

G-protein Activation Decreases Isoflurane Inhibition of N-type Ba²⁺ Currents

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Background: G-protein activation mediates inhibition of N-type Ca²⁺ currents. Volatile anesthetics affect G-protein pathways at various levels, and activation of G-proteins has been shown to increase the volatile anesthetic potency for inhibiting the electrical-induced contraction in ileum. The authors investigated whether isoflurane inhibition of N-type Ba²⁺ currents was mediated by G-protein activation.

Methods: N-type Ba²⁺ currents were measured in the human neuronal SH-SY5Y cell line by using the whole cell voltage-clamp method.

Results: Isoflurane was found to have two effects on N-type Ba²⁺ currents. First, isoflurane reduced the magnitude of N-type Ba²⁺ currents to a similar extent (IC₅₀ ~ 0.28 mM) in the absence and presence of GDPβS (a nonhydrolyzable GDP analog). Interestingly, GTPγS (a nonhydrolyzable GTP analog and G-protein activator) in a dose-dependent manner reduced the isoflurane block; 120 μM GTPγS completely eliminated the block of 0.3 mM isoflurane and reduced the apparent isoflurane potency by ~ 2.4 times (IC₅₀ ~ 0.68 mM). Pretreatment with pertussis toxin or cholera toxin did not eliminate the GTPγS-induced protection against the isoflurane block. Furthermore, isoflurane reduced the magnitude of voltage-dependent G-protein-mediated inhibition of N-type Ba²⁺ currents, and this effect was eliminated by pretreatment with pertussis toxin or cholera toxin.

Conclusions: It was found that activation of G-proteins in a neuronal environment dramatically reduced the isoflurane potency for inhibiting N-type Ba²⁺ currents and, in turn, isoflurane affected the G-protein regulation of N-type Ba²⁺ currents.

DUE to their role in neuronal excitability and synaptic transmission, voltage-dependent Ca²⁺ channels are important targets for volatile anesthetic (VA) action. Blockade of voltage-dependent Ca²⁺ channels enhances anesthetic potency^{1,2} and N-type blockers act as analgesics,^{3,4} which support the relevance of N-type channels to anesthesia. In dorsal-root ganglia neurons, isoflurane does not inhibit R-type currents, whereas it inhibits T-, L-, N-, and P/Q-current types with IC₅₀ of 0.28, 0.35, 0.68, and 1.46 mM, respectively.^{5,6} However, when expressed in *Xenopus* oocytes, P/Q-, N-, L-, and R-current types are inhibited to a similar extent by isoflurane.⁷ When expressed in *Xenopus* oocytes, neuronal L-type currents do not display inactivation in both the

presence and absence of VAs,⁷ whereas L-type currents display inactivation in various neuronal cells.⁸⁻¹¹ These reports suggest that the response to VA is dependent on the cellular environment, which in turn relates to channel subunit composition and the compliment of regulatory factors involved. To examine the role of G-protein regulation, we chose to study the VA action on N-type Ba²⁺ currents in a neuronal environment by using the human SH-SY5Y cell line. In these cells, we have previously shown that halothane at clinically relevant concentrations decreased the magnitude, accelerated the inactivation rate, and slowed the activation rate of L-type Ba²⁺ currents.¹¹

VAs may specifically affect neuronal intracellular Ca²⁺ by modulating the function of N-type currents, because these currents are only found in neurons and neurosecretory cells. G-protein activation inhibits N-type Ca²⁺ currents in every type of neuron yet studied and contributes to the modulation of electrical activity and synaptic function between neighboring neurons. VAs affect G-protein pathways at different levels,¹²⁻¹⁶ and in some cases decreasing the level of G-protein activation lowers the potency of VAs.^{17,18} In this study we investigated whether the VA inhibitory action on N-type Ba²⁺ currents was mediated by VA actions on G-proteins. Interestingly, we found that G-protein activation in a neuronal environment strongly decreased the isoflurane potency for inhibiting N-type Ba²⁺ currents and, in turn, isoflurane decreased the G-protein voltage-dependent modulation of N-type Ba²⁺ currents.

Materials and Methods

The human neuroblastoma SH-SY5Y cells were grown as previously described.¹¹ For these studies, cells were differentiated with 10 μM retinoic acid for 3-6 weeks to increase the expression level of N-type Ca²⁺ channels. We used Ba²⁺ as the permeant ion to increase the current magnitude. Exposure to retinoic acid results in cells with extensive processes, which limits the quality of the voltage clamp. To decrease contributions attributable to variable voltage-clamp errors between control and treated groups, we performed control and treated current measurements in the same cells. A two-electrode voltage clamp was used (Dagan 3900; Dagan Corporation, Minneapolis, MN). Ag/AgCl electrodes made direct contact with both aqueous solutions. Output from the voltage clamp amplifier was sent to a microcomputer (12-bit resolution, 125 kHz) data acquisition interface (Labmaster; Axon Instruments, Foster City, CA).¹¹ The

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whole cell voltage clamp mode was used. Pipette resistances were 1.6–4.0 M Ω . The pipette solution contained (in mM) CsCl 120, Mg(ATP)₂ 2, EGTA 10, HEPES 10, TEA-Cl 10, pH 7.4. The bath solution contained (in mM) NaCl 110, BaCl₂ 10, MgCl₂ 2, Glucose 10, HEPES 10, pH 7.4; to it were added 1 μ M tetrodotoxin and 10 μ M nitrendipine to block the voltage-dependent Na⁺ and L-type Ca²⁺ currents, respectively. Under these conditions, over 95% of the voltage-dependent Ba²⁺ currents were blocked by 500 nM ω -Conotoxin (type GVIA), an N-type Ca²⁺ channel blocker (3–5 min exposure). Ba²⁺ currents were measured by applying two identical sets of depolarizing pulses (50 ms), one before (eliciting the P1 current) and another after a depolarizing conditioning prepulse to +80 mV (eliciting the P2 current). The conditioning prepulse relieves the level of G-protein-mediated voltage-dependent inhibition of N-type Ca²⁺ currents.¹⁹ Current measurements were done at the end of the P1 and P2 pulses, to eliminate contributions of a small-rapidly inactivating Ba²⁺ current observed in some cells and only in the P1 currents (not in the P2 currents).

