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Contrasting anesthetic sensitivities of T-type Ca^{2+} channels of reticular thalamic neurons and recombinant $Ca_v3.3$ channels

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1 Reticular thalamocortical neurons express a slowly inactivating T-type Ca^{2+} current that is quite similar to that recorded from recombinant $Ca_v3.3b$ (α1Ib) channels. These neurons also express abundant $Ca_v3.3$ mRNA, suggesting that it underlies the native current.

2 Here, we test this hypothesis by comparing the anesthetic sensitivities of recombinant $Ca_v3.3b$ channels stably expressed in HEK 293 cells to native T channels in reticular thalamic neurons (nRT) from brain slices of young rats.

3 Barbiturates completely blocked both $Ca_v3.3$ and nRT currents, with pentobarbital being about twice more potent in blocking $Ca_v3.3$ currents. Isoflurane had about the same potency in blocking $Ca_v3.3$ and nRT currents, but enflurane, etomidate, propofol, and ethanol exhibited 2–4 fold higher potency in blocking nRT vs $Ca_v3.3$ currents.

4 Nitrous oxide (N_2O; laughing gas) blocked completely nRT currents with IC_{50} of 20%, but did not significantly affect $Ca_v3.3$ currents at four-fold higher concentrations. In addition, we observed that in lower concentration, N_2O reversibly increased nRT but not $Ca_v3.3$ currents.

5 In conclusion, contrasting anesthetic sensitivities of $Ca_v3.3$ and nRT T-type Ca^{2+} channels strongly suggest that different molecular structures of Ca^{2+} channels give rise to slowly inactivating T-type Ca^{2+} currents. Furthermore, effects of volatile anesthetics and ethanol on slowly inactivating T-type Ca^{2+} channel variants may contribute to the clinical effects of these agents.
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Keywords: Isoflurane; enflurane; barbiturates; propofol; etomidate; nitrous oxide; ethanol

Abbreviations: DRG, dorsal root ganglion; LVA, low-voltage activated; N_2O, nitrous oxide; nRT, nucleus reticularis thalami

Introduction

The mechanisms by which general anesthetics produce clinical effects are not well understood, but it is likely that they involve specific effects on multiple classes of ion channels that control excitability of nerve cells. Neuronal voltage-gated Ca^{2+} channels play a central role in control of cellular excitability and transmitter release. While subtypes of high voltage-activated (HVA) Ca^{2+} channels play important roles in synaptic transmission in CNS neurons (Miller, 1998), the main proposed function of low voltage-activated (LVA) or transient (T-type) Ca^{2+} channels include promotion of Ca^{2+}-dependent burst firing, generation of low-amplitude intrinsic neuronal oscillations, elevation of Ca^{2+} entry, and boosting of dendritic signals, which contribute to the pacemaker activity of CNS neurons, wakefulness, and seizure susceptibility (reviewed in Huguenard, 1996; Perez-Reyes, 2003).

Whereas it has been known that HVA Ca^{2+} currents arise from multiple forms of Ca^{2+} channels with distinct pharmacological properties, the extent to which T-type Ca^{2+} current arises from multiple Ca^{2+} channel subtypes became clear more recently with the cloning of three genes encoding α1 subunits of T channels, named α1G ($Ca_v3.1$; Perez-Reyes *et al*., 1998), α1 H ($Ca_v3.2$; Cribbs *et al*., 1998), and α1I ($Ca_v3.3$; Lee *et al*., 1999). These distinct genes encode channels with unique biophysical and pharmacological properties, and their region-specific expression contributes to the heterogeneity of T-type Ca^{2+} currents observed in native cells (Herrington & Lingle, 1992; Todorovic & Lingle, 1998; Perez-Reyes, 2003).

$Ca_v3.3$ is selectively expressed in brain regions such as the thalamus (only in reticular nucleus but not relay nuclei), cerebellum, and olfactory cortex (Talley *et al*., 1999). Unlike $Ca_v3.1$ and $Ca_v3.2$, $Ca_v3.3$ T-type Ca^{2+} channels have unusually slow inactivation kinetics and activate over a broader range of membrane potentials. Native and recombinant channels arising from $Ca_v3.1$ and $Ca_v3.2$ genes give rise to T currents that inactivate faster, with inactivation time constant between 20 and 30 ms, while $Ca_v3.3$ currents have up to three-fold slower inactivation (Perez-Reyes, 2003). These distinct kinetic features enable $Ca_v3.3$ channels to mediate slow and sustained influx of Ca^{2+} ions into neurons, which in turn increases their excitability by generating high frequency bursts (Kozlov *et al*., 1999; Chemin *et al*., 2002). It has been shown that volatile general anesthetics block native T currents in hippocampal (Study, 1994), dorsal root ganglion (Todorovic & Lingle, 1998), thalamic relay (Ries & Puil, 1999) neurons, as well as recombinant $Ca_v3.1$ and $Ca_v3.2$ (Todorovic *et al*., 2000) T-type Ca^{2+} currents, at clinically relevant concentrations.

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This suggests that anesthetic depression of neuronal T-type Ca^{2+} currents may contribute to the inhibitory effects of anesthetics in neuronal pathways. Therefore, we wished to investigate, under identical recording conditions, whether the anesthetic sensitivities of the kinetically distinct, slow $Ca_v3.3$ T-type channel variant are similar to the sensitivity of the faster inactivating T currents encoded by $Ca_v3.1$ and $Ca_v3.2$ genes. Furthermore, we describe contrasting pharmacological sensitivities to anesthetics and ethanol of slowly inactivating native T currents in thalamus and recombinant $Ca_v3.3$ currents using the most abundantly expressed 'b' splice variant (Murbartián *et al*., 2002).

Methods

Cell preparation

HEK-293 cells (A293, ATCC, Manassas, VA, U.S.A.) were transfected with the rat $Ca_v3.3b$ cDNA (α1Ib; AY128644) contained in the vector pcDNA3. Stable colonies were isolated in DMEM media supplemented with 1 mg ml^{-1} G418 (Invitrogen, Carlsbad, CA, U.S.A.). The Ca^{2+} currents from three stable lines were characterized, and found to be similar to that observed in the transiently transfected cells (Murbartián *et al*., 2002), so line rat Ib-17 was chosen for this study. Cells were typically used 1 to 2 days after plating. Average cell capacitance (C_m) was 12.9 ± 5.7 pF (mean $\pm$ s.d.), and the average series resistance (R_s) was 5.29 ± 2.08 ($N = 101$).

Electrophysiological methods

Recordings were made with standard whole-cell voltage-clamp techniques. Electrodes were fabricated from microcapillary tubes (Drummond Scientific Company, Broomall, PA, U.S.A.), and fire-polished. Pipette resistances were 2–5 MΩ. Voltage commands and digitization of membrane currents were carried out with Clampex 8.2 of the pClamp software package (Axon Instruments, Foster City, CA, U.S.A.) running on an IBM-compatible computer. Membrane currents were recorded with an Axopatch 200A patch-clamp amplifier (Axon Instruments). Typically, cells were held at −90 mV and depolarized to −30 mV every 20 s to evoke inward currents. Data were analyzed using Clampfit (Axon Instruments) and Origin 7.0 (Origin Lab Corporation, Northhampton, MA, U.S.A.). Currents were filtered at 5 kHz. Reported series resistance values were taken from the reading of the amplifier. All experiments were carried out at room temperature (20–23°C). In most experiments, leak subtraction was performed online with a P/5 protocol for on-line leakage subtraction.

Analysis of current blockade

The percent reduction in peak T current at a given anesthetic concentration was used to generate concentration–response curves. For each concentration–response curve, all points are averages of multiple determinations obtained from at least five different cells. In most of the cells, we tested at least two different concentrations of the same anesthetic. On all plots, vertical bars indicate standard errors (s.e.). Mean values on concentration–response curves were fit to the following function:

$$PB([DRUG]) = PB_{max}/(1 + (IC_{50}/[DRUG])^n)$$

where PB is the percent block of the peak T current, PB_{max} is the maximal block, IC_{50} is the concentration that produces 50% of maximal inhibition, and n is the apparent Hill coefficient for blockade. Fitted values are typically reported with 95% linear confidence limits.

