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Research report

Volatile anesthetic inhibition of neuronal Ca channel currents expressed in Xenopus oocytes

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Abstract

The genes encoding the α_{1A}, α_{1B}, α_{1C} and α_{1E} subunits of neuronal high voltage-gated Ca channels (HVGCCs) were separately expressed with β_{1B} and α_2/δ subunits in Xenopus oocytes to determine the effects of volatile anesthetics (VAs) on currents through each specific channel. VA effects were determined on currents carried by Ba^{2+} (I_{Ba}) using the two electrode voltage clamp technique. Although time to peak was unaffected, both halothane (0.59 mM) and isoflurane (0.70 mM) reversibly inhibited peak I_{Ba} by 25–35% and late current (at 830 ms) by 50–60%. A hyperpolarizing shift in steady-state inactivation of α_{1E}-current was found which could contribute up to one third of observed decrease in the peak current. The rate of inactivation of I_{Ba} seen with α_{1A}, α_{1B} and α_{1E}-type Ca channels was consistently increased by halothane and isoflurane. To more clearly quantify these effects, I_{Ba} inactivation was fit by a single exponential function. The anesthetics depressed both the inactivating and non-inactivating residual components of I_{Ba} and decreased the time constant of inactivation. In the case of I_{Ba} through α_{1C}-type channels, inactivation was minimal; however, the average current was inhibited by VAs. Similar inhibition of all these HVGCCs by halothane and isoflurane suggests that a common structural component may be involved. Furthermore, the inhibition of such neuronal HVGCCs in situ could alter synaptic neurotransmitter release and contribute to the anesthetic state.

Keywords: Volatile anesthetic; Halothane; Isoflurane; Ca channel; Ca current; Xenopus oocyte

1. Introduction

Volatile anesthetics (VAs) have relatively low potency and only partial steric specificity compared with most other central nervous system acting drugs. They may therefore be expected to have multiple actions on the brain thereby contributing to the general anesthetic state [20,25]. These agents enhance and/or mimic the action of the inhibitory neurotransmitter GABA [19,23] and an apparent anesthetic-specific region on $GABA_A$ receptor subunits has recently been described [30]. In addition, high voltage-gated calcium channels (HVGCCs) also represent sites of VA action. Employing guinea-pig ventricular cells, it was first reported as early as 1988 that VAs inhibited Ca^{2+} currents [53,54]. This result was substantiated by several authors using myocardial tissue [3,26,35], as well as neural [21,40,49,50] and secretory cells [16,27,36].

While VA depression of Ca^{2+} currents would be expected to alter cell excitability and neurotransmitter release, selective effects of VAs on the specific neuronal HVGCC classes, such as P/Q, N, L and R-types [47,60] have yet to be determined. Recent developments in molecular biology have made it possible to study and characterize the different types of HVGCCs in isolation. The pore-forming region of these channels is composed of a single α_1 subunit with four domains, each containing six transmembrane segments. Auxiliary β and α_2/δ subunits appear to modulate the kinetics of channel opening and closing as well as increase the number of functional HVGCCs in artificial expression systems such as Xenopus oocytes or human embryonic kidney cells (HEK 293) [6,55]. Five distinct HVGCCs α_1 subunit messages (α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}) have been identified in the nervous system and also in the periphery (with an additional α_{1S} in skeletal muscle), each of which encodes a Ca channel subtype with distinct physiological and pharmacological characteristics. α_{1A} encodes a channel which can exhibit properties similar to either P- and Q-type [48]; α_{1B} en-

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codes N-type channels [57]; α_{1C} or α_{1D} represent the L-type channels [44], while the α_{1E} subunit appears to encode the toxin-resistant R-type channel [45,55,58,60]. Two of the α_1 subunits of T-type Ca channels, belonging to the low voltage-gated Ca channels (LVGCCs) have recently been cloned and designated as α_{1G} (T-1) and α_{1H} (T-2), respectively (see Ref. [38] for a review).

All of these Ca channel types have been reported to be present in the brain as well as in the periphery and are involved in synaptic neurotransmitter release in addition to other functions [12,18,32,39,41,47,59]. Any interference with synaptic neurotransmitter release may be relevant to the anesthetic state. In support of this hypothesis, a possible mechanism for modulation of neurotransmitter release is the perturbation of Ca currents. Evidently, VAs have been shown to depress mixed [21,49] as well as specific T-, L-, and N-type Ca currents [27,35,49,50] in cultured neurons. However, literature documenting the effects of VAs on specific types of HVGCCs in isolation is lacking, hence, such an investigation would be of help to better understand the relation between HVGCCs, the anesthetic state and the action of VAs. Furthermore, study of isolated currents permits determination of VA effects on the specific kinetic behavior of each. Therefore, we expressed α_{1A}, α_{1B}, α_{1C} and α_{1E} HVGCC subunits in Xenopus oocytes (in combination with β_{1B}, α_2/δ) and examined the effects of two widely studied VAs viz., halothane and isoflurane.

2. Materials and methods

2.1. Oocyte harvesting and microinjection

Mature female Xenopus laevis frogs were obtained from Xenopus I (Ann Arbor, MI), housed in an established frog colony, and fed regular frog brittle twice weekly. For the removal of oocytes, a frog was anesthetized in 500 ml of 0.2% 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO) in water until unresponsive to a painful stimulus. The anesthetized frog was placed supine on ice and an incision of $\sim$1.5 cm in length was made through both the skin and muscle layers of one lower abdominal quadrant. A section of the ovary was exteriorized and a lobule of oocytes ($\sim$200) was removed. The wound was closed in two layers and the animal was allowed to recover from anesthesia, kept in a separate tank overnight, and returned to the colony the following day. The oocytes were washed twice in calcium-free OR2 solution (in mM: NaCl 82.5, KCl 2, $MgCl_2$ 1.8, HEPES 5, pH 7.5) and transferred to OR2 solution containing 1 mg/ml of collagenase (type 1A; Sigma, St. Louis, MO). The oocytes were agitated in collagenase solution for a period of 2–3 h at room temperature in order to remove the follicular cell layer. Defolliculation was confirmed by microscopic examination. The oocytes were washed twice in OR2 solution and transferred to modified Barth's solution (in mM: NaCl 88, KCl 1, $NaHCO_3$ 2.4, $CaCl_2$ 0.41, $MgSO_4$ 0.82, HEPES 15, pH 7.4) containing 2.5 mM sodium pyruvate and 10 μg/ml gentamicin sulfate. They were allowed to recover for 3–10 h before cDNA injection. Nuclear (germinal vesicle) injection was performed (Drummond 'Nanoject', Drummond Scientific, Broomall, PA, USA) using 9.2 nl of a 1:1:1 mix (molar ratio; not exceeding a total of 3 ng of cDNA) of one type of α_1 subunit (rat brain), with β_{1B} and α_2/δ cDNA subunits subcloned in the mammalian expression vector pMT2 [46]. The oocytes were returned to Barth's solution and incubated at 16°C for 7–8 days before current recording.

