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$Ca_V3.2$ Channel Is a Molecular Substrate for Inhibition of T-Type Calcium Currents in Rat Sensory Neurons by Nitrous Oxide

SLOBODAN M. TODOROVIC, VESNA JEVTOVIC-TODOROVIC, STEVEN MENNERICK, EDWARD PEREZ-REYES, and CHARLES F. ZORUMSKI

Departments of Anesthesiology (S.M.T., V.J.-T.) and Psychiatry (S.M.T., V.J.-T., S.M., C.F.Z.), Washington University School of Medicine, St. Louis, Missouri; and Department of Pharmacology, University of Virginia, Charlottesville, Virginia (E.P.-R.)

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ABSTRACT

Although nitrous oxide (N_2O; laughing gas) remains widely used as an anesthetic and analgesic in clinical practice, its cellular mechanisms of action remain inadequately understood. In this report, we examined the effects of N_2O on voltage-gated Ca^{2+} channels in acutely dissociated small sensory neurons of adult rat. At subanesthetic concentrations, N_2O blocks low-voltage-activated, T-type Ca^{2+} currents (T currents), but not high-voltage-activated (HVA) currents. This blockade of T currents was concentration dependent, with an IC_{50} value of 45 ± 13%, maximal block of 38 ± 12%, and Hill coefficient of 2.6 ± 1.0. No desensitization of the response or change in current kinetics was observed during N_2O application. The magnitude of T current blockade by N_2O does not seem to reflect any use- or voltage-dependent properties. In addition, T current blockade was not altered when intracellular GTP was replaced with guanosine 5′-(γ-thio)triphosphate or guanosine 5′-0-(2-thiodiphosphate) suggesting a lack of involvement of G-proteins in the inhibition. N_2O selectively blocked currents arising from the $Ca_V3.2$ but not $Ca_V3.1$ recombinant channels stably expressed in human embryonic kidney (HEK) cells in a concentration-dependent manner with an apparent affinity and potency similar to native dorsal root ganglion currents. Analogously, the block of $Ca_V3.2$ T currents exhibited little voltage- or use-dependence. These data indicate that N_2O selectively blocks T-type but not HVA Ca^{2+} currents in small sensory neurons and $Ca_V3.2$ currents in HEK cells at subanesthetic concentrations. Blockade of T currents may contribute to the anesthetic and/or analgesic effects of N_2O.

Nitrous oxide has been widely used in clinical practice for more than 150 years because of effective analgesic properties that are achieved at concentrations below those required for general anesthesia. These analgesic effects coupled with rapid onset and short duration of action have made N_2O the oldest inhalational anesthetic used in clinical anesthesia and analgesia. Although great progress has been achieved in the last decade in understanding the cellular actions of other general anesthetics (Franks and Lieb, 1994), the cellular mechanisms of N_2O remain less clear, mostly because of the great difficulty in working with this agent in vitro. We recently reported that like the dissociative anesthetic ketamine, N_2O blocks the NMDA subtype of glutamate receptors (Jevtovic-Todorovic et al., 1998) and NMDA receptor-mediated excitatory synaptic currents in hippocampal microcultures (Mennerick et al., 1998). Others have reported similar effects of N_2O on currents from cloned NMDA receptors as well as neuronal nicotinic receptors (Yamakura and Harris, 2000). However, effects of N_2O upon voltage-gated Ca^{2+} channels that influence cellular excitability in sensory neurons involved in processing nociceptive information are not known.

Both low-voltage-activated (or T-type) and high-voltage-activated (HVA) types of Ca^{2+} currents are expressed in sensory neurons and are well characterized (Carbone and Lux, 1984; Fox et al., 1987; Nakashima et al., 1998; Todorovic and Lingle, 1998). While HVA channels play prominent roles in synaptic transmission (Miller, 1998), T-type channels are thought to play a unique role in regulating the excitability of CNS neurons (Llinas, 1988; Huguenard, 1996). Major proposed roles for T-type channels in neurons include promotion of Ca^{2+}-dependent burst firing, low-amplitude intrinsic neuronal oscillations, promotion of Ca^{2+} entry and boosting of synaptic signals (Huguenard, 1996). Furthermore, T-type

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; HVA, high-voltage-activated; T-type currents, T currents; HEK, human embryonic kidney; DRG, dorsal root ganglion; CgTx, conotoxin; DMSO, dimethyl sulfoxide; GTPγs, guanosine 5′-(γ-thio)triphosphate; GDPβS, guanosine 5′-0-(2-thiodiphosphate); CNS, central nervous system; MAC, minimum alveolar concentration; phenytoin, 5,5-diphenylhydantoin.

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currents (T currents) seem to play a role in seizure susceptibility and initiation (Tsakiridou et al., 1995). Recent studies also indicate that T-type currents in sensory neurons play an important role in modulating peripheral nociception (Todorovic et al., 2001).

Despite the fact that in some preparations T currents can be isolated from other Ca^{2+} current components by virtue of their unique biophysical properties, it is now clear that T currents in native cells are complex, reflecting the contribution of multiple channel isoforms. The T-type channel family is encoded by three genes, and the channels are named $Ca_v3.1$ (α1G), $Ca_v3.2$ (α1H), and $Ca_v3.3$ (α1I) (Cribbs et al., 1998; Perez-Reyes et al., 1998; Lee et al., 1999; Ertel et al., 2000). Subsequent work has revealed that nodose sensory neurons express both $Ca_v3.1$ and $Ca_v3.2$ channels (Lambert et al., 1998). In these sensory neurons, $Ca_v3.2$ dominates and contributes about 50% of the Ca^{2+} that enters neurons during action potentials (Lambert et al., 1998). $Ca_v3.1$ and $Ca_v3.2$ have kinetic features similar to native channels described in DRG cells but their pharmacological separation in native cells is difficult because of the lack of selective antagonists. Here we show that nitrous oxide selectively blocks low-voltage activated (T-type) but not HVA Ca^{2+} currents in acutely dissociated sensory neurons of adult rat at concentrations that are comparable with those used for clinical analgesia. Furthermore, up to 80% N_2O blocks only $Ca_v3.2$ but not $Ca_v3.1$ currents in HEK cells. Thus, N_2O may serve as a tool to study currents arising from $Ca_v3.2$ channels in native cells.