Tetrodotoxin, ω -Conotoxin, pertussis toxin (PTX), the nonhydrolyzable GTP analog GTP γ S (Guanosine 5'-[3-Thiotriphosphate] Tetralithium Salt), and the nonhydrolyzable GDP analog GDP β S (Guanosine 5'-[2-thiodiphosphate] Trilithium Salt) were purchased from Sigma (St. Louis, MO). Cholera toxin (CTX) was purchased from Calbiochem (San Diego, CA). The bath solutions were applied by using syringe pumps with 10-ml glass syringes connected to a Teflon manifold solenoid valve (Cole-Parmer, Niles, IL) that was connected to a 0.24-ml chamber (Model RC-24; Warner Instruments, Hamden, CT) through a tubing with a total dead space of 40 μ l (15-cm length, ID 0.58 mm).¹¹ The chamber was filled to about 0.15 ml. Throughout the experiment bath solutions were continuously perfused at a rate of 24 ml/h, except during data acquisition when cells were not perfused to reduce the noise level of the current traces. Isoflurane solutions were prepared on the day of the experiment from a saturated solution prepared by equilibrating buffer over isoflurane overnight at room temperature. The isoflurane concentrations were measured by gas chromatography, in parallel experiments, at 1 min after isoflurane application. Isoflurane concentrations could not be measured from the buffer of the cells from which current data were collected, because buffer sampling would disrupt the experiment due to the small volume of our chamber. Bath solutions containing isoflurane were applied for a period of 3 min (corresponding to \sim 7.7 volume changes). Experiments were conducted at room temperature (23–26°C). At 25°C the hypothetical isoflurane aqueous concentration corresponding to one minimal alveolar anesthetic concentration is 0.47 mM.^{20,21} The membrane capacitance and series resistance were compensated by about 80%. The background conductance was subtracted, but not

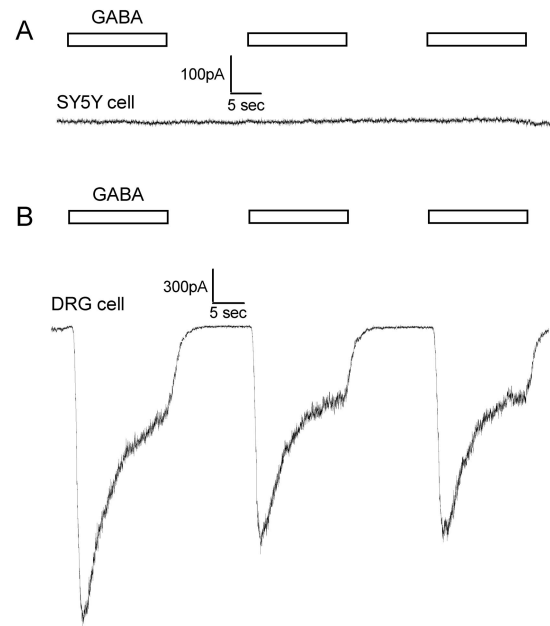


Fig. 1. Dorsal root ganglia neurons, but not SH-SY5Y cells, display GABA-evoked chloride currents. (A) Brief (15-s) application of GABA (30 μ M, open bars) directly on top of SH-SY5Y cells did not evoke current changes. (B) Identical GABA application on top of dorsal root ganglia neurons (DRG) produced the well-known Cl⁻ current increase that was reversible on washout. The membrane potential was -60 mV.

the residual capacitance transient. During acquisition, current traces were filtered at 10 kHz and digitized at 50 kHz; for data analysis, current traces were filtered at 2 kHz. For figures, the data for current traces was reduced by a factor of 8 (by taking the first of every 8 data points). Data were acquired and analyzed by using Pclamp8 (Axon Instruments, Foster City, CA) and GraphPad Prism 2.01 (GraphPad Software Inc. San Diego, CA) software.

We know of no studies describing GABA-A receptors or their Cl⁻ currents in SH-SY5Y cells. Because volatile anesthetics have been shown to increase Cl⁻ currents through activation of GABA-A receptors,^{22,20} we tested whether SH-SY5Y cells had functional GABA-A receptors. In SH-SY5Y cells, application of GABA (30 μ M) directly on top of the cell through a millimanifold (MLF-4 from ALA Scientific, NY; dead space < 50 μ l at 2.8 μ l/sec flow rate) produced no ionic current changes (11 cells, V_h = -60 mV) (fig. 1, A). Identical GABA application on adult rat cultured dorsal root ganglia neurons produced the well-known Cl⁻ current increase that was reversible on washout in all seven cells tested (fig. 1, B). Apparently SH-SY5Y cells do not have detectable functional GABA-A receptors.