2.2. Electrophysiological recording

Macroscopic currents were recorded using the two-electrode voltage-clamp technique with an Oocyte Clamp OC-725C amplifier (Warner Instrument, Hamden, CT) linked via an interface with an IBM-PC compatible computer equipped with pClamp software (version 5.6; Axon Instruments, Foster City, CA) for data acquisition. Leak currents were subtracted using the P/4 procedure. Microelectrodes were filled with 3 M CsCl; typical resistances were 0.5 to 2.5 MΩ. KCl-Agar bridges were used as ground electrodes to minimize any junction potential attributable to changes in ionic composition of the bath solution. The oocytes were placed in a recording chamber (300 μl volume) superfused continuously with a solution containing in mM: $Ba(OH)_2$ 40, NaOH 50, KOH 2, HEPES 5, pH 7.4 with methanesulfonic acid. Ba^{2+} was used as charge carrier in order to avoid Ca^{2+}-induced inactivation of HVGCCs [10] and activation of the intrinsic Ca^{2+}-activated Cl currents [$I_{Cl(Ca)}$]. Ba^{2+}-current (I_{Ba}) was recorded for a duration of 850 ms by depolarizing the oocytes to 0 mV from a holding potential of -80 mV. However, in the case of N-type channels ($\alpha_{1B}\beta_{1B}\alpha_2/\delta$), the oocytes were depolarized using $+20$ mV as the test potential. Current/voltage relationships for α_{1E}-type channels were determined from a holding potential of -80 mV using 350 ms depolarizing step potentials from -50 to 100 mV in 10 mV increments. All the oocytes exhibiting significant I_{Ba} underwent control, anesthetic treatment and wash protocols. Currents during control and wash conditions were recorded while the oocytes were continuously superfused with solution bubbled with air. More than one anesthetic concentration was applied to the same oocyte if the current returned to $>$90% of control after washout of agent. The recovery current amplitude after washout was considered the control value for the subsequent treatment.

2.3. Anesthetic administration

Output from a calibrated anesthetic-specific vaporizer was bubbled through a reservoir filled with 40 ml superfusion solution, allowing 7–10 min for equilibration. The

solution from the reservoir was then superfused through the recording chamber at a rate of approximately 5 ml/min, and current recording was performed 2 min after the oocyte was perfused with the anesthetic-equilibrated solution. Gas concentrations of 0.75, 1.5, and 2.25% halothane and 1.3, 2.5, and 4.0% isoflurane were used, which are equivalent potencies of the anesthetics and represent approximately 1, 2, and 3 times the inspired anesthetic concentration which prevents movement in response to a painful stimulus in 50% of subjects (minimum alveolar concentration, or MAC). Based upon the aqueous/gaseous partition coefficients [15], these gas phase concentrations resulted in aqueous concentrations at 25°C of 0.33, 0.59, and 1.19 mM halothane and 0.35, 0.70, and 1.07 mM isoflurane, respectively. To quantify the anesthetic concentrations in the recording chamber, triplicate samples from the chamber were equilibrated with air and analyzed in a gas chromatograph (Aerograph 940; Varian Analytical Instruments, Walnut Creek, CA) calibrated with standards for halothane and isoflurane.

2.4. Data analysis

The data are shown as mean ± S.E.M., unless otherwise indicated. The data were analyzed using either the PCS program [34] or Clampfit, version 6.0.2 (Axon Instruments). The Ba^{2+} conductance G_{Ba} was calculated as $I_{Ba}/(V - V_{rev})$ using the reversal potential (V_{rev}), as determined by interpolation over the voltage step at which current changed from inward to outward. The voltage-dependence of activation of G_{Ba} was then described by a Boltzmann equation of the form:

$$G_{Ba} = G_{Ba,max}\{1 + \exp[(V - V_n)/k_n]\}^{-1},$$

where V_n was the voltage of half maximal activation and k_n was the slope factor determined from a least squares fit (Sigma Plot, Jandel Scientific, San Rafael, CA). The inactivating component of I_{Ba} was described by a single exponential component with R^2 values consistently exceeding 0.98 for a least squares fit. This behavior was described by the formula:

$$I_{Ba} = A_1 \cdot \exp(-t/\tau_{inact}) + R,$$

where A_1 is the inactivating component, R is the residual non-inactivating component and τ_{inact} is the time constant of inactivation of I_{Ba}. Since descriptions employing two exponential components rarely provided improvements in the fit, only a single exponential was employed as done previously when α_1 subunits were expressed with the β_{1B} subunit [61]. As the α_{1C}-current inactivated slowly and also without a defined peak, the above kinetic parameters (A_1, τ_{inact}, and R) could not be analyzed. Therefore, I_{Ba} during the total period of depolarization for this subunit was averaged. Statistical significance was analyzed using paired t-test and a value of < 0.05 was considered significant.

3. Results

3.1. Properties of I_{Ba} through the expressed Ca channels

Whereas uninjected oocytes exhibited no currents upon depolarization (not shown), an inward current was induced in oocytes injected with HVGCCs α_{1A}, α_{1B}, α_{1C} and α_{1E} subunits when they were individually expressed with β_{1B} and α_2/δ subunits. Expression of the α_1-subunit alone led to a significantly smaller current, typically 2–10% of that seen with co-expression of the β_{1B} subunit. Coexpression of the α_2/δ subunit had a less significant effect (not shown). The basic properties of a typical inward I_{Ba} obtained with the various types of α_1 subunit in combination with β_{1B} and α_2/δ subunits are shown in Table 1. The maximum amplitude of I_{Ba} indicates the peak current, whereas the current at 830 ms (of a total duration of 850 ms) was designated as the late current and shows the inactivation status of the channels. The peak amplitude of I_{Ba} varied considerably among the types of HVGCCs being expressed. It cannot be determined if any selective expression of subunits or oocyte-derived factors may contribute to this variation. I_{Ba} through the dihydropyridine (DHP)-resistant channels (α_{1A}, α_{1B}, and α_{1E}) showed a defined peak that declined (inactivated) gradually over the course of the depolarization. Analysis of the inactivation component of I_{Ba} revealed that more than 75% of the peak I_{Ba} was the inactivating component A_1, while the non-inactivating R component comprised less than 25%. Cur-

Table 1
Properties of the expressed neuronal Ca channel currents in Xenopus oocytes

Subunits	Peak (nA)	Peak at (ms)	Late (nA)	A_1 (nA)	R (nA)	τ_{inact} (ms)	n
α_{1A}	−2307 ± 624	75 ± 9	−800 ± 341	−1747 ± 485	−532 ± 183	366 ± 59	6
α_{1B}	−1049 ± 171	64 ± 1	−166 ± 22	−864 ± 348	−139 ± 49	195 ± 34	11
α_{1E}	−432 ± 29	80 ± 3	−90 ± 7	−370 ± 25	−70 ± 5	290 ± 10	40
α_{1C}	−697 ± 220	–	–	–	–	–	9