Materials and Methods

Cell Preparation. HEK cells were stably transfected with either rat $Ca_v3.1a$ (cell line Nr2+) or human $Ca_v3.2$ constructs (cell lines AH-13 or Q31) as described previously (Lee et al., 1999). Cells were typically used 1 to 3 days after plating. For DRG neurons, 100- to 400-g male rats (Sprague-Dawley) were used as described elsewhere (Todorovic et al., 1998; Todorovic and Lingle, 1998). Animals were anesthetized with halothane, rapidly decapitated, and 8 to 10 DRG from thoracic and upper lumbar regions were dissected and incubated at 36°C for 60 to 90 min in Tyrode's solution (140 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, adjusted to pH 7.4 with NaOH) supplemented with 5 mg/ml collagenase (type I; Sigma Chemical Company, St. Louis, MO) and 5 mg/ml dispase II (Roche Molecular Biochemicals, Indianapolis, IN). Single neuronal cell bodies were obtained by trituration in Tyrode's solution at room temperature. Cells were kept at room temperature and used for electrophysiology within 4 to 6 h after dissociation. For recordings, neuronal cell bodies were plated onto a glass cover slip and placed in a culture dish that was perfused with external solution. All data were obtained from smaller diameter DRG neurons (21 to 27 μm) without visible processes.

Electrophysiological Methods. Recordings were made with standard whole-cell, voltage-clamp techniques (Hamill et al., 1981). Electrodes were fabricated from microcapillary tubes (Drummond Scientific Company, Broomall, PA), coated with Sylgard (Dow Corning, Midland, MI), and fire-polished. Pipette resistances were 2 to 5 MΩ. Voltage commands and digitization of membrane currents were done with Clampex 6.0 of the pClamp software package (Axon Instruments, Foster City, CA) running on an IBM-compatible computer. Membrane currents were recorded with an EPC 7 patch-clamp amplifier (List Medical Instruments, Darmstadt, Germany). Reported series resistance and capacitance values were taken from the reading of the amplifier. For HEK cells, the average uncompensated series resistance (R_s) was 5.3 $\pm$ 2.0 MΩ and average capacitance (C_m) was 21.2 $\pm$ 6.1 pF (mean $\pm$ S.E., N = 34). Series resistance typically was compensated 60 to 80% without significant oscillations in the current trace. The average C_m for DRG cells was 14.6 $\pm$ 2.5 pF, and average R_s was 6.4 $\pm$1.0 MΩ (mean $\pm$ S.E., N = 51).

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, Foster City, CA) and Origin 4.5 (Microcal Software, Northhampton, MA). Currents were filtered at 5 kHz. All experiments were done at room temperature (20–23°C). In most experiments, leakage subtraction was used with a P/5 protocol for on-line leakage subtraction.

Analysis of Current Blockade. The percentage reduction in peak T current at a given N_2O concentration was used to generate concentration-response curves. Because it is not possible to measure actual concentrations of dissolved N_2O in solutions, we determined an apparent maximal block indicated by the response to 80% N_2O in all cells included in concentration-response curves. For each concentration-response curve, all points are averages of multiple determinations obtained from at least five different cells. All concentrations were applied to the cells until an apparent steady state effect was achieved. On all plots, vertical bars indicate standard errors. Mean values on concentration-response curves were fit to the function: $PB([N_2O]) = PB_{max} / [1 + (IC_{50} / [N_2O])^{nH}]$, where PB_{max} is the maximal percent block of peak T current, the 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.

The voltage-dependence of peak conductance activation and steady-state inactivation was described with a Boltzmann distribution: $I(V) = I_{max} / (1 + \exp[-(V - V_{50}) / k])$, where I_{max} is maximal activatable current, V_{50} is the voltage where half of the current is activated or inactivated, and k (units of millivolts) represents the voltage dependence of the distribution. The time course of T current recovery from inactivation was examined using a single exponential fit. Curve fitting was done with Origin 4.5.

Solution Exchange Procedures. The solution application system consisted of multiple, independently controlled glass capillary tubes with flow 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 minimize evaporation and loss of N_2O). Changes in Ca^{2+} current amplitude in response to N_2O containing solutions or ionic changes were typically complete in 20 to 40 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 Ca^{2+} currents in DRG cells contained: 160 mM TEA-Cl, 10 mM HEPES, 5 to 10 mM $BaCl_2$, adjusted to pH 7.4 with TEA-OH; osmolarity, 316 mOsM. Cells were generally maintained in a Tyrode's solution until seal formation, at which time the bath solution was switched to the Ba^{2+} saline. Internal solution consisted of 110 mM Cs-methane sulfonate, 14 mM phosphocreatine, 10 mM HEPES, 9 mM EGTA, 5 mM Mg-ATP, and 0.3 mM tris-GTP, pH adjusted to 7.15 to 7.20 with CsOH (standard osmolarity, 300 mOsM). When this internal saline was used for recording T currents in DRG cells, most of the HVA current in these cells was blocked by preincubating cells with 1 μM ω-CgTx-GVIA, 2 μM ω-CgTx-MVIIC, and 5 μM nifedipine in the external solution, to block N-, P-, Q-, and L-type HVA currents, respectively. Because in control experiments the effect of this cocktail was irreversible for up to 60 min, we routinely preincubated every slide with these toxins and recorded within this time frame. In most cells included in this study, blockade of L- and N-, P-, and Q-type currents was sufficient to allow investigation of T current in practical isolation. Because of the possibility of some residual HVA current contamination, all measurements of T current amplitude in DRG cells were made from the peak of the inward current to the current remaining at the end of a 200-ms test step. Typically, the residual HVA current at 200 ms was indistinguishable from leak current.