In the absence (fig. 2, A) and presence (figs. 2, B and C) of GTP γ S, P1 and P2 currents display a decay/run-down on assuming the whole cell conformation; therefore, the isoflurane data were compared with control data collected at equivalent times from initiation of the

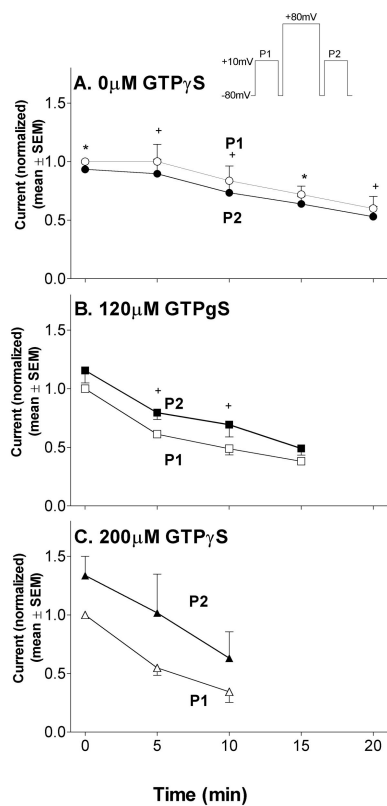


Fig. 2. N-type Ba^{2+} currents display a slow rundown in the absence and presence of $\text{GTP}\gamma\text{S}$. Current traces were measured at +10 mV by using the following voltage protocol: from a holding potential (Vh) of -80 mV (10 s), the membrane potential was depolarized to +10 mV for 50 ms (inducing the P1 current), back to Vh for 10 ms, then a depolarizing prepulse to +80 mV for 80 ms, back to Vh for 10 ms before a second 50 ms depolarization to +10 mV (inducing the P2 current) (inset). For each condition, P1 and P2 current levels were normalized to the P1 current level measured at time zero. Time zero corresponds to ~ 30 sec from formation of whole cell conformation, the time required for compensating the membrane capacitance and series resistance. Data were collected in the absence (A) P1 (\circ) and P2 (\bullet) and presence of $120 \mu\text{M}$ (B) P1 (\square) and P2 (\blacksquare) and $200 \mu\text{M}$ (C) P1 (\triangle) and P2 (\blacktriangle) $\text{GTP}\gamma\text{S}$ (n as in Fig. 3, D). Significant difference between P1 and P2 current values + $P < 0.05$, paired t test. The absolute current values at zero time were: 187 ± 25 pA ($n = 8$) for $0 \mu\text{M}$ $\text{GTP}\gamma\text{S}$, 160 ± 42 pA ($n = 6$) for $120 \mu\text{M}$ $\text{GTP}\gamma\text{S}$, and 43 ± 18 pA ($n = 6$) for $200 \mu\text{M}$ $\text{GTP}\gamma\text{S}$.

whole cell conformation. The cells exposed to $\text{GTP}\gamma\text{S}$ were allowed to equilibrate for 7 min after whole cell conformation was obtained, and the time interval between collecting control and isoflurane data was the same in the absence and presence of $\text{GTP}\gamma\text{S}$ (5 min). During this 5-min period (0–5 min for non- $\text{GTP}\gamma\text{S}$ group, 7–12 min for $\text{GTP}\gamma\text{S}$ group), there was a small current decay that was comparable for both groups ($\pm \text{GTP}\gamma\text{S}$).

Data Analysis

Comparison between different groups was performed using a two-tailed t test, paired or unpaired depending on the experimental design, by means of Sigma software (Jandel Scientific Corp., San Rafael, CA).