Solution exchange procedures

The solution application system consisted of multiple, independently controlled glass capillary tubes driven by gravity. During an experiment, solution was removed from the end of the chamber opposite the glass capillary tubes with the use of constant suction. Switching between solutions was accomplished by manually controlled valves. Test solutions were maintained in closed, weighted all-glass syringes (to avoid saline evaporation and loss of volatile agents). Changes in Ca^{2+} current amplitude in response to rapidly acting drugs or ionic changes were typically complete in 10–20 s. Switching between separate perfusion syringes, each containing control saline, resulted in no changes in Ca^{2+} current.

Solutions and current isolation procedures

The standard extracellular saline for recording of $Ca_v3.3$. Ca^{2+} current contained (in mM): 160 TEA-Cl, 10 HEPES, 2 $CaCl_2$, adjusted to pH 7.4 with TEA-OH, (316 mOsm). Internal solution consisted of (in mM): 110 Cs-methane sulfonate, 14 phosphocreatine, 10 HEPES, 9 EGTA, 5 Mg-ATP, and 0.3 Tris-GTP, pH adjusted to 7.20 with CsOH (300 mOsm).

Drugs and chemicals

Etomidate powder and isoflurane were obtained from Abbott (Abbott Park, IL, U.S.A.). All other chemicals were obtained from Sigma or Aldrich Chemicals (Milwaukee, WI, U.S.A.). Stock solutions of propofol (100 mM) and etomidate (300 mM) were prepared in dimethyl sulfoxide (DMSO) and kept at 4°C until use. DMSO (0.3%) had no effect when tested alone on $Ca_v3.3$ Ca^{2+} currents ($n = 3$). All barbiturates were prepared in stock solutions in 0.1 N TEA-OH; the pH of the final extracellular solution was adjusted with HCl to 7.4. Isoflurane and enflurane solutions were prepared from saturated saline solutions (Todorovic & Lingle, 1998; Todorovic *et al*., 2000), and the final concentration in the bath was determined by gas chromatography for each experiment (Evers *et al*., 1986).

For experiments with nitrous oxide (N_2O), the extracellular solution was bubbled with air or N_2O/O_2 mixtures using a bubbling stone (Todorovic *et al*., 2001). The bubbling container was sealed with Parafilm and was punctured with a small escape perforation. The solution was equilibrated with gas for at least 30 min, during which time gas-equilibrated solution was drawn into a closed glass syringe. The syringe served as a solution reservoir for the gravity-fed local perfusion system. For most experiments, 80% N_2O/20% O_2 was used so that bottled air 80% N_2/20% O_2 could be used as a control. All test solutions were prepared on the day of the experiment by diluting the stock solutions with the appropriate amount of extracellular saline.

In vitro *slice tissue preparation*

Most of experiments were performed with 200–250 μm thick transverse rat brain slices taken through the middle portion of the nucleus reticularis thalami (nRT) (stereotaxic levels 4.6–5.82 mm below bregma; (Paxinos & Watson, 1982). Gravid Sprague–Dawley rats were housed in a local animal facility, and used in accordance with guidelines (NIH Guide for the Care and Use of Laboratory Animals) and protocols approved by the University of Virginia Animal Use and Care Committee. Young rats, aged 7–14 days, were anesthetized with halothane and decapitated, and the brains rapidly removed and placed in chilled (4°C) cutting solution consisting of (in mM): 2 $CaCl_2$, 260 sucrose, 26 $NaHCO_3$, 10 glucose, 3 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, equilibrated with a mixture of 95% O_2 and 5% CO_2. A block of the tissue containing the thalamus was glued to the chuck of a vibrotome (TPI, St Louis, MO, U.S.A.) and 200–250 μM slices were obtained in a transverse plane. The slices were incubated in oxygenated saline at 36°C for 1 h prior to placing in a recording chamber that had been modified for superfusion at a rate of 1.5 cc/min. Incubating saline consisted of (in mM): 124 NaCl, 4 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $MgCl_2$, 10 glucose, 2 $CaCl_2$, equilibrated with a mixture of 95% O_2 and 5% CO_2. Slices were maintained at room temperature in the recording chamber and remained viable for at least up to 1 h under these conditions.

Recording procedures We recorded Ca^{2+} currents with whole-cell patch clamp technique in coronal thalamic slices from young rats from a total of 122 visually identified neurons ($R_s < 20$ MΩ) of nRT at room temperature. Whole-cell recordings were obtained from nRT visualized with an IR DIC camera (Hammamatsu, C2400) on the Zeiss 2 FS Axioscope (Carl Zeiss, Jena). Electrodes were fabricated from microcapillary tubes with thin walls, and were not fire-polished, with final resistances of 3–6 MΩ. The standard extracellular saline for recording of T-type Ca^{2+} currents consisted of (in mM): 2 $CaCl_2$, 130 NaCl, 2.5 $MgCl_2$, 10 glucose, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 CsCl, 10 TEACl, 1 4-AP and 1 μM TTX to block voltage-gated Na^+ currents. To isolate T-type from HVA Ca^{2+} currents, an internal solution was used that contained (in mM): 135–140 TMAOH, 10 EGTA, 40 HEPES, and 2 $MgCl_2$, titrated to pH 7.15–7.25 with hydrofluoric acid (Todorovic & Lingle, 1998). In some experiments, we used the same internal solution as in experiments with HEK cells and this did not influence the effects of pentobarbital, ethanol, or N_2O ($n = 3$ for each experiment, data not shown). For recordings of T currents from nRT, cells were held at –100 mV and depolarized to –50 mV to avoid small contaminating component of HVA current that was resistant to intracellular F^-. For the data presented in this study, correction of 10 mV in voltage-clamp experiments was made for liquid junction potentials.

Since voltage control is compromised in slices due to the presence of extensive cell processes, we included in our results only cells where voltage-dependent current activation was smooth; no excessive delay in the onset of current was observed and the onset and offset kinetics depended on voltage but not on the amplitude of current. All measurements of amplitudes from holding, peak, and steady-state currents are made at time points sufficient to ensure reasonably well-clamped current conditions.

Similarly, anesthetic delivery *in vitro* could be compromised in slices due to decline in drug concentration along the length of the bath and diffusion through the slice tissue. Highly lipid-soluble anesthetic agents pose challenges to many standard solution application methods. We have found that propofol and etomidate are readily absorbed into silicon and some other types of tubing within minutes. To minimize this problem, glass or metal tubing is used. We have found that a system consisting of multiple pieces of glass tubing connected with small PVC joints allows reliable and consistent delivery of drugs to the bath. Furthermore, most of the recordings are carried out from neurons in the superficial 50 μm of slices to facilitate equilibration of anesthetics and slice tissue. We demonstrate on Figure 4 that prolonged applications of etomidate, propofol, and pentobarbital do not suppress more currents after first 3 min of application. This argues that our recording conditions are adequate to perform proposed experiments and that an apparent equilibrium is reached in all experiments when measurements were taken.

Results

Isolation of currents

Figure 1(a and b) depicts average traces of T currents from 13 nRT cells and 11 HEK cells stably transfected with $Ca_v3.3$ constructs. It is evident that both currents in similar recording conditions slowly inactivate with a single exponential inactivation τ of about 56 ± 5 ms for nRT (Figure 1c) and about 78 ± 5 ms for $Ca_v3.3$ currents (Figure 1d) ($P < 0.01$).