Oocytes injected with the various Ca channel subunits were clamped 7–8 days after their injection. They were held at −80 mV and depolarized to 0 mV except α_{1B} channels which were tested at 20 mV. In case of α_{1C}-channels, the current obtained was averaged for the entire period of depolarization. The peak current denotes maximum amplitude during the depolarization, while the current seen at 830 ms was arbitrarily taken as the late current. A_1, its τ_{inact}, and R were determined using a least squares non-linear regression.

rents through the α_{1B} channel exhibited the fastest inactivation, with the α_{1E} and α_{1A} channels showing a slower decline. In contrast, current through the α_{1C}-channels, the only member of the DHP-sensitive group examined here, showed minimal inactivation and lacked a defined peak current (Fig. 1). Hence, parameters such as A_1, R, and τ_{inact} were not determined for α_{1C}-channel current. In this case, the lack of inactivation is due to the use of Ba^{2+} as a charge carrier which does not reproduce the Ca^{2+}-induced inactivation observed in L-type HVGCCs. To determine if halothane and isoflurane exhibited similar depressant actions of I_{Ba} across relevant membrane potentials, oocytes expressing α_{1E}-type channel were depolarized for a period of 350 ms to step potentials starting from −50 mV in 10 mV increments (Fig. 2). In the resulting current–voltage (I–V) relationships, I_{Ba} was first activated at −30 mV

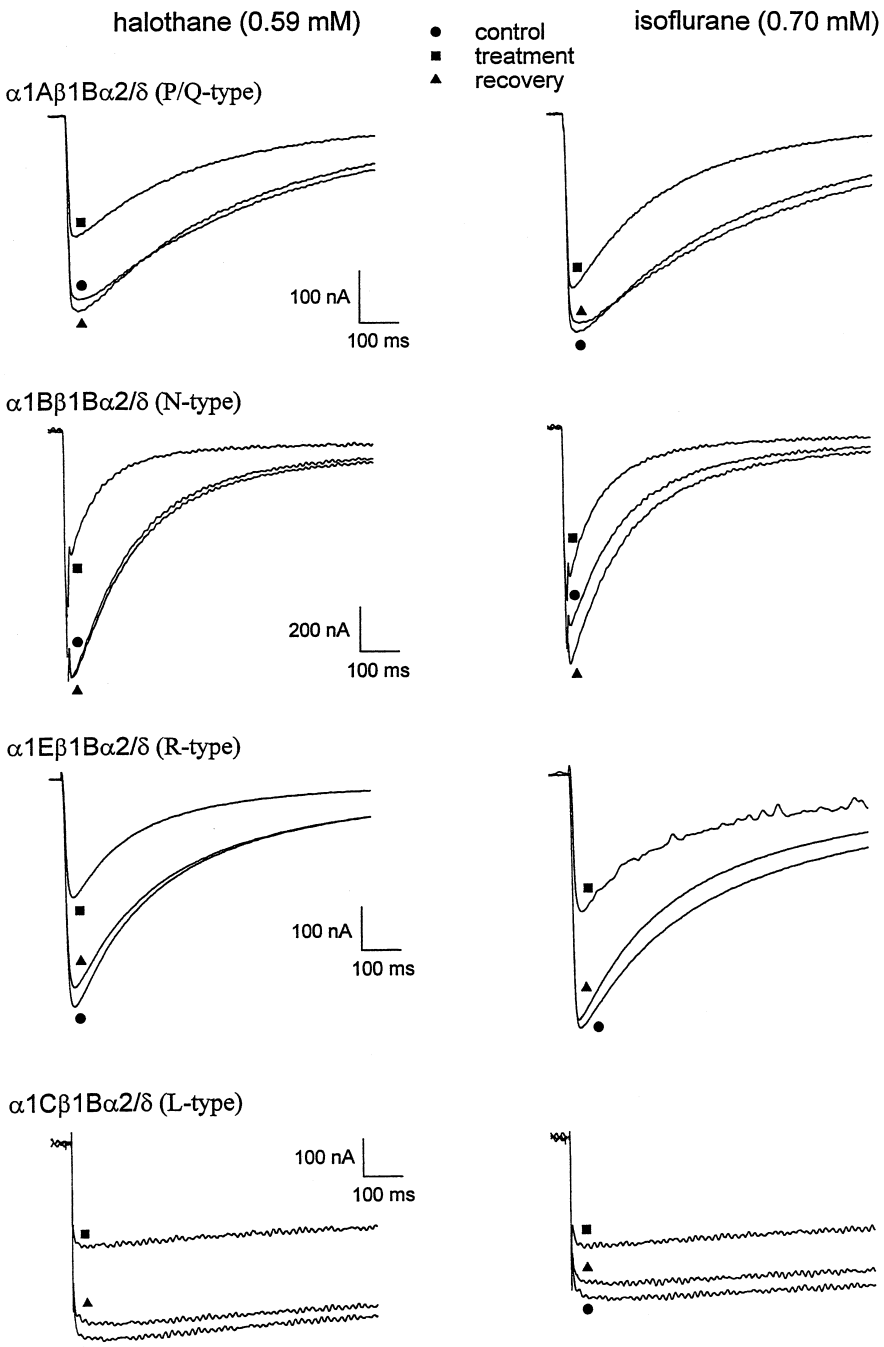

Fig. 1. Effect of 0.59 mM halothane (left panels) or 0.70 mM isoflurane (right panels) on I_{Ba} in oocytes expressing the indicated neuronal Ca channels. The oocytes were held at −80 mV and depolarized to 0 mV in the presence or absence of anesthetic and after washout. In case of α_{1B}-channels, the oocytes were held at −80 mV and depolarized to 20 mV. The currents were recorded after at least 2 min exposure to the anesthetics. Nearly complete recovery was seen 5 min after washout.