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Drugs and Chemicals. ω-CgTx-GVIA and ω-CgTx-MVIIC were obtained from RBI/Sigma (Natick, MA). All other chemicals were obtained from Sigma or Aldrich Chemicals (Milwaukee, WI).

Drug Preparation. For addition of gas, the extracellular solution was bubbled with air or N_2O/O_2 mixtures using a bubbling stone. The bubbling container was sealed with Parafilm and was punctured with a small escape hole. The solution was equilibrated with gas for at least 30 min, at 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 with its tip positioned 100 μm from the cell. For most experiments, 80% N_2O/20% O_2 was used, and bottled air (80% N_2/20% O_2) was used as control. Lower concentrations of N_2O were achieved by diluting an 80% solution in extracellular saline, which resulted in lower concentrations of N_2O but kept O_2 content constant. All solutions containing N_2O were used for experiments within 1 h of bubbling. A stock solution of phenytoin (600 mM) was prepared in dimethyl sulfoxide (DMSO) and was kept at 4°C until use. DMSO (0.5%) had no effects when tested alone in DRG cells or HEK cells transfected with Ca_V 3.1 constructs.

Results

Effects of N_2O on Voltage-Gated Ca^{2+} Channels in Rat Sensory Neurons. Nitrous oxide blocked T-type currents partially and reversibly in all small DRG cells tested (N = 51) (Fig. 1A). In contrast, HVA currents were not affected significantly by up to 80% N_2O (Fig. 1B). The average amplitude of HVA Ca^{2+} current in the presence of 80% N_2O was 95.6 ± 4.6% (mean ± S.E., N = 12 cells) of the control response. Even when tested on the same cell (Fig. 1C), 80% N_2O blocked only T-type, but not HVA Ca^{2+} currents. In five cells, application of extracellular solution equilibrated with air did not affect the amplitude of T currents (Fig. 1D). No desensitization of response was observed when the same concentration of N_2O was applied repeatedly (Fig. 1D) or when N_2O was applied for up to 5 min (N = 4, data not shown). No change in T current kinetics was observed during N_2O application (Fig. 1A). In 13 small DRG cells, the 10 to 90% rise time of T current was 10.8 ± 3.0 ms before and 10.9 ± 3.0 ms during application of 80% N_2O at a test potential of −30 mV (mean ± S.D., not significant by t test). Similarly, the T current inactivation time constant (τ) in the same cells was not significantly altered by 80% N_2O (control, 33.6 ± 5.9 ms; N_2O, 38.7 ± 9.7 ms; N = 13).

N_2O blockade of T currents was concentration dependent, as shown in Fig. 1E. At all concentrations, the onset and offset of block was rapid. N_2O blocked DRG T currents with an IC_{50} value of 45 ± 13%, a maximal block of 38 ± 12%, and a Hill coefficient of 2.9 ± 1.1 (Fig. 1F). We were unable to test N_2O at concentrations > 80% because DRG cells did not tolerate the hypoxia.

Mechanisms of Blockade of T-type Ca^{2+} Currents in Rat Sensory Neurons. A paired-pulse protocol was used to assess whether N_2O affects the voltage dependence of inactivation of T channels (Todorovic and Lingle, 1998). A family of currents evoked by this protocol is depicted in Fig. 2A before and during application of 80% N_2O. In Fig. 2B, average results from five experiments similar to the one in Fig. 2A are plotted, and the solid line indicates the best fit to the data with a Boltzmann equation. These experiments indicate that 80% N_2O had little effect on the voltage dependence of inactivation of DRG T currents.

Similarly, there was little effect of N_2O on the voltage dependence of activation (N = 4, Fig. 2C) and time course of recovery from inactivation (N = 3, Fig. 2D). The magnitude of blockade by N_2O was identical at stimulation frequencies of 1/20 or 1/5 sec (Fig. 2E, N = 10), indicating that the magnitude of T current blockade by N_2O does not seem to reflect any use dependence.

Because some T-channel antagonists (e.g., mibefradil) compete for channel binding sites with permeant ions (Martin et al., 2000), we examined whether changing the charge carrier concentrations or replacing Ba^{2+} with Ca^{2+} in the external solution influences N_2O effects on T currents. The amplitude of blockade of T currents by 80% N_2O was not altered when Ba^{2+} was replaced with equimolar Ca^{2+} or when the concentration of divalent charge carrier was 1, 2, 5, or 10 mM (N = 3 for each condition, data not shown).