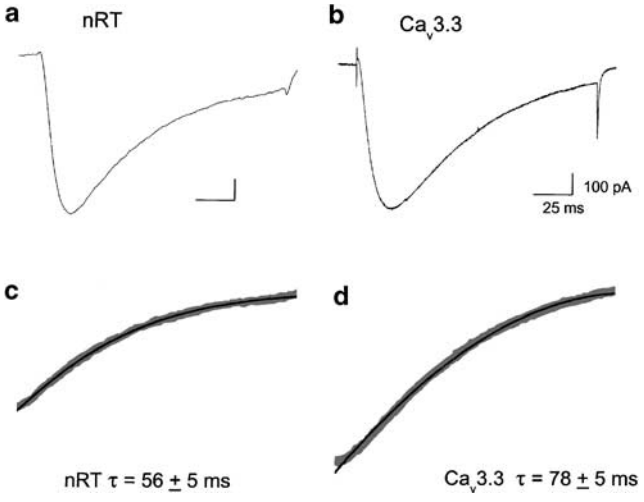

Figure 1 Both recombinant $Ca_v3.3$ and native nRT T currents have slow inactivation kinetics. (a) Traces evoked from V_h –100 mV and V_t –50 mV from 13 nRT cells are averaged. (b) Similar currents averaged from 11 HEK cells transfected with $Ca_v3.3$ constructs are evoked from V_h –90 mV to V_t –30 mV. Bars indicate calibration. (c) A single exponential fit (dark line) of inactivating portion of the nRT current from panel (a) gave an average inactivation τ of 56 ± 5 ms. (d) A single exponential fit (dark line) of inactivating portion of the $Ca_v3.3$ current from panel (b) gave an average inactivation τ of 78 ± 5 ms.

Effects of barbiturates on $Ca_v3.3$ current

Barbiturates have been used clinically for induction of general anesthesia, as anticonvulsant drugs, and also as neuroprotective agents. Here, we examined the effects on $Ca_v3.3$ currents of three barbiturates that we used previously in our studies with recombinant channels. Figure 2 depicts the concentration-dependent effects of pentobarbital, thiopental, and phenobarbital on recombinant $Ca_v3.3$ currents. All barbiturates blocked currents completely and reversibly, with pentobarbital and thiopental being about 4–5 times more potent than phenobarbital (Figure 2d). Figure 2b also illustrates the stability of current when recorded over a prolonged period of time. Pentobarbital depressed peak inward Ca^{2+} currents with an IC_{50} of $180 \pm 42\,\mu$M and thiopental blocked currents with IC_{50} of $260 \pm 52\,\mu$M, and phenobarbital blocked with IC_{50} of $1000 \pm 200\,\mu$M. All barbiturates depressed the peak of the current with very little effect on current activation and inactivation kinetics. The time course of current activation was assessed by measuring 10–90% rise time, and inactivation time constant (τ) by fitting the decaying portion of the current with a single exponential function before and during application anesthetic at a concentration that blocks $\sim$50% of the peak current. Overall, the three barbiturates did not significantly

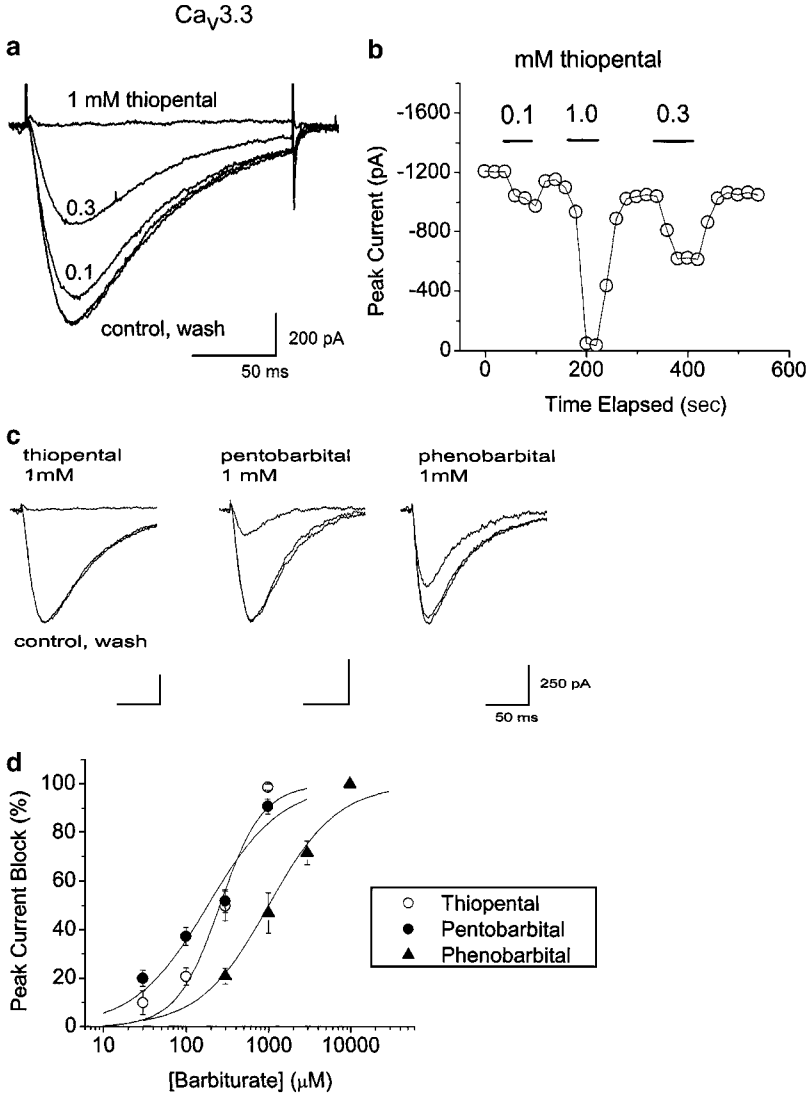

Figure 2 Barbiturates block $Ca_v3.3$ currents. (a) T currents are reversibly blocked by three different concentrations of thiopental. Inactivation τ changed from 80 ms in control to 62 ms in the presence of 0.3 mM thiopental. (b) Time course of an experiment showing the block of peak currents by thiopental (same cells as in panel (a). Peak inward current is plotted as function of time and horizontal solid bars indicate times of application. Note fast onset and offset, as well as near complete recovery from thiopental blocking action. (c) Representative traces from cells where 1 mM of thiopental, pentobarbital, and phenobarbital are used. Note that this concentration of thiopental and pentobarbital blocked almost completely, while phenobarbital blocked only 42% of peak inward current. (d) Concentration–response curves for three barbiturates are shown, with each point being the average of 6–9 different cells. Symbols indicate different anesthetic as indicated on this figure. Vertical lines are s.e. the solid lines are best fits with the Hill equation where the steepness of the slope is described by the coefficient h. IC_{50} values for block of $Ca_v3.3$ current were $180 \pm 42\,\mu$M ($h = 1.0 \pm 0.2$, $n = 17$) for pentobarbital, $260 \pm 52\,\mu$M ($h = 1.64 \pm 0.5$, $n = 9$) for thiopental, and $1000 \pm 200\,\mu$M ($h = 1.0 \pm 0.2$, $n = 12$) for phenobarbital.

change ($P>0.05$) the 10–90% rise time or inactivation rate (τ) in these cells ($n=5$–8 cells for each agent, data not shown).

Effects of propofol and etomidate on $Ca_v3.3$ currents

Propofol (2,6 diisopropylphenol) is a new intravenous anesthetic agent that has gained widespread use. Figure 3a, c and e indicate that propofol blocked $Ca_v3.3$ currents in a concentration-dependent manner with an IC_{50} of $60\pm12\,\mu$M. Propofol caused a significant increase in current inactivation rate that was evidenced by up to three-fold decrease in inactivation τ ($n=5$, $P<0.001$).

Etomidate is an intravenous anesthetic that blocks native T channels in DRG neurons (Todorovic & Lingle, 1998) and recombinant $Ca_v3.1$ and $Ca_v3.2$ currents in HEK cells (Todorovic *et al*., 2000) without affecting their apparent rate of current activation and inactivation. In contrast, etomidate blocked $Ca_v3.3$ currents at lower concentrations, and caused a significant increase in the rate of current decay (Figure 3b). Etomidate (100 μM) decreased the apparent inactivation τ by $56\pm7\%$ ($n=7$ cells, $P<0.05$), with change in the 10–90% rise time of only $12\pm5\%$ ($P>0.05$). Block of the peak current was reversible and concentration-dependent with an IC_{50} of $90\pm11\,\mu$M (Figure 3b, d and f).