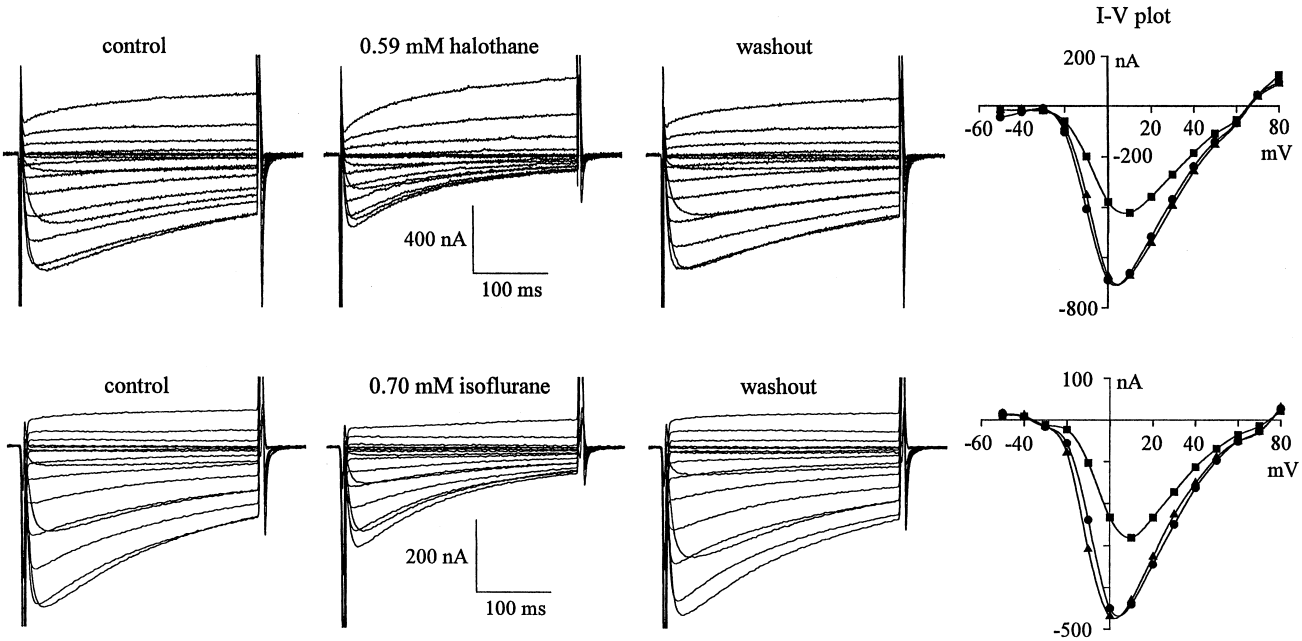

Fig. 2. Effect of 0.59 mM halothane (top panel) and 0.70 mM isoflurane (bottom panel) on I_{Ba} in oocytes expressing $\alpha_{1E}\beta_{1B}\alpha_2/\delta$-type Ca channels. Oocytes were held at −80 mV and depolarized in step potentials from −50 mV to 100 mV with increments of 10 mV using 40 mM Ba^{2+} as the charge carrier. The data for halothane and isoflurane came from two different oocytes. The control (circle), treatment (square) and washout (triangle) were obtained from the same oocyte. Recording solution equilibrated with the respective VA was administered by means of continuous perfusion. The current was recorded 2 min after the beginning of perfusion with anesthetic-equilibrated solution. The washout was recorded 5 min after perfusion with the control solution.

and typically peaked between −10 and 10 mV, with the presence of either anesthetic causing relatively uniform depression of I_{Ba}. Although the anesthetics shifted the potential at which peak I_{Ba} occurred in Fig. 2, this was not observed in all cases.

3.2. Inhibition of peak, late, and average currents by VAs

In spite of the differences in baseline kinetics of the different subunits (Table 1), both halothane and isoflurane clearly inhibited peak and late I_{Ba} in all types of neuronal Ca channels in a reversible manner (Figs. 1–3). This inhibition is concentration-dependent as evidenced by the increased depression of I_{Ba} at increasing anesthetic concentrations using α_{1E}-types of channels (Fig. 3). At the intermediate concentration, both halothane (0.59 mM, 1.5%) and isoflurane (0.70 mM, 2.5%) depressed the peak I_{Ba} by ~30% in all the channels studied (Figs. 1–3). For example, halothane depressed the α_{1E} peak I_{Ba} at 0 mV from 463 ± 60 to 333 ± 46 nA (recovery, 450 ± 61 nA; $n = 11$), although the time to peak was not significantly decreased (control, 82 ± 4 ms; halothane, 76 ± 4 ms). Similarly, isoflurane decreased α_{1E} peak I_{Ba} at 0 mV from 466 ± 57 to 301 ± 40 nA (recovery, 405 ± 56 nA; $n = 9$) with no change in time to peak (control, 77 ± 3 ms; isoflurane, 74 ± 2 ms). The mean depression of α_{1A}, α_{1B}, α_{1E} and α_{1C} subunit currents by the intermediate concentrations of halothane and isoflurane is plotted in Fig. 3. It is evident that both halothane and isoflurane depressed the late current about twice as much as the peak current, reflecting the acceleration of inactivation (Figs. 1–3). Since α_{1C}-channels displayed little inactivation, the anesthetic depression of the average current alone is indicated. Furthermore, at the intermediate concentration these two agents had similar effects on the current–voltage relationship, inhibiting the I_{Ba} at all voltages examined (Fig. 2).

3.3. Effects of VAs on A_1, R, and τ_{inact} of neuronal Ca channels

To better characterize the changes induced by the anesthetics on channel kinetics, control and anesthetic treated I_{Ba} were analyzed and the A_1, its τ_{inact}, and R were determined for the three subunit currents displaying inactivation. Both A_1 and R were reduced in α_{1A}, α_{1B} and α_{1E}-types of channels to a variable extent (Fig. 4). For example, in α_{1E}-channels, calculated as a percent of control, A_1 was reduced $25.5 \pm 4.5\%$ by 0.59 mM halothane and $31.5 \pm 2.6\%$ by 0.70 mM isoflurane; in contrast R was inhibited nearly twice as much by halothane and isoflurane being reduced by 48.2 ± 5.4 and $50.8 \pm 4.4\%$, respectively. Furthermore, this inhibition of A_1 and R currents in α_{1E}-channels was dose-dependent as greater inhibition of this current was seen with increasing concen-

