Differential Sensitivity of Expressed L-Type Calcium Channels and Muscarinic M₁ Receptors to Volatile Anesthetics in *Xenopus* Oocytes

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ABSTRACT

Since volatile anesthetics inhibited high voltage-gated calcium channels and G-protein-coupled M₁ muscarinic signaling, their effects upon M₁ receptor-induced modulation of L-type (α₁C) calcium channel was investigated. Voltage-clamped Ba⁰⁺ currents (I_{Ba}) were measured in *Xenopus* oocytes coexpressed with L-type channels and M₁ muscarinic receptors. M₁ receptor agonist, acetyl-β-methylcholine (MCh) inhibited the peak and late components of I_{Ba} in a dose-dependent manner. Analysis of I_{Ba} after the treatment with M₁ or volatile anesthetics revealed that the inactivating component, its time constant, and the noninactivating current were all decreased by these agents. M₁-induced inhibition followed a second messenger pathway that included G-proteins, phospholipase C, inositol-1,4,5-trisphosphate, and intracellular calcium [Ca²⁺]. Although halothane or isoflurane further inhibited I_{Ba}, their effect was not mediated through these intracellular second messengers. By using volatile anesthetics and M₁ sequentially, and in various combinations, the susceptibility of L-type currents and their modulation by M₁ receptors to volatile anesthetics were investigated. When M₁ and volatile anesthetics were administered together simultaneously, a pronounced inhibition that was approximately equal to the sum of their individual effects was seen. Halothane or isoflurane further inhibited the I_{Ba} when either volatile anesthetic was administered following the inhibition produced by prior administration of M₁. However, when M₁ was administered following either volatile anesthetic, its effect was significantly reduced. Thus, whereas volatile anesthetics appear to directly inhibit L-type channels, they also interfere with channel modulation by G-protein-coupled receptors, which may have functional implications for both neuronal and cardiovascular tissues.

In addition to ligand-gated channels, such as GABAₐ (Mihic et al., 1997), nicotinic (Scheller et al., 1997), N-methyl-D-aspartic acid (Keita et al., 1999), and serotonin (Sanna et al., 1999) receptors, G-protein-coupled receptors (Anthony et al., 1999; Durieux, 1995; Magyar and Szabo, 1996) and high voltage-gated calcium channels (HVGCCs) (Krnjevic and Puil, 1988; Pancrazio, 1996; Kamatchi et al., 1999) also represent prime molecular targets of volatile anesthetics. At concentrations relevant to general anesthesia, HVGCCs are inhibited and their steady-state activation and steady-state inactivation curves were shifted to depolarizing and hyperpolarizing directions, respectively, by volatile anesthetics (Kamatchi et al., 1999). Interference with calcium 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 (Takenoshita and Takahashi, 1987). Although an earlier study showed that volatile anesthetics depress HVGCC current present in neurons (Krnjevic and Puil, 1988), more recent studies have characterized volatile anesthetic-induced inhibition of specific P/Q-, L-, N-, and R-types of HVGCCs (Pancrazio, 1996; Kamatchi et al., 1999).

Recent developments in molecular biology identified that the HVGCC is a multisubunit complex comprising α₁, β, α₂/δ, and γ subunits. Although the α₁ subunit is responsible for the expression of the calcium-selective channel pore and sensitivity to calcium agonists and antagonists, the auxiliary subunits modulate the amplitude of calcium current and its kinetics when expressed in artificial systems, *Xenopus* oocytes, or HEK 293 cells. The various α₁ subunits (α₁A, α₁B, α₁C/D/S, and α₁E) correspond, respec-

Abbreviations: HVGCC, high voltage-gated calcium channel; AT₁A, angiotensin receptor; [Ca²⁺], intracellular calcium; IP₃, inositol-1,4,5-trisphosphate; PLC, phospholipase C; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; I_{Ba}, Ba⁰⁺ current; I-V, current-voltage; MCh, acetyl-β-methylcholine; GDP-β-S, guanosine-5’-O-(2-thiodiphosphate) triphlum; BAPTA, 1,2-bis-(o-aminophenoxo)ethane-N,N’N’-tetraacetic acid sodium; G_{Ba}, Ba⁰⁺ conductance; GABAₐ, γ-aminobutyric acid, type A.

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tively, to the HVGCCs responsible for the P/Q-, N-, L-, and R-type calcium currents defined by pharmacological and electrophysiological characterizations. The L-type channels are the most common, and the α1C subunit is the α1 variant present in cardiac, vascular, and neuronal tissue (see, for a review, Stea et al., 1995; Krizanova, 1996). These channels participate in their own modulation and thereby in calcium homeostasis, because a calcium sensor present in the cytoplasmic region of the α1C subunit regulates calcium entry through a negative feedback inhibition (Zhou et al., 1997; Zuhlke and Reuter, 1998).

Both receptor-regulated processes, as well as volatile anesthetics, alter HVGCCs and Ca$^{2+}$ homeostasis. For example, application of halothane or activation of angiotensin receptors (AT$_1$A) or odd-numbered muscarinic receptors (M$_1$, M$_3$, and M$_5$) released stored intracellular calcium [Ca$^{2+}$]$_i$ (Griendling et al., 1986; Caulfield, 1993; Lynch and Frazer, 1994; Pajewski et al., 1996) and also inhibited L-type channels in Xenopus oocytes or mammalian cells (Pancrazio, 1996; Pemberton and Jones, 1997; Oz et al., 1998; Kamatchi et al., 1999). Activation of AT$_1$A receptors induced release of calcium from inositol-1,4,5-trisphosphate (IP$_3$) receptor-gated calcium stores by following a second messenger pathway that involved G-proteins, phospholipase C (PLC), and IP$_3$. Since the contribution of these intermediaries in the inhibition of L-type channels by M$_1$ receptor activation and anesthetics is not clear, it was investigated.