Effects of etomidate, propofol, and pentobarbital on slowly inactivating nRT currents

We tested the hypothesis that the slowly inactivating T-type currents from nRT are carried by $Ca_v3.3$ channels by

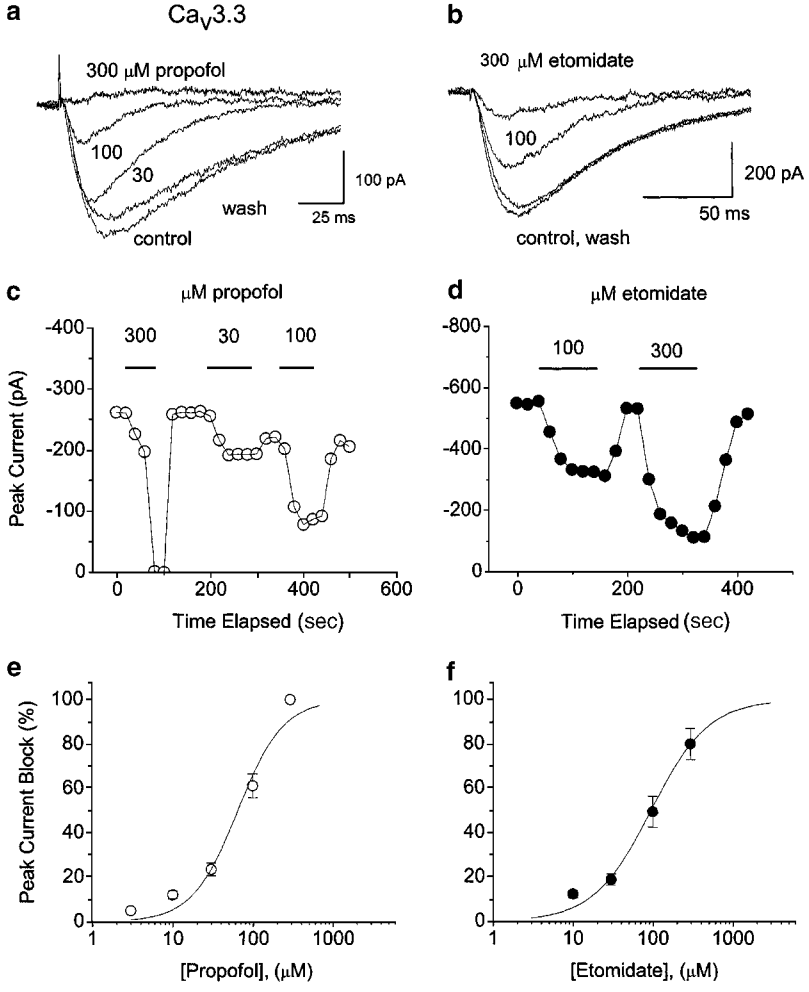

Figure 3 Effects of propofol and etomidate on $Ca_v3.3$ currents. (a) Experiments from an HEK cell show effects of three different concentrations of propofol on $Ca_v3.3$ currents. Propofol increased the apparent inactivation of current by decreasing inactivation τ (from 65 ms in control saline, to 27 ms with application of 30 μM propofol). Note that propofol blocked current nearly completely when higher concentrations are used. (b) This panel depicts representative traces showing reversible block of $Ca_v3.3$ currents with 0.1 and 0.3 mM etomidate. Similar to propofol, etomidate increased inactivation of the current by decreasing inactivation τ by about 50% (from 78 ms in control saline to 40 ms with anesthetic application). (c) The time course of block by randomly applied multiple concentrations of propofol is plotted for the same cell as in (a). Bars indicate time of application. (d) Time course of etomidate-induced $Ca_v3.3$ current blockade is depicted (same cell shown in panel b). Horizontal bars indicate time of application of 0.1 and 0.3 mM etomidate. (e) The concentration dependence for block of $Ca_v3.3$ currents by propofol (open circles, $n=13$ cells, IC_{50} $60\pm12\,\mu$M, $h=1.5\pm0.4$). (f) Concentration–response curve for etomidate block of $Ca_v3.3$ currents from total of 13 cells. Each symbol represents the average from at least five different cells. The solid line represents the fit to the average data: IC_{50} of $90\pm14\,\mu$M and $h=1.54\pm0.34$.

comparing their sensitivity to intravenous anesthetics. Figure 4 summarizes our findings from these experiments. At lower concentration, all anesthetics in most of the cells partially blocked peak current evoked from −100 to −50 mV (Figure 4a, c and e). However, at higher concentrations of 0.1–1 mM, all agents induced a complete and reversible block of nRT currents (Figures 4b and d). *Pentobarbital* as a representative barbiturate was about two times less potent in blocking nRT than $Ca_v3.3$ currents with an IC_{50} of about 264 μM, while *etomidate* and *propofol* were 2–4 times more potent in blocking nRT than $Ca_v3.3$ currents with IC_{50}s of about 25 μM. (Figure 4f). In contrast to their effects on $Ca_v3.3$ currents, neither of the tested anesthetics significantly affected inactivation kinetics. For example, average control inactivation τ's in these experiments were 53 ± 5 ms ($n = 17$) and 54 ± 4 ms ($n = 6$, $P > 0.05$), 63 ± 14 ms ($n = 6$, $P > 0.05$), and 68 ± 8 ms ($n = 5$, $P > 0.05$) for pentobarbital, etomidate, and propofol, respectively. Overall, our data indicate that different classes of intravenous anesthetics block native nRT currents differently than recombinant channels.