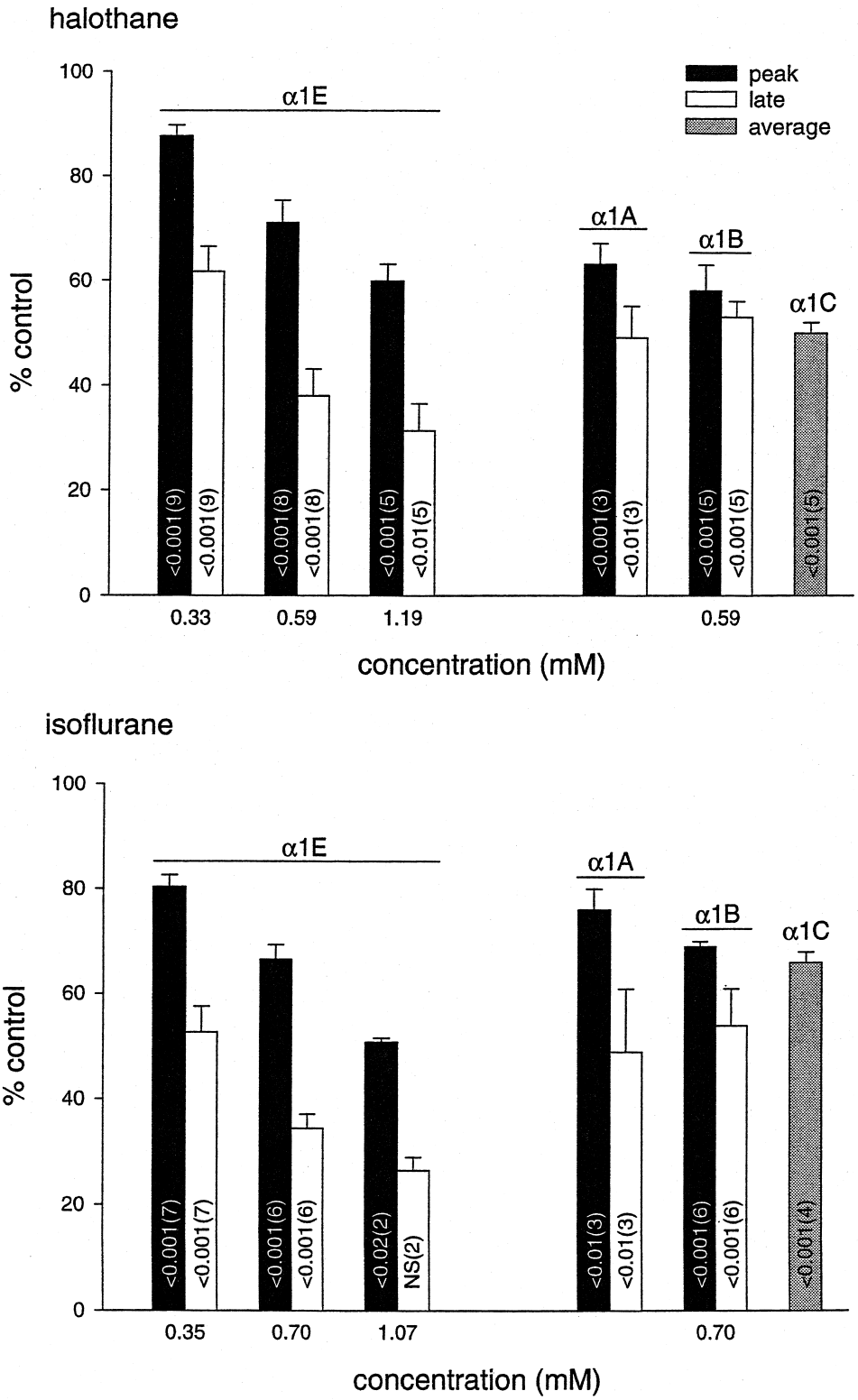

Fig. 3. Inhibition of peak, late, and average currents through expressed neuronal Ca channels by halothane or isoflurane. Dose-dependent inhibition of peak and late currents were determined for $\alpha_{1E}\beta_{1B}\alpha_2/\delta$-type Ca current with halothane (top panel) or isoflurane (bottom panel). Percent control is shown for the currents present upon depolarization to 0 mV from a holding potential of −80 mV in the presence of VA. In case of α_{1B}-channels, the oocytes were held at −80 mV and depolarized to 20 mV. For the α_{1C}-subunit, the current obtained at 0 mV was averaged for the entire period of depolarization. Only the equianesthetic concentrations of halothane (0.59 mM) or isoflurane (0.70 mM) were used to examine α_{1A}, α_{1B}, and α_{1C}-currents. Values are mean ± S.E.M. and numbers in parentheses indicate 'n'. Values inside the bars indicate significance when compared to respective control values.

