas it produced asystole at exceedingly high concentrations (≥500 μM). In a subsequent study by Alphin et al. (1995), propofol slowed the atrial rate, with a reported EC50 of 9.1 ± 0.5 μM. Our data demonstrate that propofol produces slowing in the clinically relevant concentration range and produced ventricular fibrillation progressing to asystole with prolonged application of a supraclinical concentration (100 μM; data not shown). Our data both confirm the original observations and, more importantly, expand on them with respect to the addition of the QT interval analysis. The fact that propofol at concentrations less than 10 μM had no significant effect on the QTc indicates that the observed slowing was not due to propofol-mediated inhibition of the cardiac IKs current, which would prolong QTc because of delayed repolarization (Daleau and Turgeon, 1994), consistent with previous reports of a much lower sensitivity of IKs channels to propofol (IC50 of 250 μM) (Heath and Terrar, 1997). Thus, the slowing of sinus rate observed both by us and others with low micromolar concentrations of propofol is probably due to propofol impairment of HCN channel function. This interpretation is supported by our observations that, over the same concentration range, propofol modulates HCN channel gating in both oocyte and HEK 293 cells.

HCN channel distribution is variable across species. In rabbits, for example, HCN1 and HCN4 proteins and transcripts are abundant in the sinoatrial node, whereas HCN2 protein expression is weak and HCN3 transcript is absent (Ishii et al., 1999; Shi et al., 1999). In contrast, HCN4 transcripts predominate in the mouse sinoatrial node, whereas HCN2 levels are moderate and HCN1 is the least detected (Moosmang et al., 2001). In the human heart, HCN2 may be the predominant isoform (Ludwig et al., 1999b) but detailed information on its precise distribution is lacking. Thus, the
effects of propofol on heart rate in different species are probably a product of the relative sensitivities of the different HCN subunit types to propofol and the relative contributions of each HCN subunit type to cardiac pacemaker currents. Furthermore, heteromeric HCN channels may generate cardiac $I_h$ in some instances; future studies are necessary to understand the effect of heteromerization on HCN channel sensitivity to propofol.

In conclusion, the present study provides evidence that the intravenous general anesthetic propofol inhibits and slows the activation of HCN1, HCN2, and HCN4 channels. Because of the importance of the $I_h$ current in contributing to neuronal and cardiac pacemaker cell excitability and autorhythmicity, these effects could provide novel insights into the hypnotic, amnestic, and bradycardic properties of propofol. Of equal importance, these results provide a framework for the development of propofol derivatives that retain desirable anesthetic properties but lack adverse cardiac side effects.

Acknowledgments
We thank Dr. Harunori Ohmori for generously sharing the HCN1 clone and Dr. Bina Santoro for the HCN1, HCN2, and HCN4 clones as well as many useful comments.

References


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