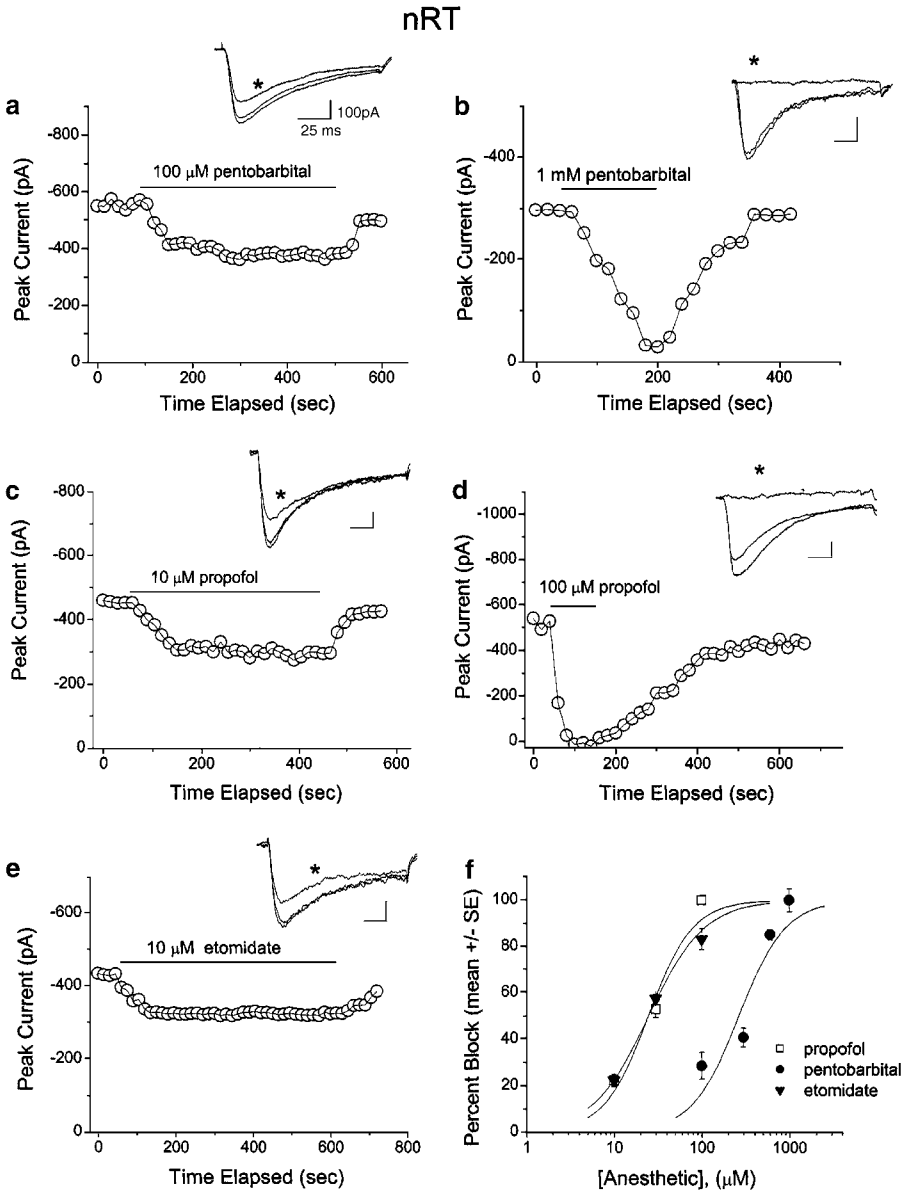

Figure 4 Pentobarbital, etomidate, and propofol block slow thalamic T currents. (a–e). Time course of block of peak T current in different nRT cells in slices perfused with different concentrations of pentobarbital, etomidate, and propofol. Bars indicate time of application. Insets depicts traces of T current evoked by depolarizing to −50 from −100 mV for the given experiments where time course of block is presented. An asterisk (*) marks currents obtained while drugs were applied. Two control traces indicate currents before and after drug application. Calibration for T current in top left panel applies for all T currents. (f) Average concentration–response curves for propofol (open squares, $n = 17$ cells), etomidate (filled triangles, $n = 18$ cells), and pentobarbital (filled circles, $n = 19$ cells). Solid lines indicate best fit for average data giving IC_{50} of 25 ± 2, 25 ± 4 and 264 ± 76 μM for etomidate, propofol, and pentobarbital, respectively. Slope of the curve indicating steepness of concentration–response relationship was 1.7 ± 0.5, 1.6 ± 0.7, and 1.3 ± 0.2 for propofol, pentobarbital, and etomidate, respectively.

Differential sensitivities of ethanol on $Ca_v3.3$ and nRT T-type Ca^{2+} currents

Similar to many general anesthetics, ethanol depress neuronal activity by enhancing inhibitory $GABA_A$ currents or blocking excitatory NMDA currents (Catlin *et al*., 1999). However, recent reports indicate that ethanol modulates HVA voltage-gated Ca^{2+} channels in the same concentration range found to affect ligand-gated currents (Walter & Messing, 1999), Since effects of ethanol on T-type Ca^{2+} channels are not well characterized, we examined effects of ethanol on slowly inactivating T-type Ca^{2+} channel variants. We found that nRT currents were completely blocked with 50–200 mM ethanol (Figure 5a and b) and $Ca_v3.3$ currents were relatively resistant to these concentrations of ethanol (Figure 5c and d). At 200 mM, ethanol blocked only about 26% of $Ca_v3.3$ currents, while this concentration blocked slow nRT T currents reversibly and almost completely (84%) (Figure 5a–d). Concentration–response relationship for effects of ethanol upon nRT T currents gave an IC_{50} of about 100 mM and $Ca_v3.3$ currents were about four times less sensitive (IC_{50} 365 mM) (Figure 5e). Ethanol blocked completely and reversibly both recombinant and native T currents but did not change inactivation τ of either $Ca_v3.3$ or nRT currents.

Effects of volatile anesthetics isoflurane, enflurane, and N_2O on $Ca_v3.3$ currents

The frequently used potent volatile anesthetic isoflurane blocks faster-inactivating ($Ca_v3.1$ and $Ca_v3.2$) T-type Ca^{2+} channel variants at subanesthetic concentrations (Todorovic *et al*., 2000), but the sensitivity of slowly inactivating variants

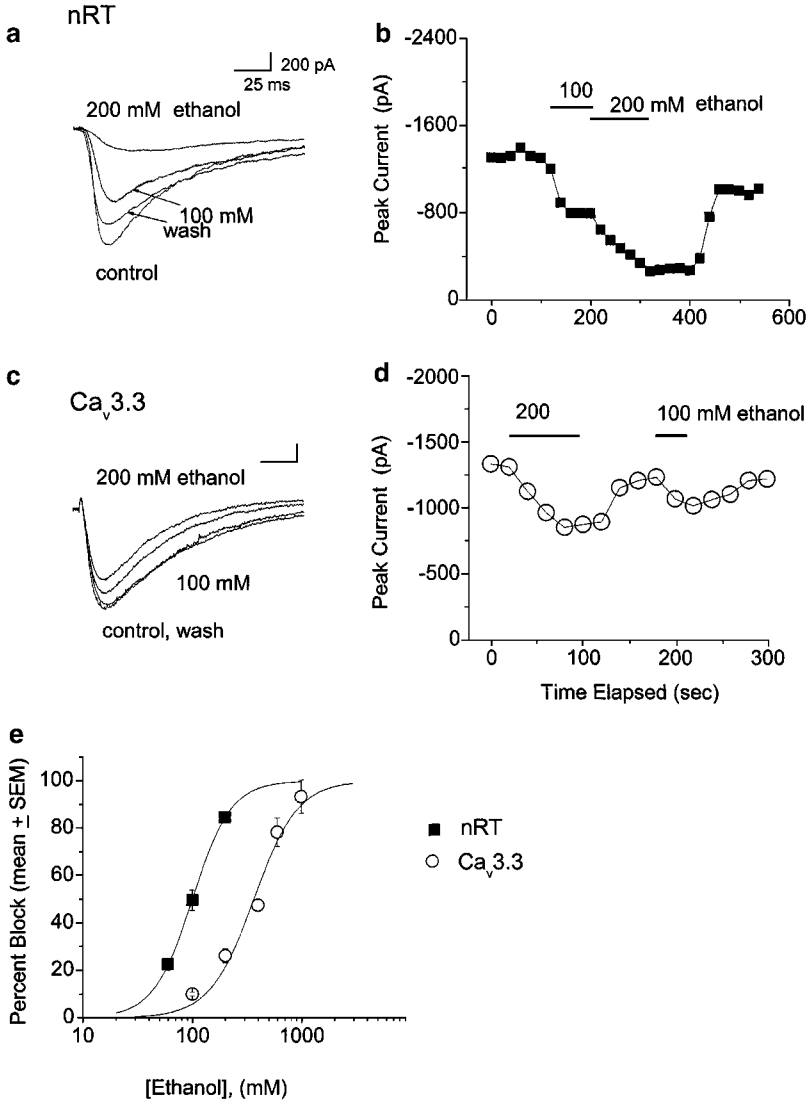

Figure 5 Ethanol blocks $Ca_v3.3$ and nRT T currents. (a) Traces show nRT currents activated at −50 mV in the presence and absence of 2 concentrations of ethanol. (b) Time course from the same experiment showing reversibility of ethanol's effects on nRT currents. (c) Traces showing very little block of $Ca_v3.3$ currents by 100 and 200 mM ethanol. Bars indicate the calibration. (d) Percent block of peak nRT current is plotted as a function of time. Bars indicate the time of application. Note that 200 mM ethanol blocked only about 37% of $Ca_v3.3$ current while almost completely blocked nRT current (panel b). (e) Comparison of sensitivity to ethanol of $Ca_v3.3$ (open circles) and nRT currents (solid squares). All symbols are averaged from multiple determinations ($n=9$ cells for $Ca_v3.3$ and $n=25$ cells for nRT). Solid lines represent fit to the average data: $Ca_v3.3$, IC_{50} of 365 ± 32 mM, $h=2.2\pm0.4$; and for nRT, IC_{50} of 100 ± 1 mM and $h=2.4\pm0.1$.

like $Ca_v3.3$ is not known. Therefore, we examined effects of isoflurane and its isomer enflurane on $Ca_v3.3$ currents in HEK cells. We found that both volatile anesthetics blocked $Ca_v3.3$ currents completely and reversibly in a concentration-dependent manner (Figure 6a–d). Both anesthetics had a similar potency with IC_{50}s of 260 ± 23 and $360\pm21\,\mu$M, for isoflurane and enflurane, respectively. Similar to etomidate and propofol, the two volatile anesthetics had very little effect on the time course of current activation, but significantly increased current inactivation rate more than two-fold. Isoflurane decreased inactivation τ by $66\pm6\%$ ($n=6$, $P<0.01$), and enflurane decreased inactivation τ by $62\pm8\%$ ($n=5$, $P<0.001$).