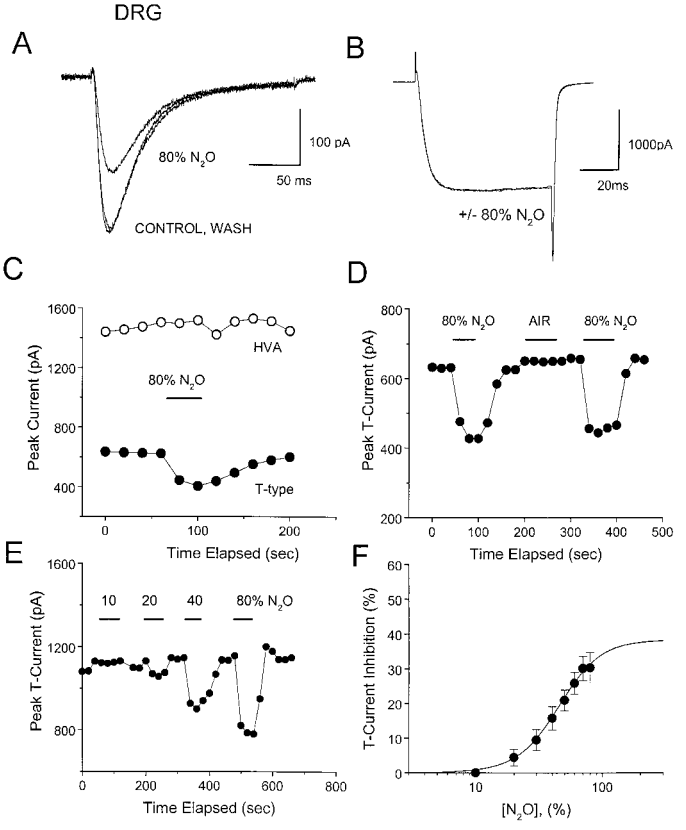

Fig. 1. Nitrous oxide selectively blocks T-type Ca^{2+} currents in rat DRG neurons in a concentration-dependent manner. A, T currents evoked at a test potential (V_t) of −30 mV from a holding potential (V_h) −90 mV are reversibly blocked by 80% N_2O. Note that most of the inward current inactivates during 200-ms test pulse. B, representative traces of HVA (high-voltage-activated) Ca^{2+} currents that are evoked from V_h −60 mV and V_t −10 mV in another DRG cell were not affected by 80% N_2O. C, peak inward current is plotted as a function of time in another DRG cells that had both HVA and T currents. Currents were isolated with double-pulse voltage-clamp protocols: T currents V_h −90 mV for 20 s and V_t −40 mV for 200 ms, HVA currents V_h −50 mV for 100 ms and V_t 0 mV for 40 ms. Note that N_2O application (indicated by horizontal bar) reversibly reduced about 30% of T current (●) but had very little effect on peak HVA current (open symbols). D, time course of the peak T current from another cell in which 80% N_2O and air were applied. Note that air had no effect, whereas N_2O reproducibly depressed the peak T current with fast onset and offset. E, Temporal record showing the effect of 4 escalating concentrations of N_2O as indicated by horizontal bars on peak T current in the same DRG neuron. F, The average concentration-response curve generated from experiments similar to one presented in E. Points are average of at least five different cells (total N = 12 cells). Vertical bars indicate S.E., and solid line indicates best fit using unrestricted dose response equation, giving 38 ± 12% for maximal block, IC_{50} of 45 ± 13%, and Hill coefficient 2.6 ± 1.0.

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The cellular effects of some other volatile anesthetics (e.g., halothane) seem to be mediated by G-protein-dependent processes (Johns, 1998). We examined whether G-proteins could be involved in the block of T current in DRG cells using known activators and inhibitors of G-proteins. In one set of experiments, inhibition of G-protein–mediated signaling was achieved by introduction of 2 mM GDPβS into the recording pipette. This antagonist of G-protein activation (Holz et al., 1986) failed to alter the response of DRG cells to 80% N_2O (Fig. 3A). In a second set of experiments, the recording pipette contained 100 μM GTPγS, which activates G-proteins persistently and prevents subsequent effects of G-protein-dependent agonists. However, neither baseline T currents nor responses to repeated applications of 80% N_2O were affected in rat DRG cells (Fig. 3B). Overall, 80% N_2O inhibited 35 ± 2% of T currents in the presence of GDBβS ($N = 5$) and 34 ± 1% in the presence of GTPγS ($N = 6$). In control conditions with GTP in recording pipette, this concentration of N_2O inhibited 31 ± 2 ($N = 11$) of T current. Previously, we found that these two compounds inhibited muscarinic receptor-mediated blockade of HVA Ca^{2+} currents in DRG cells, indicating that these agents can be used to probe G-protein-dependent processes in these cells (Nakashima et al., 1998).

Effects of N_2O on Cloned T-type Ca^{2+} Channels. The partial effects of N_2O on T currents in DRG cells could result from selective effects on T-channel subtypes. Several isoforms of α1 subunits of T-channels have been cloned recently (Cribbs et al., 1998; Perez-Reyes et al., 1998) and are referred