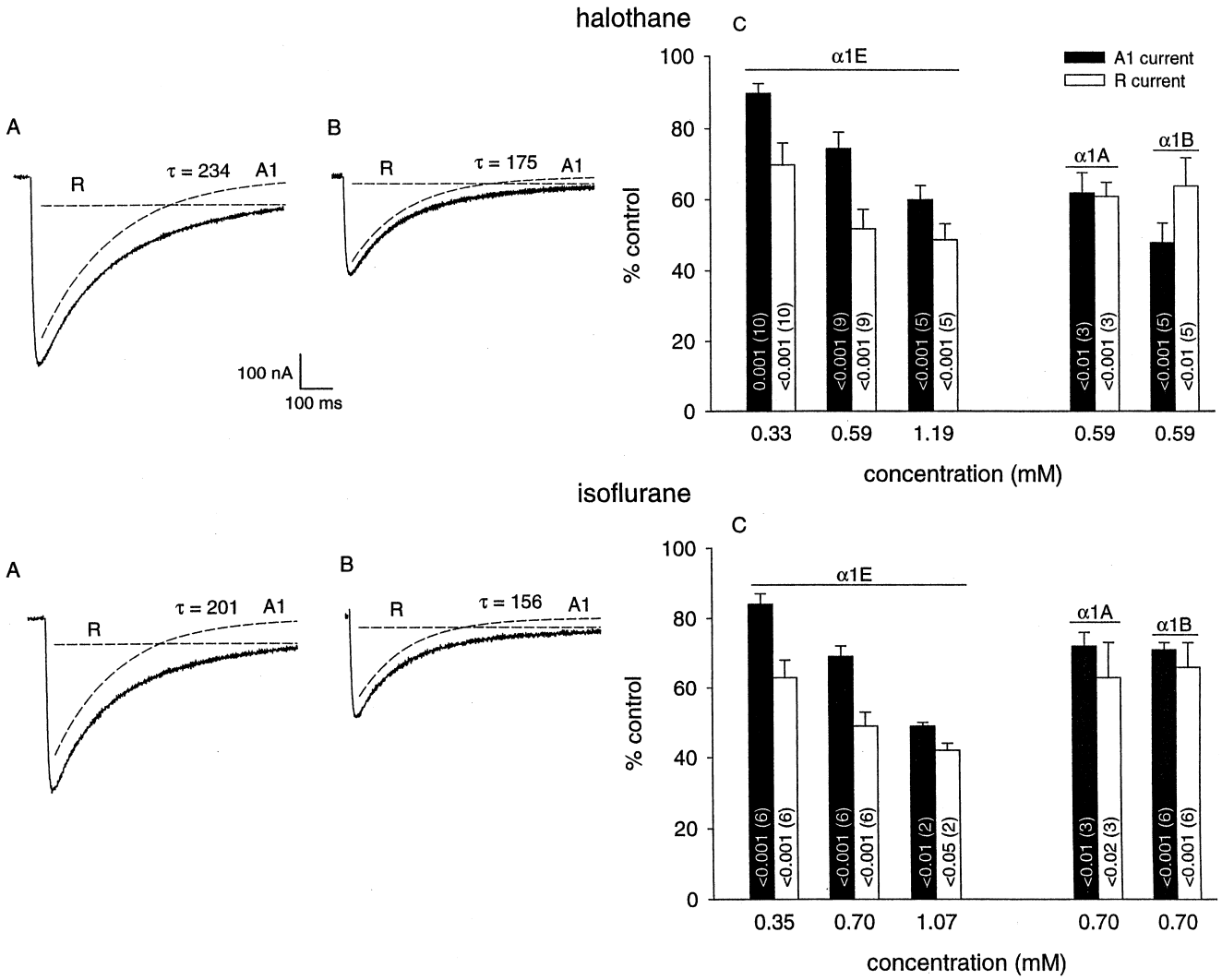

Fig. 4. The effects of VAs on the A_1 and R components of neuronal Ca currents. Currents were in response to a depolarization to 0 mV from a holding potential of −80 mV. In case of α_{1B}-channels, the oocytes were held at −80 mV and depolarized to 20 mV. The dashed lines represent A_1 and R components of I_{Ba} determined by a least squares non-linear regression. (A) Shows control α_{1E}-current and the calculated A_1, its time constant, τ_{inact} (in ms) and R. (B) Shows α_{1E}-current and the calculated A_1, its time constant and R in the presence of 0.59 mM halothane (top panel) or 0.70 mM isoflurane (bottom panel). (C) Displays the summary of the inhibition of A_1 and R at various concentrations for halothane (top left panel) and isoflurane (bottom left panel) in α_{1E}-channels. The right panels show the inhibition obtained for both α_{1A} and α_{1B}-type channels with halothane or isoflurane. The data are the mean ± S.E.M.; the numbers inside the bars indicate the significance (compared with control) while the 'n' values are shown within parentheses.

trations of VAs. However, such a difference in anesthetic sensitivity to A_1 and R components did not achieve significance in α_{1A} and α_{1B}-channels (Fig. 4).

Acceleration of inactivation by VAs was quantitated in Fig. 5, where the reduction in τ_{inact} is plotted for the various HVGCCs types. For α_{1E}-current in which concentration-dependence was determined, the τ_{inact} at 0 mV decreased with increasing VA concentrations being reduced by 21–25, 31–33, and 54–55% by the low, intermediate and high anesthetic concentrations, respectively. In a similar fashion, the intermediate anesthetic concentrations also reduced τ_{inact} for α_{1A} and α_{1B} by 30–40%. The very modest decline in the α_{1C} subunit I_{Ba} over 800 ms suggests that inactivation is extremely slow. In the presence of the anesthetics, not only did α_{1C} peak I_{Ba} decline, but there may also have been a slightly greater decline during the depolarization; however, this was too small to permit appropriate quantitation.

3.4. Effects of VAs on voltage-dependence of activation and steady-state inactivation

To determine the effect, if any, of VAs on voltage-dependence of activation, the conductance was calculated in α_{1E}-channels at test potentials from −30 to 30 mV and the voltage-dependence described by the Boltzmann equation, fitting V_n and k_n. Under control conditions, V_n was −8.4 ± 3.6 mV and k_n was 5.7 ± 0.5 mV for all oocytes

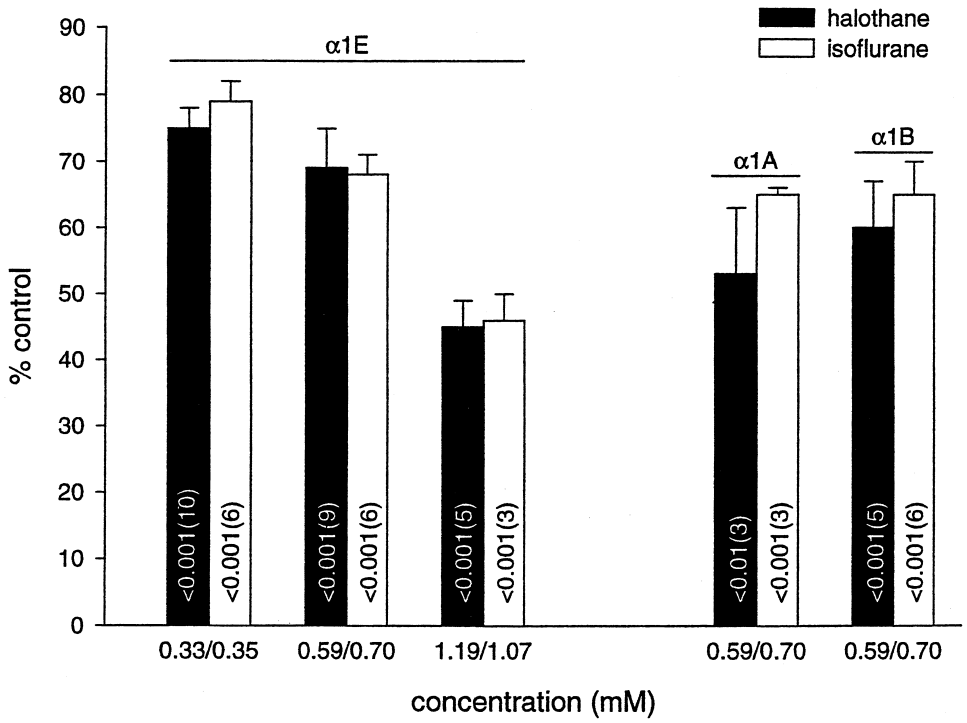

Fig. 5. The effects of halothane and isoflurane on the τ_{inact} of the inactivating component of current (A_1) of neuronal Ca channels. The dose-dependent inhibition with halothane or isoflurane is shown for $\alpha_{1E}\beta_{1B}\alpha_2/\delta$-type Ca currents only. The effects of equianesthetic concentrations of halothane (0.59 mM) and isoflurane (0.70 mM) are shown for α_{1A} and α_{1B}-type currents. All the values were obtained by depolarizing to 0 mV from a holding potential of −80 mV. In the case of α_{1B}-channels, the oocytes were held at −80 mV and depolarized to 20 mV. Values are mean ± S.E.M. and numbers in parentheses indicate 'n'. Values inside the bars indicate the significance when compared to respective control values.

studied ($n = 19$). For the specific case of 0.70 mM isoflurane as an example, the Boltzmann curve describing the anesthetic responses was shifted by 2.4 ± 0.5 mV in the depolarizing direction compared to prior controls, with a