N_2O (laughing gas) has been used for almost two centuries in medical and dental practice because of effective analgesic properties that are achieved at subanesthetic concentrations. While cellular targets for N_2O were not known for a long time due to difficulties in working with this agent *in vitro*, it was recently reported that, unlike most of the other general anesthetics that augment GABA currents, N_2O blocks NMDA currents in CNS neurons (Jevtovic-Todorovic *et al*., 1998). N_2O blocks potently native DRG and $Ca_v3.2$ T-type Ca^{2+} currents but not $Ca_v3.1$ currents in HEK cells (Todorovic *et al*., 2001). Here, we found that 80% N_2O (maximal concentration that could be used in *in vitro* experiments) had small blocking effect on peak $Ca_v3.3$ currents ($8\pm2\%$, $n=6$). However, this was not significantly different from effects of air ($6\pm2\%$ block, $n=4$, data not shown) which was used as a control ($P>0.05$). Cells that were not sensitive to N_2O had the usual sensitivity to isoflurane (Figure 6e and f).

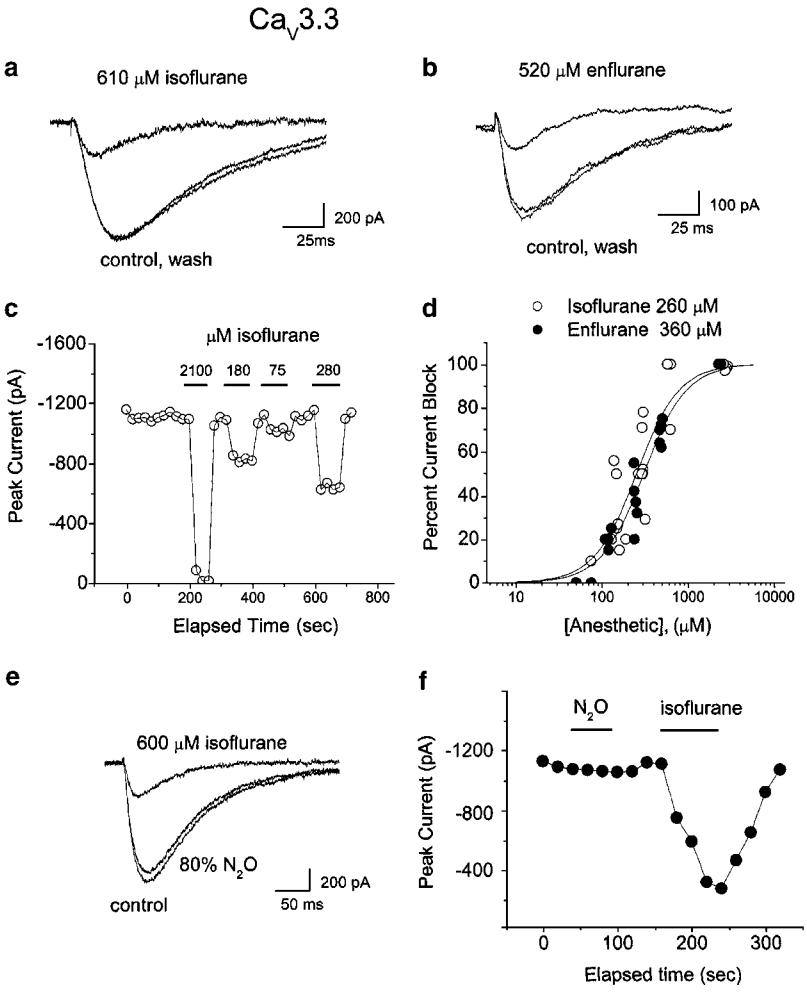

Figure 6 Isoflurane and enflurane block $Ca_v3.3$ currents in subanesthetic concentrations. (a) Traces show $Ca_v3.3$ currents activated at -30 mV in the presence and absence of isoflurane. Similar to propofol and etomidate, isoflurane increased the apparent inactivation rate (inactivation τ was 87 ms in control conditions, and 32 ms after isoflurane was applied). (b) Traces from another experiment where isoflurane's isomer, enflurane was applied. Enflurane (520 μM) blocked 75% of the peak current and decreased the inactivation τ about three-fold (from 72 to 23 ms). (c) Time course of the effects of four different concentrations of isoflurane on peak $Ca_v3.3$ current is plotted. Horizontal bars indicate times of application. Note fast onset and offset of isoflurane's effects on the peak inward current. (d) Percent block of peak $Ca_v3.3$ current is plotted as a function of isoflurane (open circles) and enflurane (solid circles) concentrations ($n=8$ cells for enflurane and $n=9$ cells for isoflurane). Solid lines represent fit to the average data: isoflurane, IC_{50} of $260\pm23\,\mu$M, $h=1.7\pm0.3$; and for enflurane, IC_{50} of $360\pm21\,\mu$M and $h=1.7\pm0.2$. (e) Traces show minimal effect (about 7% block) of 80% N_2O on peak $Ca_v3.3$ current. In contrast, 600 μM isoflurane blocked about 70% of the peak current. (f) Time course of the experiment depicted on panel (e) of this figure is presented. Horizontal bars indicate times of application of 80% N_2O and 600 μM isoflurane.

Effects of fluorinated volatile anesthetics and N_2O on nRT T currents

We reported that volatile anesthetics isoflurane and enflurane block nRT currents completely with IC_{50} of 281 and 148 μM, respectively (Joksovic *et al*., 2004).

We found that at low concentrations of 8%, N_2O consistently and reversibly increased peak T current by about $30\pm9\%$ in six out of eight tested cells (Figure 7a and b). The increase in T current by N_2O was not accompanied in changes in apparent current kinetics or holding current. In three cells, 8% N_2O increased current while 80% N_2O blocked peak T current in the same cells (data not shown). This indicates that an increase in peak T current by low concentrations of N_2O does not reflect action on a distinct subpopulation of thalamic cells but strongly suggests that it may be a dual mechanism of T channel modulation by N_2O in nRT neurons.

N_2O at higher concentrations (20–80%) reversibly inhibited nRT currents with a maximal current block of $82\pm4\%$ at 80% N_2O and an apparent IC_{50} of about 20% N_2O (Figure 7e, $n=9$). Unlike isoflurane and enflurane (Joksovic *et al*., 2004), this blocking effect was not accompanied by significant change in inactivation kinetics. Average inactivation τ was 57 ± 7 ms in control conditions and 83 ± 11 ms in the presence of 40% N_2O ($P>0.05$, $n=9$). This suggests that N_2O and fluorinated general anesthetics block slow T current by different mechanisms. In control experiments, air did not significantly affect peak T current ($n=6$ cells, data not shown). Therefore, these data indicate that T currents in nRT are more sensitive to N_2O than any other T-current variant previously tested.