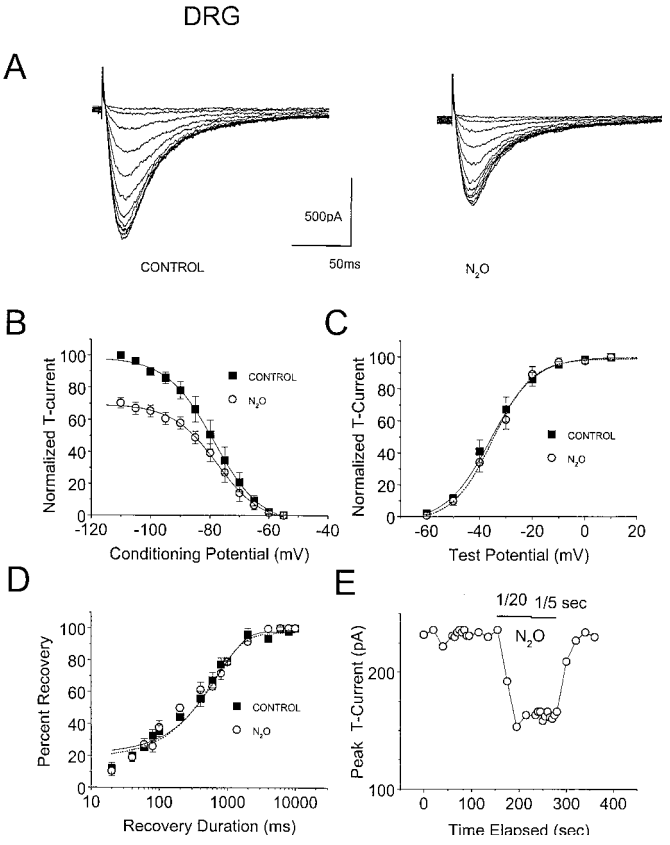

Fig. 2. Nitrous oxide has little effect on the voltage-dependence of inactivation and activation of T current in DRG cells. A, the traces depict T currents evoked by steps to −30 mV after 5-s prepulses to potentials ranging from −110 to −55 mV before (left) and during application of 80% N_2O (right). B, peak amplitudes from experiments similar to the one depicted in A are plotted as a function of voltage. Each point is the average of 5 cells with vertical lines indicating S.E. Data were normalized to the control at −110 mV. Solid line is best fit of the data using a Boltzmann equation. In control conditions (■), half-maximal availability occurred at −78.6 ± 0.5 mV with a slope factor of 7.9 ± 0.5. For N_2O (open symbols) half-maximal availability was at −77.6 ± 0.5 mV and a slope factor of 7.6 ± 0.5. C, nitrous oxide does not affect voltage-dependence of T current activation in DRG cells. All points on this figure are averages of four different cells and solid line represents the best fit of the Boltzmann equation before and during application (dotted line) of 80% N_2O. To obtain these data, we measured tail currents at the end of a 20-ms pulse to activate channels at various test potentials indicated on *x*-axis (V_h −90 mV). Under these conditions, the tail current amplitude is directly proportional to the number of channels that are open at the end of activating pulse. The best fit of the Boltzmann equation for control (■) gave half-maximal activation at −36.0 ± 1.5 mV and a slope factor of 8.5 ± 1.0 (solid line). In the presence of 80% N_2O (○ and dotted line) half-maximal activation was −35.0 ± 1.2 mV with a slope factor of 7.8 ± 1.0. D, Recovery from inactivation is not affected by N_2O in small DRG cells. Recovery from inactivation was examined in three cells before and during N_2O application with a paired-pulse protocol in which a 100-ms step to −30 mV was first used to inactivate most T current. After a variable recovery interval from 20 to 10,000 ms at −90 mV, a second test step to −30 mV was used to determine the amount of T current that had recovered from inactivation during the recovery period. The best fit with a single exponential curve indicated half-recovery in 587 ± 55 ms for controls (solid line) and 625 ± 83 ms (dotted line) in the presence of N_2O. As in C, all points are normalized to the maximal response in either control conditions or during the application of 80% N_2O. E, T current was elicited by steps to −30 mV from a V_h of −90 mV either every 20 s or every 5 s. The magnitude of peak current block by 80% N_2O is similar in both cases indicating lack of use-dependent blockade.

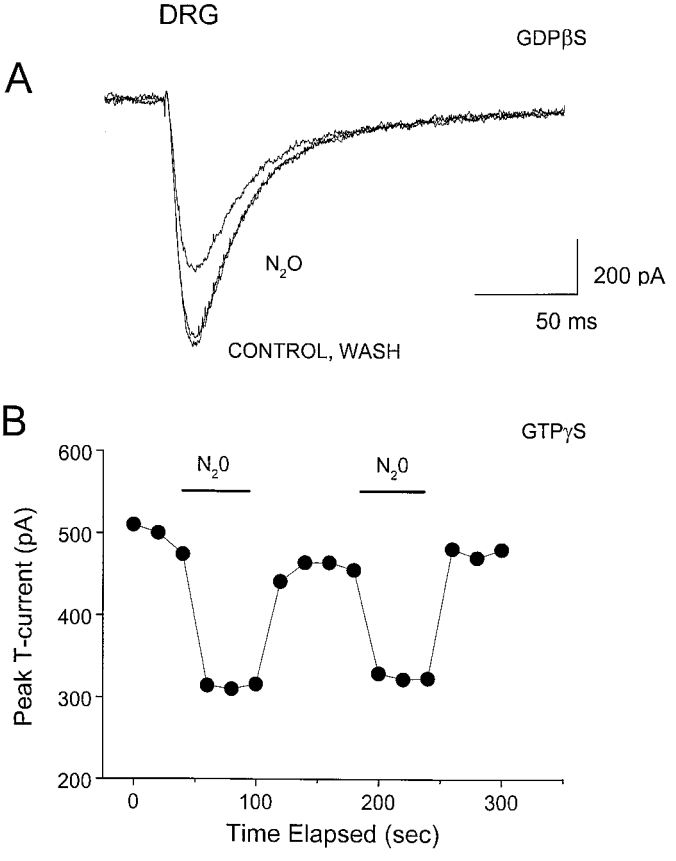

Fig. 3. Effects of nitrous oxide on T currents are not G-protein-mediated. A, representative traces from an experiment with 2 mM GDPβS in the recording pipette. 80% N_2O reproducibly and reversibly depressed peak T current in the presence of this inactive GTP analog. Bars indicate calibration. (V_h −90 mV, V_t −30 mV). B, the temporal record of peak T current. GTP in recording pipette was replaced with 100 μM GTPγS, which tonically activates G-proteins. Neither peak T current nor maximal blocking effect of N_2O was affected.

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to as $Ca_v3.1$ (α1G), $Ca_v3.2$ (α1H), and $Ca_v3.3$ (α1I). $Ca_v3.1$ and $Ca_v3.3$ channel variants are mostly expressed in the brain (Talley et al., 1999). $Ca_v3.3$ can be distinguished readily from other cloned variants and native sensory T currents by much slower kinetics and activation and inactivation at more depolarized potentials (Lee et al., 1999). Molecular studies have shown that mRNAs for all of these isoforms are present in smaller DRG cells; $Ca_v3.2$ mRNA has the highest expression (Talley et al., 1999). Similarly, $Ca_v3.2$ is thought to be the most abundant subtype in nodose sensory ganglia (Lambert et al., 1998). However, because of similar kinetic features of $Ca_v3.1$, $Ca_v3.2$ and currents in native sensory neurons and lack of selective antagonists for these isoforms, it is not possible to pharmacologically determine the relative contribution of T-channel subtypes to T currents in native sensory neurons. This led us to examine the effects of N_2O on cloned T-channel subtypes in HEK cells, focusing on $Ca_v3.2$, the predominant channel in nociceptors, and $Ca_v3.1$, the predominant channel in cerebellum and other CNS regions (Talley et al., 1999). Figure 4 illustrates that N_2O, at a concentration that maximally blocked DRG T currents, had little effect on currents mediated by $Ca_v3.1$ channels. Figure 4B demonstrates that in the same cell that had little response to N_2O, the anticonvulsant phenytoin (300 μM) blocked the current almost completely. In eight cells, we found that 80% N_2O produced only 3.8 ± 2.3% change in $Ca_v3.1$ currents.