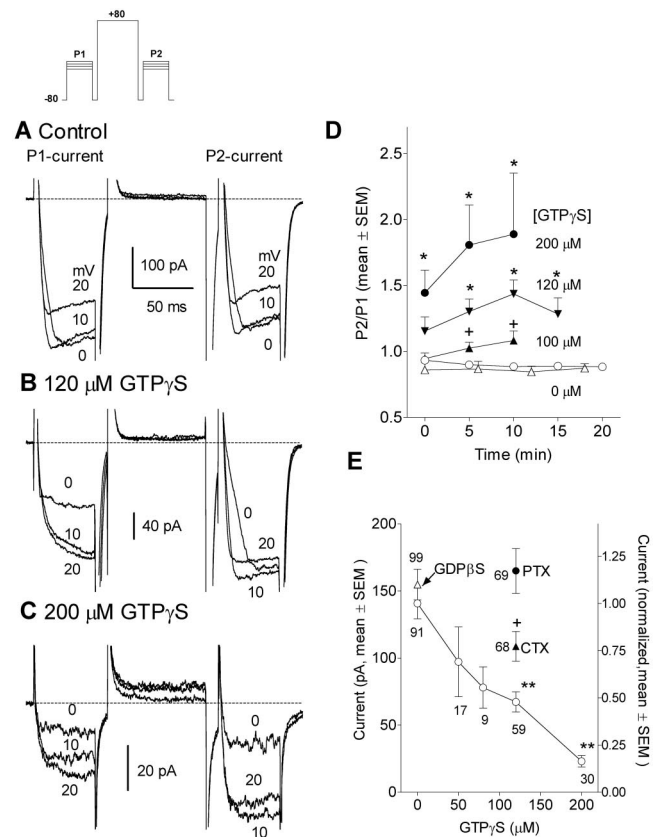


Fig. 3. $\text{GTP}\gamma\text{S}$ decreases the magnitude and increases the P2/P1 ratio of N-type Ba^{2+} currents in SH-SY5Y cells. Current traces were recorded at various membrane potentials using the voltage protocol described in Fig. 2 (inset). A family of current traces for three different cells, in the absence (A) and presence of $120 \mu\text{M}$ (B) and $200 \mu\text{M}$ (C) $\text{GTP}\gamma\text{S}$ are shown for 0, +10, and +20 mV. (D) The P2/P1 current ratio (at +10 mV) was calculated and plotted with respect to time, in either the absence (\circ , $n = 7$ for 0 and 5 min, $n = 5$ for 10 and 15 min, $n = 3$ for 20 min) and presence of $100 \mu\text{M}$ (\blacktriangle , $n = 7$ for 0 and 5 min, $n = 6$ for 10 min), $120 \mu\text{M}$ (\blacktriangledown , $n = 6$ for 0, 5, and 10 min, $n = 4$ for 15 min), and $200 \mu\text{M}$ (\bullet , $n = 5$ for 0 min, $n = 4$ for 5 min, $n = 3$ for 10 min) $\text{GTP}\gamma\text{S}$; and in the presence of $120 \mu\text{M}$ $\text{GTP}\gamma\text{S}$ group, * $P < 0.01$; + $P < 0.05$, nonpaired t test. (E) P1 current magnitude measured at 7 min from forming whole cell recording; n values are indicated. Significantly different from the $0 \mu\text{M}$ $\text{GTP}\gamma\text{S}$ group, ** $P < 0.00$; + $P < 0.05$. Left Y-axis shows absolute current values and right Y-axis shows current values normalized to the mean value of the $0 \mu\text{M}$ $\text{GTP}\gamma\text{S}$ group.

Results

GTPγS Reduces the Magnitude and Increases the P2/P1 Ratio of N-type Ba^{2+} Currents in SH-SY5Y Cells

G-protein mediated inhibition of N-type Ba^{2+} currents was measured by applying two sets of depolarizing pulses, one before (eliciting the P1 current) and the other after a depolarizing prepulse to +80 mV (eliciting the P2 current) (fig. 3). The prepulse to +80 mV, partially relieves the G-protein-mediated voltage-dependent block in the subsequent P2 pulse.¹⁹ The level of the G-protein-mediated voltage-dependent block was mea-

sured as the change in the P2/P1 current ratio.¹⁹ In the absence of G-protein activators the magnitude of P1 and P2 currents and kinetics are similar (fig. 3, A), suggesting that in the whole cell conformation the level of endogenous G-protein activation is minimal. In fact, similar P2/P1 current ratio values were found in the absence of the G-protein activator GTP γ S (fig. 3, D, open circles) and in the presence of the G-protein deactivator GDP β S (fig. 3, D, open triangles). In the absence of G-protein activation, the P2 current magnitude tended to be slightly lower than the P1 current magnitude (fig. 3, A); hence, the P2/P1 current ratio was about 0.9 rather than unity (fig. 3, D, open symbols). A possible reason for the P2/P1 ratio being lower than unity under these conditions could be due to incomplete removal of N-type current inactivation before measuring the P2-current. In the absence of G-protein activation the P2/P1 current ratio was constant over the observation time (fig. 3, D, open symbols), indicating that the P2/P1 current ratio is not affected by the slow rundown of N-type Ba²⁺ currents (fig. 2, A).

Addition of GTP γ S to the pipette solution slowed the activation kinetics of P1 currents with respect to that of P2 currents (figs. 3, B and C, vs. fig. 3, A), increased the P2/P1 current ratio (fig. 3, D), and decreased the N-type Ba²⁺ current magnitude (fig. 3, E) in a dose-dependent manner. These effects are characteristic of G-protein-mediated inhibition of N-type currents.^{23,19} The current magnitude in these cells is normally distributed (not shown) and highly variable (fig. 3, E). To reduce the effect of this variability when measuring the isoflurane effects, we collected control and isoflurane data from the same cells.

GTP γ S Decreases the Isoflurane Potency for Reducing N-type Ba²⁺ Currents

In the absence of GTP γ S, 0.3 mM isoflurane decreased the magnitude of N-type Ba²⁺ currents (fig. 4, A) without affecting their reversal potential (fig. 4, B), which is consistent with the absence of activation of other ionic currents. Isoflurane reduced the N-type Ba²⁺ currents in a dose-dependent manner with an apparent IC₅₀ of about 0.28 mM for both the P1 and P2 current (fig. 4). Reversal of the isoflurane-induced current reduction was very slow, ranging from 4–34% after a 3-min washout (corrected for rundown; data not shown).

To investigate the effects of isoflurane in the presence of GTP γ S, we selected 120 μ M GTP γ S, because at this concentration G-protein activation is not maximal (previous section, fig. 3, E). Therefore, we could test whether the isoflurane-induced reduction in N-type Ba²⁺ currents was due to enhancement of G-protein-mediated channel block. Interestingly, we found that in the presence of 120 μ M GTP γ S, isoflurane at 0.3 mM did not decrease but increased the P1 current magnitude in five out of six cells. The increase in P1 current magnitude