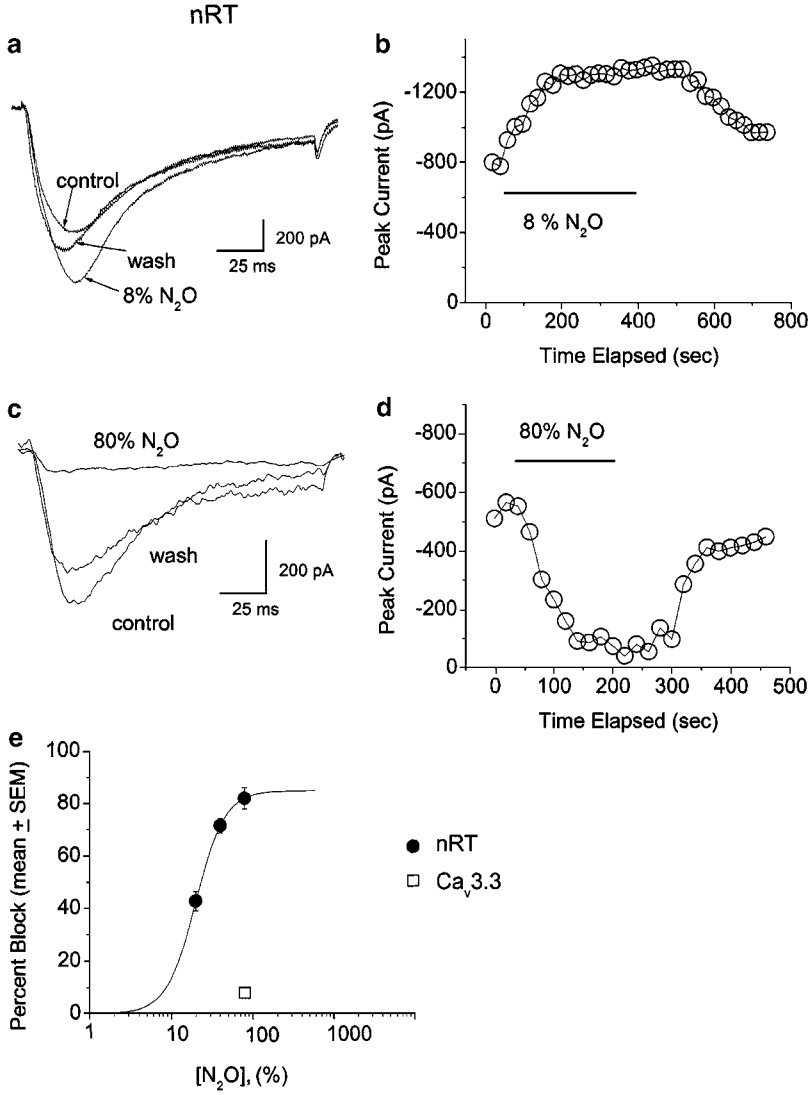

Figure 7 Effects of N_2O on T currents in nRT cells. (a) Representative traces showing effects 8% N_2O on an nRT cell. (b) Time course from the same experiments depicted on panel (a) indicate reversible increase of about 50% of peak current. (c) At concentrations of 80% N_2O almost completely blocked nRT current as indicated on this cell. (d) Time course from the same experiment depicted on panel (c) indicate fast onset and offset of block by N_2O. (e) Filled circles indicate average block of nRT currents by 20, 40, and 80% N_2O ($n=25$ cells). Solid line is best fit of the data giving IC_{50} of $20.0\pm0.1\%$, fitted maximal block of $85\pm1\%$ and h of 2.4 ± 0.1. Open symbol indicates average block of $Ca_v3.3$ currents by 80% N_2O.

Comparison of anesthetic sensitivities of different T-type Ca^{2+} channel variants

Table 1 describes the similarity and difference of the blocking effects of tested anesthetics on $Ca_v3.3$ as well as $Ca_v3.1$ and $Ca_v3.2$ currents in HEK cells, under identical recording conditions (Todorovic *et al*., 2000). It appears that all three cloned T channels, despite their different kinetic properties, have similar sensitivities to the most tested anesthetics. The exception is that $Ca_v3.3$ currents are resistant to N_2O like $Ca_v3.1$ currents, more sensitive to etomidate and pentobarbital, and somewhat less sensitive to propofol. In contrast, nRT T current were less sensitive to pentobarbital, but 2–4-fold more sensitive to etomidate, propofol, and N_2O. When compared to $Ca_v2.3$ currents in HEK cells (Nakashima *et al*., 1998) or oocytes (Kamatchi *et al*., 1999), it is evident that T-type Ca^{2+} channels variants are more sensitive to anesthetics, particularly volatile anesthetics like isoflurane (e.g. four-fold difference in sensitivity). It must be noted that in our studies, for most anesthetics, we did not test for voltage- or use-dependence of block. Substantial voltage- or use-dependence of block can affect estimated IC_{50} values. However, there was no indication of slow block by any of the anesthetics we examined.

Discussion

It was recognized as early as two decades ago that volatile anesthetics block certain types of (e.g. L-type) Ca^{2+} currents in myocardial tissue (Lynch *et al*., 1981; Terrar & Victory, 1988; Bosnjak *et al*., 1991). However, multiple classes of Ca^{2+} channels exist in nerve cells and our more recent work (Todorovic & Lingle, 1998; Todorovic *et al*., 2000; 2001) and the work of others (Herrington & Lingle, 1991; Takenoshita & Steinbach, 1991; Study, 1994; McDowell *et al*., 1996) indicates that T-type voltage-gated Ca^{2+} channels in both central and peripheral neurons are inhibited by volatile general anesthetics at concentrations which occur under clinical conditions. However, previous studies did not investigate anesthetic sensitivities of kinetically distinct slow T-type Ca^{2+} channel variants. Here, we have explored the sensitivity of the recombinant rat $Ca_v3.3b$ T-channel isoform and native nRT T currents to various classes of anesthetics and compared with sensitivities of other T-channel variants ($Ca_v3.1$ and $Ca_v3.2$), as well as one class of channels that contributes to native R-type HVA currents ($Ca_v2.3$).

Anesthetic sensitivities of various LVA currents

We find that, in general, sensitivities of most classes of anesthetics among different variants of recombinant T channels do not vary greatly (i.e. less than two-fold). Exceptions are N_2O, propofol, and etomidate. Propofol, is about 2–4-fold less potent in blocking $Ca_v3.3$ current than $Ca_v3.1$ and $Ca_v3.2$ currents (Table 1). On the contrary, etomidate is about two-fold more potent in blocking $Ca_v3.3$ currents than other recombinant T-channel isoforms. N_2O blocks potently recombinant $Ca_v3.2$ and related native DRG at subanesthetic concentrations, while higher concentrations are required to block $Ca_v3.1$ (Todorovic *et al*., 2001) and $Ca_v3.3$ currents (this study). This indicates that N_2O is a valuable tool as a selective blocker for $Ca_v3.2$-based currents in recombinant and native systems.

We also compared the sensitivities of slow and fast T-channel variants to the sensitivities of $Ca_v2.3$ isoform, which in native cells gives rise to R-type HVA currents (Piedras-Renteria & Tsien, 1998). However, in certain instances, $Ca_v2.3$- and R-based currents may resemble T currents because of somewhat similar kinetic features, similar permeation properties, and similar sensitivity to nickel (Randall & Tsien, 1997; Nakashima *et al*., 1998). We found that $Ca_v3.3$, as well as other T-channel variants are in most instances at least two-fold more sensitive to anesthetic blockade than $Ca_v2.3$ currents (Table 1). Therefore, our data suggest that anesthetics,

Table 1 Comparison of sensitivities of native nRT T currents and recombinant T-type currents