In contrast, the two upper panels of Fig. 5 demonstrate that 80% N_2O blocked almost half of the total current mediated by $Ca_v3.2$ channels. In 11 HEK cells, 80% N_2O blocked 41 ± 2% of $Ca_v3.2$ total current (mean ± S.E.). As in DRG cells, no desensitization was observed with applications of N_2O up to 5 min (Fig. 5B, $N = 6$). The effects of N_2O on $Ca_v3.2$ current were concentration-dependent (Fig. 5, C and D) with an IC_{50} value of 58 ± 17%, maximal block of 66 ± 16%, and Hill coefficient of 2.1 ± 0.7 (Fig. 5E). In control experiments, external saline bubbled with air had very little effect on the amplitude of

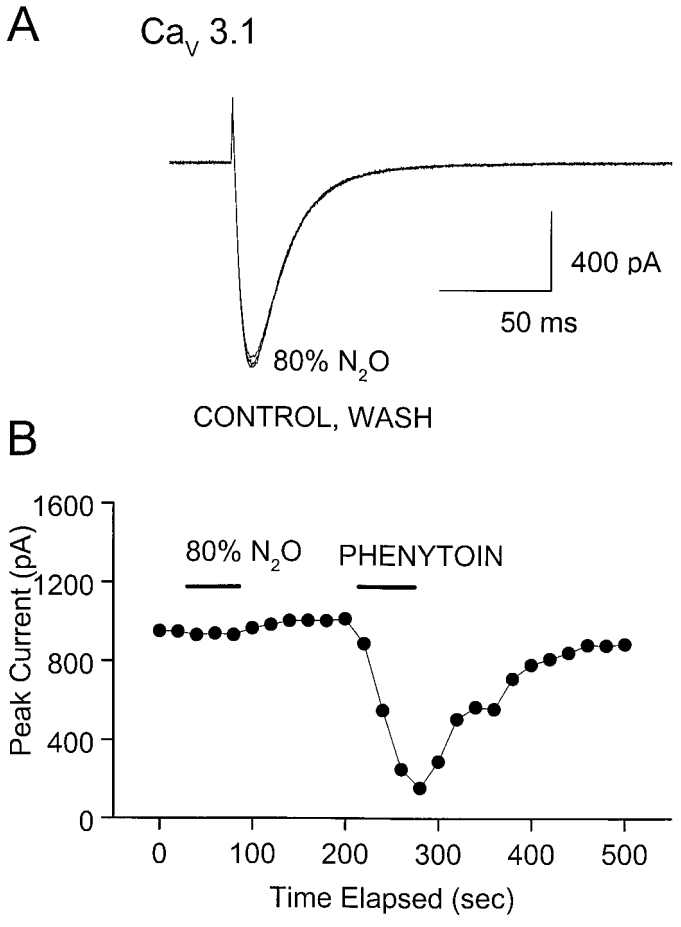

Fig. 4. The lack of effect of nitrous oxide on $Ca_v3.1$ currents in HEK cells. A, Inward currents evoked in HEK cells stably transfected with rat $Ca_v3.1a$ subunit of T-channels (V_h −90 mV, V_t −30 mV) are little affected by 80% N_2O. B, Time course from the above experiment where the peak amplitude of current is plotted against time. Although N_2O had very little effect on peak current amplitude, the anticonvulsant phenytoin (0.3 mM) blocked most of the current in these cells.

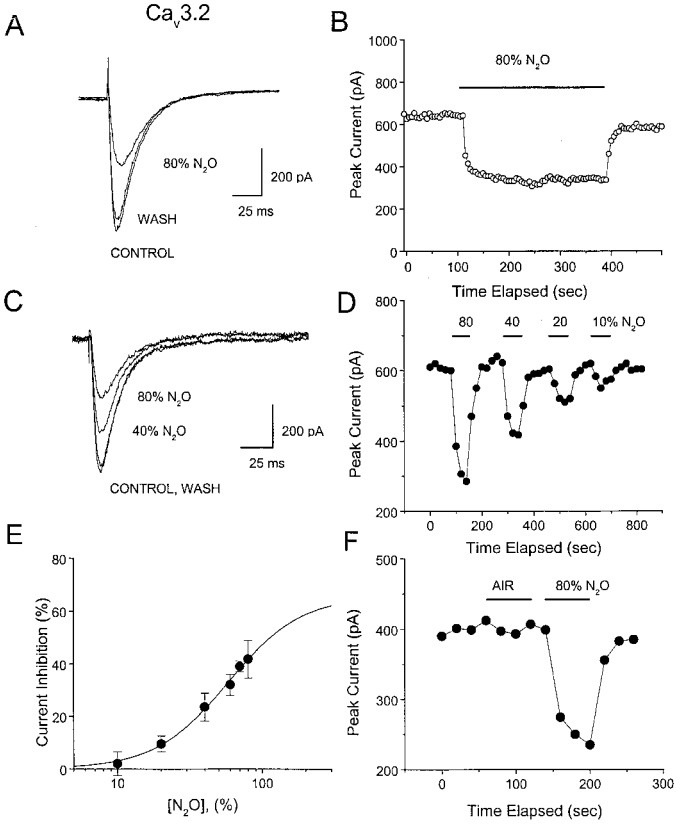

Fig. 5. Nitrous oxide blocks $Ca_v3.2$ currents in a concentration-dependent manner. A, The traces (V_h −90 mV, V_t −30 mV) from an experiment illustrating that in contrast to $Ca_v3.1$ currents, $Ca_v3.2$ −based currents in HEK cells were reversibly blocked when exposed to 80% N_2O. Eighty percent N_2O blocked reversibly about 45% of inward current. B, the time course of N_2O effect on $Ca_v3.2$ currents in the same cell depicted on A of this figure. The peak of the current evoked every 5 s was plotted showing fast onset and offset of blockade by 80% N_2O that was applied for 5 min. Note that this prolonged application of N_2O did not show desensitization of response. C, the concentration-dependent blockade of $Ca_v3.2$ current in a HEK cell. The traces show blockade of $Ca_v3.2$ currents by 40 and 80% N_2O. D, in the same cell shown in C of this figure, the time course of concentration-dependent block of N_2O is illustrated. Horizontal bars indicate progressively smaller concentrations of N_2O obtained by dilution of 80%-gassed saline. E, the average concentration-response curve generated from experiments in $Ca_v3.2$ transfected HEK cells. Points are averages of at least five different cells (total $N = 11$ cells). Vertical bars indicate S.E., solid line is best fit of the Hill equation giving maximal block was 66 ± 16%, IC_{50} = 58 ± 17%, and Hill n_H is 1.7 ± 0.3. F, The time course from an experiment in HEK cell showing minimal effect of air and fast onset and offset of nitrous oxide-induced blockade of inward $Ca_v3.2$ currents carried by 10 mM Ba^{2+} ions. Horizontal bars indicate times of applications.