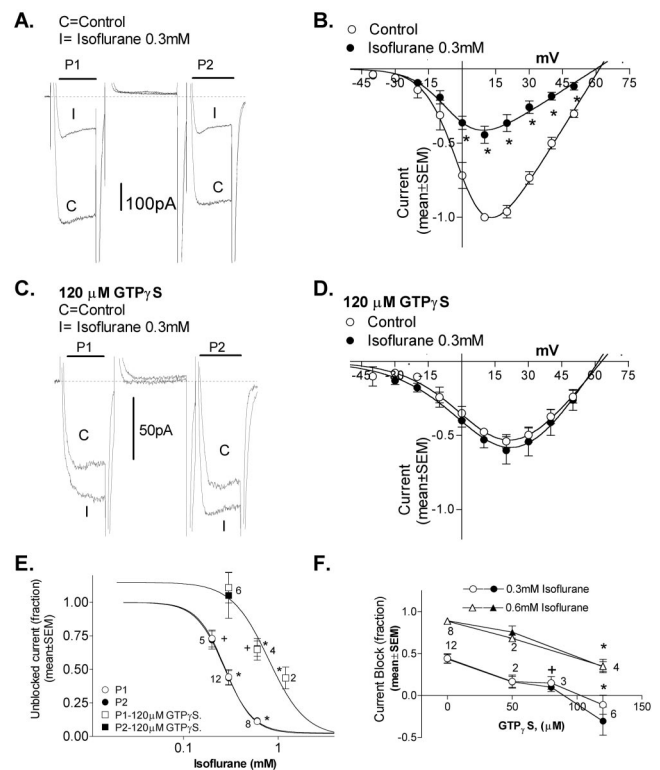


Fig. 4. Isoflurane effect on N-type Ba²⁺ currents in the absence and presence of 120 μ M GTP γ S. (A) Current traces at +10mV and (B) current-voltage relationship in absence of GTP γ S before and after the addition of isoflurane. Voltage protocol as in Fig. 3. Data were normalized to current level at +10 mV during control measurement and fit to a two-state Boltzmann distribution. $I_n = G_n \cdot (x - V_r) / (1 + \exp(-K \cdot (x - V_a) / 25.44))$, where I_n = normalized current, G_n = normalized slope conductance, V_r = reversal potential, V_a = midpoint potential. Control, $V_r = 61.5$ mV, $V_a = 0.6$ mV; isoflurane, $V_r = 59.9$ mV, $V_a = -3.9$ mV, $n = 12$. * $P < 0.001$, paired t test. (C) Current traces at +10mV and (D) current-voltage relationship in presence of 120 μ M GTP γ S before and after addition of isoflurane. Control current level at +10 mV was considered equal to 0.478, based on population current reduction observed with 120 μ M GTP γ S (Fig. 3, E). Control, $V_r = 62.9$ mV, $V_a = 11.5$ mV; isoflurane, $V_r = 61.4$ mV, $V_a = 14.8$ mV, $n = 6$. (E) Dose response of isoflurane-induced reduction of N-type Ba²⁺ currents. Data were normalized to either the P1 or the P2 current level measured in absence of isoflurane at time zero and fitted to a Hill function. In absence of GTP γ S, midpoint value = 0.28 mM, Hill coefficient = 2.94. In presence of 120 μ M GTP γ S (squares), a line was drawn by using midpoint value = 0.79 mM, Hill coefficient = 2.22, maximum current value = 1.15. Significantly different from control (no isoflurane), * $P < 0.01$; + $P < 0.05$, paired t test. (F) Fraction of isoflurane-induced block of N-type Ba²⁺ currents was measured in absence and presence of various concentrations of GTP γ S (P1, open symbols; P2, filled symbols) at two isoflurane concentrations. Significantly different from 0 μ M GTP γ S group, * $P < 0.01$; + $P < 0.05$ unpaired t test. In (E) and (F), n values are indicated.

was high in some cells (fig. 4, C), but on average the increase did not reach statistical significance (fig. 4, D). Elimination of the 0.3 mM isoflurane-induced current block and the tendency for isoflurane to increase the current magnitude was also observed in the P2 current (figs. 4, C and E). In the presence of 120 μ M GTP γ S, isoflurane was approximately three times less potent in inhibiting N-type Ba²⁺ currents (fig. 4, E, squares). The

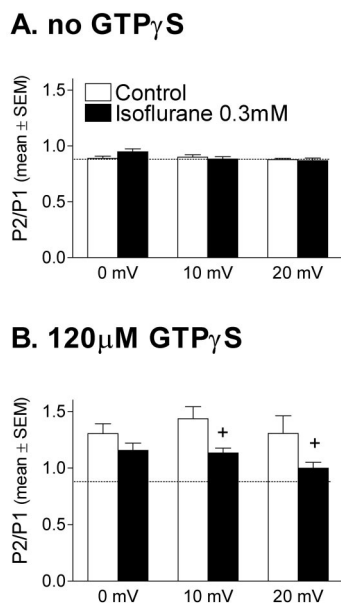


Fig. 5. Isoflurane decreases the P2/P1 current ratio magnitude in the presence but not in the absence of 120 μ M GTP γ S. The isoflurane effect on the P2/P1 current ratio magnitude in the absence (A) and presence (B) of 120 μ M GTP γ S. In the absence of GTP γ S, control n = 8; isoflurane n = 11 for 0 mV and n = 12 for 10 and 20 mV. In the presence of GTP γ S, control and isoflurane n = 4 at 0 mV and n = 6 for 10 and 20 mV. Significant difference between the control and isoflurane groups, + $P < 0.05$ unpaired t test.

effect of GTP γ S on reducing the isoflurane potency for inhibiting N-type Ba $^{2+}$ currents was dose-dependent (fig. 4, F).

The P2/P1 current ratio was not changed by isoflurane in the absence of GTP γ S (fig. 5, A), but it was significantly reduced by isoflurane in the presence of 120 μ M GTP γ S (fig. 5, B). The isoflurane-induced reduction in the P2/P1 current ratio seems to result from a slightly larger increase in the P1 current relative to the P2 current (fig. 4, C). The magnitude of the isoflurane-induced reduction in the P2/P1 current ratio values was observed in all cells at +20mV and in most cells at +10mV. In the presence of GTP γ S, the isoflurane reduction in the P2/P1 current ratio was completely reversed after 3 min of washout (data not shown).