	$Ca_v3.1$ ($\alpha 1G$)[a,b]	*$Ca_v3.2$ ($\alpha 1H$)*[a,b]	*$Ca_v2.3$ ($\alpha 1E$)*[c]	*NRT*[d]	*$Ca_v3.3$*[d] *($\alpha 1I$)*
Anesthetic			*$IC_{50} \pm$s.e.m., maximal block*		
Pentobarbital	IC_{50} 310±40 μM max. 100%	IC_{50} 345±94 μM max. 100%	IC_{50} 600 μM max. 100%	IC_{50} 264±76 μM max. 100%	IC_{50} 180±42 μM max. 100%
Thiopental	IC_{50} 280±40 μM max. 100%				IC_{50} 260±52 μM max. 100%
Phenobarbital	IC_{50} 1.5±0.2 μM max. 100%		IC_{50} 2.7 μM max. 100%		IC_{50} 1.0±0.2 mM max. 100%
Propofol	IC_{50} 20±2.0 μM max. 100%	IC_{50} 27±3 μM max. 100%		IC_{50} 25±4 μM max. 100%	IC_{50} 60±12 μM max. 100%
Etomidate	IC_{50} 160±46 μM max. 100%			IC_{50} 25±2 μM max. 100%	IC_{50} 90±14 μM max. 100%
Isoflurane	IC_{50} 277±24 μM max. 100%		IC_{50} 1 mM max. 100%	IC_{50} 281±40 μM max. 100%	IC_{50} 260±23 μM max. 100%
Nitrous oxide	No effect up to 80%	IC_{50} 58±17 μM max. 66%		IC_{50} 20.0±0.1% max. 85%	No effect up to 80%
Enflurane				IC_{50} 148±10 μM max. 100%	IC_{50} 360±21 μM max. 100%

[a]Data from Todorovic *et al*. (2000), using 2 mM Ca^{2+} as charge carrier.
[b]Data for N_2O are from Todorovic *et al*. (2001), using 10 mM Ba^{2+} as a charge carrier.
[c]Data from Nakashima *et al*. (1998) using HEK-293 cells, except isoflurane data which is an estimate of IC_{50} from Kamatchi *et al*. (1999) using Xenopus oocytes. Both studies used 10 mM Ba^{2+} as charge carrier.
[d]This study used 2 mM Ca^{2+}, data for isoflurane and enflurane from Joksovic *et al*. (2004).

particularly volatile anesthetics like isoflurane, may be used to differentiate native currents arising from $Ca_v3.3$ and $Ca_v2.3$ genes.

An interesting feature of anesthetic-induced blockade of $Ca_v3.3$ currents is that two mechanisms of channel blockade may occur. Volatile anesthetics like isoflurane and enflurane share the ability to increase the rate of inactivation of both recombinant and native T currents. On the contrary, neither of the barbiturates, N_2O, nor ethanol significantly affected current kinetics of $Ca_v3.3$ or nRT T channels. Similarly, we noticed this effect on current inactivation for isoflurane in our experiments with $Ca_v3.1$ and $Ca_v3.2$ recombinant channels (Todorovic *et al*., 2000). Halothane produces a similar increase in inactivation rate of T currents in native GH3 cells (Herrington & Lingle, 1991; 1992). This suggests that inhibition of both native and recombinant T currents can occur by either of the two mechanisms, either one of which can produce complete inhibition of T current.

We noticed that N_2O at lower concentrations enhances nRT currents. Similar dual mechanism of modulation of T currents by ethanol was recently reported in thalmocortical neurons in slice preparation (Mu *et al*., 2003). Even though the mechanism of this dual modulation is not clear, this suggests that thalamic neurons may be uniquely regulated by general anesthetics and ethanol.

Contribution of T channels to clinical effects of volatile anesthetics

The block of recombinant $Ca_v3.3$ and nRT currents by volatile anesthetics at concentrations believed to exist in brain tissue during anesthesia states suggests that this block may be clinically relevant. For volatile anesthetics in most mammals, clinically relevant concentrations are assumed to be similar to aqueous concentrations of anesthetic in brain tissue at inspired partial pressures (minimal alveolar concentration – MAC) that prevent purposeful movement to painful stimuli, such as surgical incision in 50% of subjects. For isoflurane, 1 MAC for *in vitro* experiments at room temperature is 320 μM, and for enflurane it is 620 μM (Franks & Lieb, 1994). In our studies, IC_{50}s for inhibition of $Ca_v3.3$ currents are 260 and 360 μM for isoflurane and enflurane, respectively. Furthermore, enflurane blocks nRT T current even more potently with an IC_{50} of 148 μM (Joksovic *et al*., 2004). Therefore, it appears that suppression of both $Ca_v3.3$ and nRT, as well as other T-channel isoforms (Table 1), occurs at subanesthetic concentrations of fluorinated ether anesthetics.

Native T-channel isoforms with properties similar to recombinant $Ca_v3.3$ currents have not been characterized pharmacologically, although slow-inactivating T currents have been recorded in various brain regions such as nRT (Huguenard & Prince, 1992), lateral habenula (Huguenard *et al*., 1993), and inferior olive (Llinás & Mühlethaler, 1988), where these channels are thought to underlie long-lasting subthreshold burst firing. Furthermore, *in situ* hybridization studies indicate that these brain regions have high expression of $Ca_v3.3$ mRNA (Talley *et al*., 1999). This suggests that suppression of $Ca_v3.3$-based currents by volatile anesthetics in CNS neurons would occur during clinical anesthesia. However, our comparison of anesthetic sensitivities of $Ca_v3.3$ and nRT T currents reveals that substantial differences exist in pharmacological sensitivities to anesthetics between these kinetically similar T-current variants. Particularly, paramount sensitivity of nRT T currents to N_2O raises the possibility that these currents may arise from $Ca_v3.2$ gene. This is supported by findings that mRNA for both $Ca_v3.2$ and $Ca_v3.3$ is abundant in nRT neurons (Talley *et al*., 1999). Since expression of native T channels may be dependent on post-translational modifications and tissue-specific factors, more work is necessary to elucidate molecular structure of slowly inactivating T currents in nRT neurons. Since $Ca_v3.3$ channel may undergo post-translation modification giving rise to multiple splice variants in the CNS (Mittman *et al*., 1999; Murbartián *et al*., 2002), it is important to study in future experiments the effects of anesthetics on native cells where these splice variants are expressed.

Inhibition of $Ca_v3.3$ and nRT currents by parenteral anesthetics and ethanol

We tested a variety of parenteral anesthetics for their ability to suppress recombinant $Ca_v3.3$ and native nRT currents. It is more difficult to determine clinically relevant concentrations of parenteral anesthetics for *in vitro* experiments. This is mainly because these anesthetics are used for induction of the anesthetic state due to their rapid redistribution from CNS tissue to body lipids. It has been estimated that clinically relevant anesthetic concentrations for propofol and etomidate are in the low μM range, and 25, 50, and 100 μM for thiopental, pentobarbital, and phenobarbital, respectively (Franks & Lieb, 1994). It appears from our data that inhibition of $Ca_v3.3$ currents, as well as nRT currents, may occur during clinical use of volatile anesthetics but is less likely that this would occur during general anesthesia with parenteral anesthetics. These differential sensitivities of various T currents to general anesthetics may contribute to different clinical properties. For example, volatile anesthetics that are more potent in blocking T-type Ca^{2+} currents are also much better analgesics and more profoundly suppress sensory information processing measured with sensory evoked potentials, than parenteral anesthetics like propofol and etomidate (Barash *et al*., 1992). At least part of these effects of volatile anesthetics may be mediated by depression of T currents in peripheral sensory and central neuronal pathways.

On the other hand, clinically relevant concentrations of ethanol are 50–100 mM (Catlin *et al*., 1999; Walter & Messing, 1999). We found that $Ca_v3.3$ currents are relatively resistant to up to 100 mM ethanol, but ethanol blocked completely native nRT T currents with an IC_{50} of 100 mM. This indicates that blockade of nRT but not $Ca_v3.3$ currents may occur under clinically relevant blood levels of ethanol and reveals another important pharmacological difference among nRT and $Ca_v3.3$ currents.

In conclusion, we have examined the sensitivity of $Ca_v3.3$ and native nRT T currents to various classes of general anesthetics and ethanol. Our results indicate that in spite of similar kinetic properties, anesthetic sensitivity of these two T-channel isoforms is greatly different for most of the tested agents. Furthermore, the effects of volatile general anesthetics and ethanol on these currents may contribute to their clinical and behavioral effects.

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