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$Ca_v3.2$ currents (Fig. 5F, $N = 5$). As in DRG cells, the block by N_2O showed little voltage dependence using steady-state inactivation protocols (Fig. 6) and blocked the same amount of current when the stimulation frequency was increased 4-fold ($N = 5$, data not shown). Also, as in DRG cells there was no significant effect on time course of current activation or inactivation. In 12 HEK cells, the 10 to 90% rise time was 5.8 ± 2.7 ms and 5.3 ± 1.8 ms, and the inactivation τ was 21.5 ± 8.5 and 23.2 ± 6.9 ms (mean ± S.D.) for control and 80% N_2O, respectively.

Discussion

Blockade of T-type Ca^{2+} Channels in Sensory Neurons. We report here that N_2O selectively blocks T-type currents in rat sensory neurons at concentrations used clinically for anesthesia and analgesia. N_2O has low potency as an anesthetic with a minimum alveolar concentration (MAC) that prevents response to a surgical stimulus in half of human subjects of 105% (Hornbein et al., 1982). However, in medical and dental practice, effective analgesic concentrations are in the range of 20 to 50% inhaled gas (0.2–0.5 MAC). In rats, reported MAC values range from 150 to 180% (delivered in a hyperbaric chamber; Gonsowski and Eger, 1994), whereas the reported ED_{50} for analgesia in the tail-flick test is only 67% (Berkowitz et al., 1977) or 0.3–0.4 MAC. This suggests that analgesic effects are achieved at subanesthetic concentrations and that cellular targets involved in analgesia may be distinct from those that mediate anesthesia. N_2O blocks NMDA currents in CNS neurons (Jevtovic-Todorovic et al., 1998; Mennerick et al., 1998) and expression systems (Yamakura and Harris, 2000) at clinically relevant concentrations. This effect can probably account for anesthesia because N_2O, unlike most other general anesthetics, has very little effect on γ-aminobutyric acid-mediated currents (Franks and Lieb, 1998; Jevtovic-Todorovic et al., 1998; Mennerick et al., 1998; Yamakura and Harris, 2000). However, although central blockade of NMDA receptors can contribute to analgesia, NMDA receptors do not generally mediate pain perception in peripheral sensory neurons under physiological conditions (Todorovic et al., 2001). Similarly, central opiates (Berkowitz et al., 1977; Chapman and Benedetti, 1979) and noradrenergic descending systems (Guo et al., 1996) could participate in the analgesic effects of N_2O but no clear target has been identified in primary sensory neurons. Voltage-gated T-type Ca^{2+} channels are abundant in smaller sensory neurons, the majority of which are sensitive to capsaicin, which identifies them as polymodal nociceptors (Cardens et al., 1995). Furthermore, we have recently shown that T-channels could be present in peripheral sensory endings that modulate peripheral thermal and mechanical nociception (Todorovic et al., 2001). Various neurotransmitters and neuromodulators released in response to noxious stimulation are known to alter the influx of Ca^{2+} through ligand and voltage-gated channels in nociceptive sensory neurons (Coderre et al., 1993; Levine et al., 1993). Intracellular Ca^{2+} then influences the excitability of sensory neurons. Previously, it was shown that even partial blockade of only 20% of T currents in nociceptors by neuromodulators such as serotonin is sufficient to decrease the excitability of these neurons (Sun and Dale, 1997). We show that N_2O blocks more than 30% of T currents in sensory neurons at concentrations that are analgesic. Thus, direct blockade of T-type Ca^{2+} channels in nociceptors could account at least partly for the potent analgesic effects of N_2O observed in vivo.