Pretreatment with PTX and CTX

To further characterize the nature of the G-proteins involved in the above observations, cells were treated with either PTX or CTX. These toxins affect G-proteins by adenosine diphosphate ribosylating the G α subunits at different sites. PTX adenosine diphosphate ribosylates G α of "i, o and t types" at a cysteine, which leads to the accumulation of inactive GDP-bound G-proteins, whereas CTX adenosine diphosphate ribosylates G α of the "s and olf type" at an arginine, resulting in an initial activation followed by a strong down-regulation (1–8 h) of these G-proteins.^{24,25} Pretreatment with either PTX or CTX reduced the P2/P1 current ratio of cells exposed to

GTP γ S, toward baseline values (the value in the absence of GTP γ S) and eliminated the isoflurane-induced reduction in the P2/P1 current ratio (fig. 6, A). In contrast, pretreatment with either PTX or CTX did not significantly affect the GTP γ S-evoked protection against the isoflurane-induced reduction of N-type Ba $^{2+}$ currents (fig. 6, B), even though PTX pretreatment resulted in current values resembling those of untreated cells and CTX pretreatment resulted in a ~50% current recovery (fig. 3, E). These observations indicate that in these cells, G-protein-mediated current reduction (voltage-dependent and voltage-independent) of N-type Ba $^{2+}$ currents is mediated by PTX/CTX-sensitive G-proteins, whereas CTX/PTX-insensitive G-proteins mediate the decrease in potency of isoflurane in blocking N-type Ba $^{2+}$ currents.

Discussion

In this study we investigated whether isoflurane inhibition of N-type Ba $^{2+}$ currents was mediated by isoflurane action on G-proteins, such as an isoflurane-mediated augmentation of G-protein inhibition of N-type Ba $^{2+}$ currents. We found that isoflurane had two effects on N-type Ba $^{2+}$ currents that seem to involve different underlying cellular mechanisms. First, isoflurane reduced N-type Ba $^{2+}$ currents. This effect was considerably reduced on G-protein activation. Second, isoflurane reduced the P2/P1 current ratio only in the presence of G-protein activation.

Isoflurane-induced Reduction of N-type Ba $^{2+}$ Currents Does Not Require G-protein Activation but Is Strongly Affected by G-protein Activation

Isoflurane reduced N-type Ba $^{2+}$ currents in the absence of G-protein activation, both in the absence of GTP γ S and in the presence of GDP β S. Because GDP β S irreversibly inactivates all G-protein types, it is apparent that isoflurane does not mediate the reduction of N-type Ba $^{2+}$ currents by increasing the G-protein-mediated inhibition of N-type Ba $^{2+}$ currents.

Although isoflurane mediates a reduction of N-type Ba $^{2+}$ currents through a G-protein-independent pathway, we found the isoflurane inhibition of the N-type currents is in turn strongly affected by G-protein activation. Because both G-protein activation and isoflurane reduce N-type Ba $^{2+}$ currents, it is possible that the apparent reduction of isoflurane potency by GTP γ S is due to competitive reversal of the G-protein block of N-type Ba $^{2+}$ currents by isoflurane. Against this possibility is the observation that PTX and CTX treatment, respectively, eliminated or reduced the G-protein-mediated N-type Ba $^{2+}$ current block without eliminating the G-protein-mediated protection against isoflurane-mediated block. In addition, we approached this possibility by using competitive interaction concepts (Appendix). Our cal-