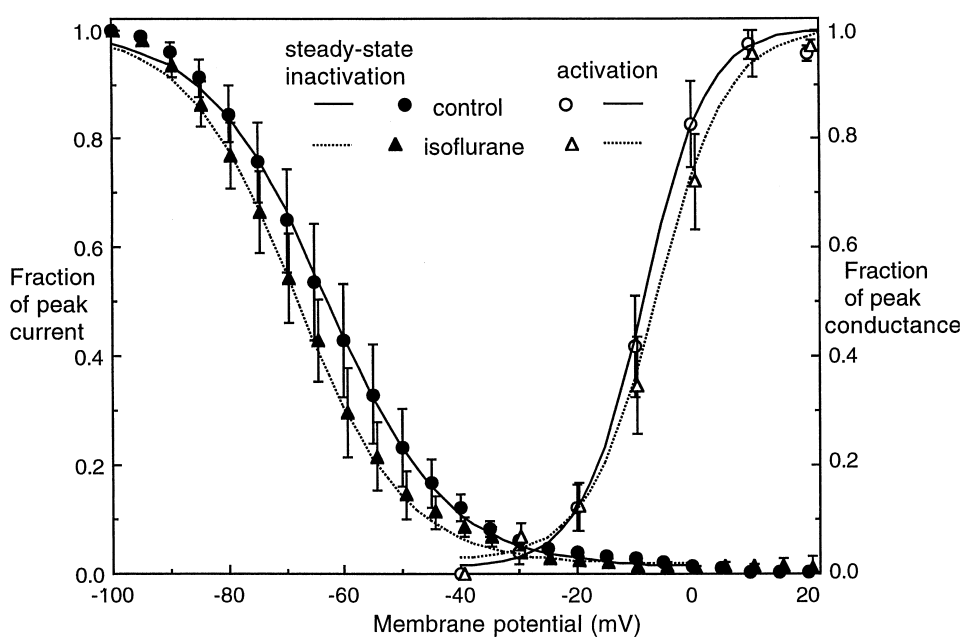

Fig. 6. Effect of 0.70 mM isoflurane on the voltage-dependence of activation (open symbols) and steady-state inactivation (filled symbols) for $\alpha_{1E}\beta_{1B}\alpha_2/\delta$-type Ca channels. The activation curves are the least squares fit to the Boltzmann equation for the normalized peak conductance. For activation, the control V_n was −8.2 ± 1.0 mV which shifted to −5.8 ± 1.1 mV in the presence of isoflurane, with a change in k_n from 5.5 ± 0.8 to 6.2 ± 0.9 mV ($n = 3$). The inactivation curves are least squares fit to the Boltzmann equation for the average fraction of peak current. For inactivation, the mean control responses were fit by $V_n = -63 \pm 1$ mV which decreased to −68 ± 1 mV in the presence of isoflurane, with calculated k_n of −10.3 ± 0.7 mV for control vs. 9.5 ± 0.6 mV, in the presence of isoflurane ($n = 4$).

$12 \pm 6\%$ increase in the k_n ($n = 3$, Fig. 6). Similar shifts in V_n (~ 1.8–2.3 mV) and increases in k_n of activation were observed for the higher and lower concentrations of isoflurane as well as for halothane. Although these shifts were reversible on washout of anesthetic, there was little concentration dependence with variable levels of significance ($P < 0.10$ to < 0.01).

The voltage-dependence of steady-state inactivation for an oocyte expressing α_{1E}-channels in which the pre-potential was changed in 5 mV increments could also be described by a Boltzmann relationship as indicated in Fig. 6. The average of the individual responses in four oocytes studied for V_n was -63.8 ± 4.6 mV with a k_n of -8.5 ± 0.4 mV. At -80 mV, the steady-state inactivation reduced the peak current to $84.5 \pm 5.3\%$ of the maximum current observed at -100 mV as shown in Fig. 6. In contrast to the slight depolarizing shift in the activation curve, 0.70 mM isoflurane shifted the voltage-dependence of steady-state inactivation by 4.9 ± 1.1 mV in the hyperpolarizing direction with no change in k_n (-8.4 ± 0.4 mV). Because of this isoflurane-induced shift, the peak I_{Ba} at a holding potential of -80 mV was $76.9 \pm 6.3\%$ of the value at -100 mV, representing significantly ($P < 0.005$, paired *t*-test) greater steady-state inactivation than in the absence of the anesthetic.

4. Discussion

Our study demonstrates that halothane and isoflurane inhibited the currents through HVGCCs formed by the combined expression of any one α_1-subunit (A, B, C or E) with β_{1B} and α_2/δ subunits. Two distinct effects were evident with all channels: (i) the peak currents were reduced in a concentration dependently manner and (ii) inactivation rate were enhanced (Figs. 3 and 4). Detailed analysis of the various components of currents, viz., A_1, R, and τ_{inact}, showed that they were also decreased. It should be stressed that all the above changes were reversible, which is a typical property of anesthetics. The 30–35% decrease in peak I_{Ba} by halothane (0.59 mM) and isoflurane (0.70 mM) is similar to that reported for I_{Ca} or I_{Ba} through native HVGCCs in situ. For L-type currents in guinea pig ventricular myocytes, 0.8 mM isoflurane or 0.45 mM halothane caused $\sim 25\%$ depression [35], while a somewhat greater ($\sim 35\%$) decrease was observed with 0.61 mM isoflurane or 0.4 mM halothane in canine ventricular myocytes using Ba^{2+} as the charge carrier [4]. In cultured hippocampal cells, Study [49] found a 50% reduction of the peak I_{Ca} by 2.0% isoflurane (~ 0.78 mM), although the same concentration caused a modest 24% depression of I_{Ca} in bovine adrenal chromaffin cells [36].

One of the candidates responsible for the inhibition of the peak I_{Ba} by VAs might be the hyperpolarizing shift in the steady state inactivation observed for α_{1E} at the holding potential of -80 mV (Fig. 6). Such a shift in the steady-state inactivation curve of HVGCCs has been observed for isoflurane in ventricular myocyte L-type current [35]. At -80 mV, the holding potential employed for most of this study, isoflurane caused about 8% more steady-state inactivation (the inhibition increased from 15 to 23%). Such an effect could only account for about $1/4$ of the $\sim 30\%$ inhibition observed in peak I_{Ba}. To account for the additional inhibition that was observed, several other factors may be considered. Though a very small positive shift in the voltage-dependence of activation was seen with both anesthetics, together with a slight increase in the k_n, it seems too small (2.4 mV) to markedly influence the peak current (Fig. 6). In addition to the effects of steady-state inactivation, the accelerated inactivation observed with a given depolarization could influence peak I_{Ba}. Evidence against a contribution of inactivation in the depression of peak current is present in the studies with α_{1C}-channels, where there was minimal evidence of inactivation over the 850 ms depolarization. Even in the absence of significant inactivation, the α_{1C}-channels still demonstrated a quantitatively similar fractional inhibition by the VAs of the average current when compared to the other channels (Figs. 1 and 3). A major factor responsible for the decrease in peak current may well be a decrease in the channel open probability as shown for halothane (1.4 mM), though with a high concentration in ventricular myocytes [35].