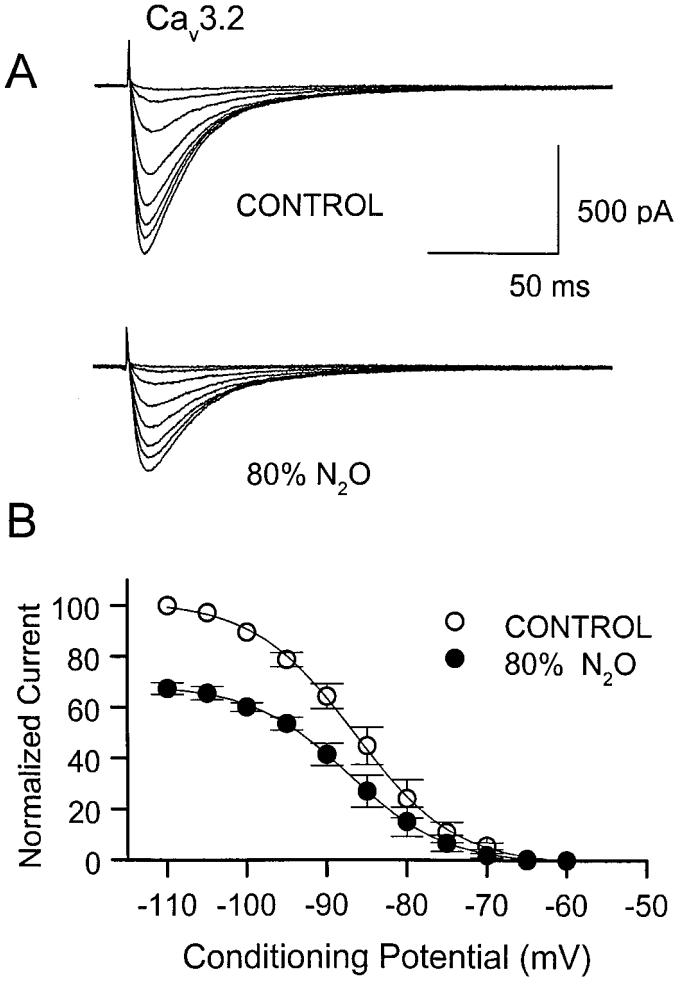

Fig. 6. Nitrous oxide has little effect on voltage-dependent inactivation of $Ca_v3.2$ currents in HEK cells. A, representative traces of inward currents elicited by steps to −30 mV after 5-s prepulses to potentials ranging from −100 to −65 mV before (top traces) and during application of solution bubbled with 80% N_2O (bottom traces). B, the peak amplitudes of inward currents from experiments like those in A are plotted as a function of voltage. Each point is the average of four cells normalized to maximal control response with vertical lines indicating S.E. Solid line is the best fit of Boltzmann distribution to the data points. In control (○), half-maximal availability was observed at −87.0 ± 0.3 mV with a slope factor of 6.3 ± 0.3. For N_2O (●) half-maximal availability was at −87.0 ± 0.2 mV and slope factor of 6.0 ± 0.2.

Mechanisms of Blockade of T-Type Ca^{2+} Currents. An interesting aspect of N_2O-induced blockade of T currents in rat sensory neurons is that only partial blockade is achieved. Many blockers are thought to inhibit ion channels by occluding the ion permeation pathways directly, inhibiting some modulatory pathways regulating channel behavior or producing allosteric changes in channel gating that favor inactivated or closed channel states. We did not observe any changes in current activation and inactivation in the presence of N_2O to account for allosteric modulation of the channel. In the case of N_2O, incomplete channel block does not result from state or use dependent properties. Similarly, this block is unlikely to be mediated by diffusible second messengers, because addition of GTPγS or GDPβS to the intracellular saline did not alter the ability of N_2O to inhibit T cur-

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rents. We also did not observe any desensitization to N_2O with repeated or prolonged applications. Inhibition of Ca^{2+} currents by G-protein–mediated signaling pathways often exhibits a characteristic desensitization (Shapiro and Hille, 1993). Partial block of other Ca^{2+} channel variants has been described and, in the case of blockade of P-type Ca^{2+} current by ω-conotoxin IIIa, it has been proposed that a partial reduction of the rate of ion permeation through the channel may account for the incomplete blocking effects (Mintz, 1994). Other general anesthetics, including the volatile anesthetics isoflurane and halothane, produce a complete blockade of DRG T currents (Todorovic and Lingle, 1998). However, a number of other compounds, including neuroactive steroids and the anticonvulsants phenytoin, valproic acid, and α-methyl-α-phenyl-succinimide, also produce incomplete blockade of T currents in rat sensory neurons, even when used at supramaximal concentrations (Todorovic et al., 1998; Todorovic and Lingle, 1998). In the case of T current blockade by anticonvulsants and neuroactive steroids, the mechanism underlying the partial blockade is unknown. It is possible that partial blockade by N_2O is only apparent because of inability to apply concentrations above 80% in vitro although concentration-response curves (Figs. 1F and 5E) predict saturation at less than complete block. It is likely that in vivo concentrations of N_2O in tissue are higher than in our experimental system, where there is some inevitable loss of volatile agents. However, to our knowledge, methods for measuring concentrations of dissolved N_2O in experimental solutions or body fluids are not yet available.

Inhibition of $Ca_v3.2$ but Not $Ca_v3.1$ Channels in HEK Cells. We tested the possibility that partial block of native DRG T-channels by N_2O reflects selective action at a T-channel isoform by examining the effects of N_2O on cloned T-type Ca^{2+} channel variants in HEK cells. Interestingly, we found that $Ca_v3.2$, but not $Ca_v3.1$ channels, are sensitive to inhibition by N_2O and that the block of $Ca_v3.2$ resembles the inhibition of T currents in DRG cells. However, the block of $Ca_v3.2$ currents, like the block of DRG T-channels, is also incomplete at N_2O concentrations up to 80%. Other general anesthetics block DRG T currents completely and block both T-channel variants in HEK cells with comparable potency (Todorovic et al., 2000). Thus, these other anesthetics do not allow determination of the relative contribution of the respective channels to the total currents in native DRG cells. At present, the reasons for incomplete inhibition of either $Ca_v3.2$ currents or native T currents by N_2O remain unclear. However, N_2O can be used as a tool to differentiate between these two variants of T-channels in native cells. $Ca_v3.2$ based currents could also be present in other sensory neurons in nociceptive pathways. Dorsal horn neurons in spinal cord express T currents (Ryu and Randic, 1990), which are present in both interneurons and spinothalamic cells (Huang, 1989). It was shown that mRNA for the $Ca_v3.2$ variant of T-channels is the most abundant isoform in superficial layers of dorsal horn (Talley et al., 1999), an area that receives most of the sensory input from peripheral polymodal nociceptors. Therefore, blockade of T currents in this area could also contribute to analgesic properties of N_2O.

In conclusion, we show that the general anesthetic N_2O blocks T-type but not HVA currents in acutely dissociated sensory neurons of adult rat. Because these neurons are involved in nociceptive processing and blockade is achieved at subanesthetic concentrations, T-channel inhibition could contribute to the analgesic properties of this widely used general anesthetic. In HEK cells stably transfected with α1 subunit constructs of T-channels, N_2O blocked $Ca_v3.2$ but not $Ca_v3.1$ currents. These data strongly suggest that $Ca_v3.2$ channels contribute significantly to the T currents in native DRG cells of small size that process nociceptive information.

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Address correspondence to: Dr. Slobodan M. Todorovic, University of Virginia Health System, Department of Anesthesiology, PO Box 800710, Charlottesville, VA 22908-0710.

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