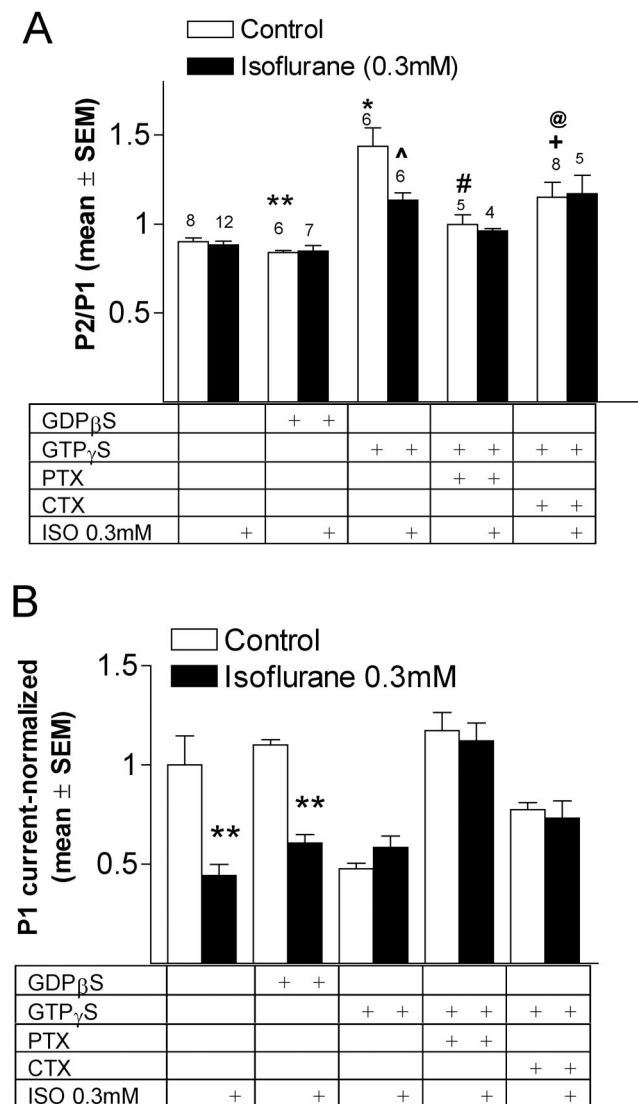


Fig. 6. Effect of PTX and CTX on the isoflurane action on N-type Ba^{2+} currents in the presence of $120 \mu\text{M}$ $\text{GTP}\gamma\text{S}$. Cells were treated overnight with either PTX or CTX (500 ng/ml), and current recordings were done in the presence of $120 \mu\text{M}$ $\text{GTP}\gamma\text{S}$ in the pipette. *n* values are indicated in (A) and are the same for (A) and (B). (A) Measured P2/P1 current ratio. In the absence of isoflurane (open bars), significant difference between the group and nothing pipette, * $P < 0.01$, + $P < 0.05$; between the group and the $\text{GTP}\gamma\text{S}$ group, ** $P < 0.001$, # $P < 0.01$, @ $P < 0.05$. Significant difference between control (open bars) and isoflurane (filled bars) in each group, $P < 0.05$, unpaired *t* test. (B) P1 current level. For each condition (nothing, $\text{GDP}\beta\text{S}$, $\text{GTP}\gamma\text{S}$, $\text{GTP}\gamma\text{S}/\text{PTX}$, $\text{GTP}\gamma\text{S}/\text{CTX}$), the mean control current level was set equal to the population value found for each condition (shown in Fig. 3, E). For each cell the first current measurement was done in the absence of isoflurane, then the second current measurement (5 min later) was done either in the absence (open bars) or presence of (filled bars) isoflurane and normalized to their corresponding first measurement. Significant difference between control and isoflurane within each condition was ** $P < 0.001$, unpaired *t* test.

calculations, based on a competitive interaction of isoflurane and activated G-protein molecules for a single binding site on the N-type channel, predict that in the presence of $120 \mu\text{M}$ $\text{GTP}\gamma\text{S}$, 0.6 mM isoflurane should

lead to a N-type Ba^{2+} current reduction of between 69–84% (Appendix), but we actually observed only a 35% current block (fig. 4, E). These calculations suggest that a competitive interaction cannot explain the $\text{GTP}\gamma\text{S}$ -evoked diminution in isoflurane-mediated inhibition of the N-type Ba^{2+} currents. One of the various alternative possibilities is that the binding of activated G-protein (at a site different from that mediating the increase in P2/P1 current ratio) leads to a conformational change in the channel and a decreased affinity of isoflurane for its binding site on the N-type channel. Another possibility is that even if two completely independent receptor sites are involved in initiating the $\text{GTP}\gamma\text{S}$ and isoflurane-evoked current reduction, their action has a late common path. In this case, one could expect that isoflurane will not produce the same level of fractional current block in the absence and presence of $\text{GTP}\gamma\text{S}$ but rather the same absolute amount of current reduction compared with untreated cells as it was found.

G-protein-mediated modulation of the isoflurane-induced current reduction may occur in some of the other voltage-dependent Ca^{2+} channels as well. It has been reported that the inhibitory effect of VA on R-type Ca^{2+} currents is prevented by strong activation of muscarinic receptors.²⁶ Because muscarinic receptors are G-protein-linked receptors, the underlying mechanism may have some similarities. Differential G-protein modulation may account in part for the different VA potency for blocking various Ca^{2+} channel types observed in dorsal root ganglia neurons^{5,6} and when expressed in *Xenopus* oocytes.⁷ Conversely, activation of G-proteins has been shown to increase the potency of VA for inhibiting the electrical-induced contraction in ileum.¹⁸ Then G-protein activation may modulate various VA effects, but the direction of the modulation (increase or decrease of the VA potency) might depend on the pathway involved.

Isoflurane Reduces the P2/P1 N-type Current Ratio in the Presence but not the Absence of $\text{GTP}\gamma\text{S}$

SH-SY5Y cells express a variety of G-proteins including PTX- and CTX-sensitive and -insensitive types.²⁷ In the presence of $120 \mu\text{M}$ $\text{GTP}\gamma\text{S}$, PTX pretreatment returned the P2/P1 current ratio to baseline (no $\text{GTP}\gamma\text{S}$), and CTX-treatment lowered the P2/P1 current ratio, suggesting that (in these cells) the voltage-dependent block of N-type currents is mediated by PTX/CTX-sensitive G-proteins. In the presence of either CTX or PTX treatment, isoflurane did not induce an additional decrease in the P2/P1 current ratio, which suggests that isoflurane affects the voltage-dependent action of PTX/CTX-sensitive G-proteins on N-type currents. The isoflurane-evoked reduction in the P2/P1 current ratio was accompanied by a tendency for a current magnitude increase (mostly in the P1 current level). The latter might explain the previously reported isoflurane-induced transient augmentation of N-type Ca^{2+} currents in rat dorsal root

ganglion.⁵ Another possibility that we cannot presently rule out is that the reduction in P2/P1 current ratio results from a slowing of the recovery for inactivation of N-type Ba²⁺ channels in the presence of GTPγS and isoflurane.

In summary, in the human neuronal SH-SY5Y cell line it was found that isoflurane blocks N-type Ba²⁺ currents in a G-protein-independent manner (does not require G-protein activation), that this isoflurane-evoked current block is drastically reduced by activation of CTX/PTX-insensitive G proteins, and that isoflurane decreases the CTX/PTX-sensitive G-protein-mediated voltage-dependent block of N-type Ba²⁺ currents. The differential dependence on G-protein activation and the apparent involvement of different G-protein types, indicate that both of the measured isoflurane effects (current reduction and P2/P1 current ratio reduction) are most likely mediated through different mechanisms. The level of activation of various G-proteins (and corresponding pathways) would be expected to modulate the VA potency between various cell types and in a given cell type between various physiologic states. Whether G-protein activation in the intact organism can lead to an alteration in volatile anesthetic sensitivity must be examined.