VAs caused a greater depression of late current than that of peak I_{Ba} in the three inactivating channels (Fig. 3). A comparable effect was observed [49], in hippocampal neurons where the inhibition of steady current was half-maximal with 1% isoflurane, whereas the peak current required about 2% isoflurane to achieve the same inhibition. Similarly, in bullfrog atrial myocytes, perfusate equilibrated with 2.5% isoflurane or 5% sevoflurane caused $\sim 55\%$ decrease in τ_{inact} of L-type Ca^{2+} currents [17]. As opposed to peak I_{Ba}, the late current was reduced by the acceleration of its inactivation rate. A similar action has previously been noted in guinea pig ventricular myocytes, where VAs increased the apparent rate of inactivation [35]. This increased inactivation rate was associated with a reduction in mean open time in that study and may be responsible for the observed reduction of A_1 as well as the R components in this study. This suggests that the inactivating channels as well as those with more sustained opening have similar sensitivity to both halothane and isoflurane. Furthermore, τ_{inact} is also decreased in a similar manner by both the VAs which is compatible with the inhibition of A_1 and a decrease in mean open time (Fig. 5).

In this study, the use of Ba^{2+} as the charge carrier prevented the appearance of Ca^{2+}-induced inactivation. Although such a Ca^{2+}-induced process is not evident in α_{1E} subunits, it is prominent in L-type channels and also present in N- and P-type HVGCCs [9,10,52]. While the use of Ba^{2+} did prevent the study of potential anesthetic effects on this Ca^{2+}-induced process, we were able to

determine anesthetic actions on voltage-dependent inactivation. Nevertheless, previous investigations with ventricular myocytes suggest that both halothane and isoflurane accelerate inactivation whether Ba^{2+} or Ca^{2+} is used as the charge carrier, suggesting that anesthetic acceleration of inactivation kinetics combines with the acceleration due to Ca^{2+}-dependent inactivation [35]. Curiously, while myocardial L-type currents showed considerable inactivation of I_{Ba} which could be enhanced by VAs [17,35], the α_{1C}-subunit currents in this study lacked any significant amount of inactivation. These differences may be attributed to the accompanying β-subunit which is known to modulate inactivation kinetics of the α_1-subunits of HVGCCs [56]. It is possible that other β-subunits were involved in the functional L-type HVGCCs of the ventricular myocytes thus contributing to their much faster inactivation kinetics than was observed with the β_{1B} subunit. In addition, the L-type HVGCCs, differ substantially from α_{1A}, α_{1B} and α_{1E}-types and may be considered a distinct class based upon their structure and amino acid sequence. While α_{1C}, α_{1S} and α_{1D} subunits have been classified under L-type of channels on the basis of DHP-binding and a short domain II–III segment ($\sim$ 100 to 150 a.a.), α_{1A}, α_{1B} and α_{1E}-types have a longer II–III segment ($>$ 400 a.a.) and are insensitive to DHP [47].

Although recent evidence suggests an anesthetic site of action on the $GABA_A$ receptor [30], HVGCCs of the CNS may well represent an additional site of anesthetic action, as they are involved in the regulation of cell excitability and release of neurotransmitters. Interference with Ca^{2+} entry into nerve terminals, either by blockade of impulse conduction or inhibition of HVGCCs, could reduce neurotransmitter release and synaptic transmission, an apparent action of various general anesthetics [22,42,51]. Determination of Ca^{2+} transients and neurotransmitter release in cultured neurons or brain synaptosomes have documented inhibition by VAs [2,28,29]. While VAs have been shown to depress mixed Ca^{2+} currents present in neurons [21,49], more recent studies have documented inhibition of specific T-, L-, and N-type currents [27,35,49,50]. The link between VAs and transmitter release is further strengthened as all the neuronal HVGCCs have been shown to be involved in neurotransmitter release in addition to other functions. Both the α_{1A}-subunit, which encodes P/Q-type of channels, and the α_{1B}-subunit, encoding N-type of channels, have been found to control transmitter release evoked by action potentials in most synapses examined thus far [12,41]. While the α_{1A}-subunit has been shown to be present in various regions of the CNS and also periphery, the α_{1B}-subunit to date has been found to be limited to the nervous system and to neuronally derived cells [47]. In addition to the peripheral sites (especially, heart and aorta), the presence of α_{1C}-subunits and their involvement in neurotransmitter release, gene expression and certain aspects of long-term potentiation has been shown in various brain regions [18,32,39]. Recently, it has been shown that R-type channels are found in the brain and contribute to neurotransmitter release, though with a lower efficacy than other types of HVGCCs [59].

Behavioral studies using laboratory animals provide a link implicating the blockade of HVGCCs in the anesthetic state. Non-specific HVGCCs blockade by Cd^{2+} as well as L-type channel block by verapamil have been found to increase the anesthetic potencies of ethanol, pentobarbitone, ketamine, etc., in mice [11,43]. Funnel-web spider toxin which blocks P-type and other high-HVGCCs can cause lethargy and stupor in mice [24,33], while blockade of N-type channels by spinally administered ω-conotoxin MVIIA (SNX-111, ziconotide) has distinct antinociceptive actions [7]. Thus, the anesthetic state caused by VAs may include this common inhibitory effect on all types of HVGCCs. Characteristic VA-induced depression of peak current and distinct acceleration of inactivation are prominent features involving the various HVGCCs, occurring not only in the variety of HVGCCs selectively expressed in oocytes here, but also in native HVGCCs in freshly isolated or cultured cells. Since the transmembrane domains show the greatest sequence similarity as well as the greatest hydrophobicity among the various HVGCC classes, they represent a possible common site for VA action. Alternately, anesthetic intercalation into the membrane headgroup region of the lipid bilayer may have a similar effect on the conformational rearrangements of these various channel types, possibly at sites of boundary of the lipid interaction with the channel transmembrane helixes. If the open state of the channel represents a conformational state which is only transiently stable (the closed state being stable at negative membrane potentials and the inactivated state being more stable at more positive potentials), the presence of VAs within the boundary lipids or within the transmembrane helixes may have a further destabilizing effect, decreasing the probability of opening, as well as the open channel lifetime by enhancing inactivation. It is perhaps noteworthy that such acceleration of inactivation is also observed with Na channels [1] (although on a much faster time scale) which share an overall similar structure and substantial homology with HVGCCs.

The compatibility between our findings and previously reported data in native cells establishes the Xenopus oocyte model as a system to study anesthetic actions on HVGCCs. Using Xenopus oocytes, it has been shown that VAs inhibited muscarinic and lysophosphatidate (LPA) signaling at clinical concentrations similar to those employed in this investigation [8,13,31]. However, one of the drawbacks associated with Xenopus oocytes is the presence of $I_{Cl(Ca)}$ [5,14,37], which, as in this study, obligates the use of Ba^{2+} as the charge carrier. Hence, it will be relevant to substantiate these findings in a mammalian expression system such as HEK 293 cells, which more readily permits examination of currents carried by Ca^{2+}. Based upon this preliminary data our laboratory is currently undertaking such an endeavor.

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