Appendix

Is the apparent reduction of isoflurane potency in the presence of GTPγS due to relief of the G-protein blocking effects on N-type currents? The receptor's affinity and binding site (channel) occupancy were estimated by:

$$Y_x = 1/[1 + 1/(K_x \cdot (X)^n)] \quad (1)$$

$$Y_x \cdot e = \{1/(1 + 1/(K_x \cdot (X)^n))\} \cdot e \quad (2)$$

where n = the number of drug molecules X that bind to one channel, Y_x = the fraction of channels occupied by "nX" number of molecules, K_x (mM^{-1}) = the equilibrium binding constant of X , ($1/K_x = K_D$, the dissociation constant), (X) = the concentration of X , $Y_x \cdot e$ = the fractional response, and e = drug efficiency.^{28,29} Because isoflurane and GTPγS can fully block N-type currents, we assume that their "e" is equal to one. We assume that GTPγS works by activation of G-proteins, and refer to channel occupancy by either isoflurane or by activated G-protein molecules. The $Y_x \cdot e$ was calculated from the inverse of the dose-responses curve and fitted with equation II (fig. 7). The data fit suggests that three isoflurane molecules have to bind to the channel to block the channel, and that each isoflurane molecule binds with a $K_D = 22 \mu\text{M}$. The estimated K_D value is lower than the measured K_D -apparent ($280 \mu\text{M}$) (fig. 4, *E*, circles), which is consistent with the idea that binding of more than one isoflurane molecule is needed for isoflurane to mediate channel block. Only one molecule of activated G-protein (~ GTPγS) is necessary to block the N-type channel with a K_D of $76 \mu\text{M}$.

We estimated how the binding site occupancy of each of these substances was affected by the presence of the other by using equations III and IV,^{28,29} where A and B refer to isoflurane and activated G-protein (~ GTPγS), respectively:

$$Y_a \cdot e = 1/(1 + 1/(K_A \cdot (A)^n) + (K_B \cdot (B))/(K_A \cdot (A)^n)) \quad (3)$$

$$Y_b \cdot e = 1/(1 + 1/(K_B \cdot (B)) + (K_A \cdot (A)^n)/(K_B \cdot (B))) \quad (4)$$

We first assumed that the binding of three isoflurane molecules to the channel was needed to displace the binding of one activated

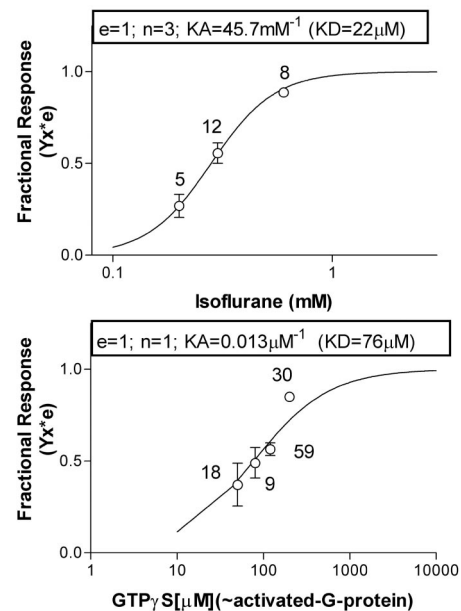


Fig. 7. Fractional response as a function of either isoflurane or GTPγS (see Appendix for details). The fractional response ($Y_x \cdot e$) was calculated from the inverse of the dose-response curves and was fitted with equation 2. The results of the fit for the fractional response for isoflurane are $K_x = 45.7 \text{ mM}^{-1}$ ($K_D = 22 \mu\text{M}$), $n = 3$ and for GTPγS are $K_x = 13.1 \text{ mM}^{-1}$ ($K_D = 76 \mu\text{M}$), $n = 1$.

G-protein molecule (3:1 competition). When present by themselves, the occupancies of 0.6 mM isoflurane (0.896) and $120 \mu\text{M}$ GTPγS (0.525) are higher than when present together ($Y_a = 0.797$, $Y_b = 0.125$). If in the presence of $120 \mu\text{M}$ GTPγS we have 67 pA , then the total current (in the absence of GTPγS) is 141 pA (fig. 3, *E*). In the presence of 0.6 mM isoflurane, $120 \mu\text{M}$ GTPγS occupies/blocks 12.5% of the channels, *i.e.*, it blocks 17.6 pA . In the presence of $120 \mu\text{M}$ GTPγS, 0.6 mM isoflurane occupies/blocks 79.7% of the channels, *i.e.*, it blocks 112 pA . In our experiments we take as the control current level the one present at $120 \mu\text{M}$ GTPγS, *i.e.*, 67 pA , and since we are left with 11 pA , then we have 84% current block. This is high compared with the actual measurement of 35% block with 0.6 mM isoflurane (fig. 4, *E*, squares).

We also considered that binding of only one isoflurane molecule is sufficient to displace the activated G-protein molecule (1:1 competition), and that all three isoflurane molecules are equally efficient in mediating such displacement. In this case, there is a higher level of displacement of the activated G-protein (by isoflurane), and displacement of activated G-protein could occur without a need of blocking the channel. In this case, the prediction was 68.8% block, which still is higher than the actual measurement of 35% block with 0.6 mM isoflurane (fig. 4, *E*). Therefore, displacement of activated G-protein by isoflurane through either a 3:1 or 1:1 competitive interaction does not account for the potency reduction of isoflurane in the presence of GTPγS.

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