Based on biochemical and electrophysiological studies, the G-protein coupled muscarinic M$_1$ receptor has long been considered a target of volatile anesthetics (Anthony et al., 1989; Durieux, 1995). However, in our previous studies, whereas the protein kinase C (PKC)-mediated effect of phorbol 12-myristate 13-acetate (PMA) was blocked by volatile anesthetics, the effect of M$_1$ receptor activation via PKC was resistant to these agents (Kamatchi et al., 2000). Considering the potential effect of volatile anesthetics on various aspects of the cell signaling pathways that modulate HVGCCs, we decided to determine how volatile anesthetics and the M$_1$ muscarinic receptor system would interact in altering L-type channel behavior. Since the M$_1$ muscarinic receptors and L-type channels are widespread in the central nervous system (Stea et al., 1995; Levey, 1996), there may be multiple anatomic locations at which volatile anesthetic could interfere with or augment these processes. This investigation was carried out in Xenopus oocytes by coexpressing the cDNAs that encode L-type HVGCCs (α1C/β1/δ subunits) with cDNA for the M$_1$ receptors. This is feasible since the oocytes endogenously express constituents of several second messenger pathways, including protein kinase A and PKC (Dascal, 1987; Snutch, 1988). This system is comparable to expression studies in mammalian cells since similar inhibition of L-type currents by [Ca$^{2+}$]$_i$ or muscarinic receptor activation has been reported using HEK 293 cells or NIH 3T3 cells (de Leon et al., 1995; Pemberton and Jones, 1997). Furthermore, the ability of oocytes to withstand the experimental conditions for longer period of time and the bigger expressed current allows the experimenter to study drug-induced modulation of currents with comparative ease.

### Materials and Methods

#### Oocyte Harvesting and Microinjection

 Mature female Xenopus laevis frogs were obtained from Xenopus I (Ann Arbor, MI), housed in a 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-amino-benzoic 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 ~1.5 cm 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 (~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 7.4) and transferred to a 100-mm Petri dish containing 1 mg/ml collagenase (type 1A; Sigma) in OR2 solution. The Petri dish containing the oocytes was agitated gently in a platform shaker for a period of 2 to 3 h at room temperature to remove the follicular cell layer. Defolliculation was confirmed by microscopic examination. This was followed by the washing of oocytes in 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. The oocytes were allowed to recover for 3 to 10 h at 16°C before cDNA injection. Nuclear (germinal vesicle) injection (Drummond Nanoject, Drummond Scientific Co., Broomall, PA) was performed using 9.2 nl of a 1:1 mix (molar ratio; not exceeding a total of 3 ng of cDNA) of rat brain α1C, β1B, and α2/δ subunits subcloned in the mammalian expression vector pMT2. For the coexpression of muscarinic M$_1$ receptor with the calcium channel, 1 ng of rat M$_1$ receptor cDNA subcloned in pcDNA 3.1 (Invitrogen, Carlsbad, CA) was included with the above mix. The oocytes were returned to Barth’s solution and incubated at 16°C for 6 to 8 days before current recording.

#### Current Recording

 Macroscopic currents were recorded using the two-electrode voltage-clamp technique with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). The amplifier was linked to an interface and an IBM PC-compatible computer equipped with pClamp software (version 5.6; Axon Instruments) for data acquisition. Leak currents were subtracted using the P/4 procedure. CsCl (3 M) containing microelectrodes with an agarose bridge in them were used to record the current; 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 300-μl volume recording chamber superfused with Tsien’s buffer (recording solution) containing (in mM): Ba(OH)$_2$ 40, NaOH 50, KOH 2, HEPES 5, niflumic acid 0.4, and neutralized to pH 7.4 with methanesulfonic acid. Ba$^{2+}$ replaces Ca$^{2+}$ as the charge-carrying ion in this Ca$^{2+}$-free environment, and niflumic acid was included in the recording solution to block intrinsic chloride channels. The Ba$^{2+}$ current (I$_{Ba}$) was elicited for a duration of 850 ms by depolarizing the oocytes to 0 mV from a holding potential of ~80 mV. The current-voltage (I-V) relationship for the peak $I_{Ba}$ for these channels was obtained by depolarizing the oocytes to step potentials starting from ~50 to 100 mV in 10-mV increments for the duration of 450 ms.

#### Equilibration and Treatment with Volatile Anesthetics

 Designated halothane or isoflurane reservoirs containing about 30 to 40 ml of the recording solution were bubbled with the respective volatile anesthetic from their calibrated individual vaporizers. Air at a flow rate of 500 ml/min was used as the carrier gas, and a minimum of 10 min of bubbling was allowed for equilibration of the recording solution with volatile anesthetic. The oocytes were perfused with this.
solution to determine the response with the volatile anesthetic. Due to possible loss of anesthetic to the atmosphere, the superfusion solution containing volatile anesthetic or its combination with other agents was perfused continuously (5 ml/min) until the current was recorded. In case of a combined treatment of volatile anesthetic with other agents, the recording solution containing the final concentration of the respective drug was bubbled with volatile anesthetic. The concentration of volatile anesthetic in the recording chamber was periodically verified by triplicate aqueous samples from the chamber that equilibrated with air (1:4, air:solution) and analyzed in a gas chromatograph (Aerograph 940; Varian Analytical Instruments, Walnut Creek, CA) calibrated with standards for halothane and isoflurane. Results were converted to concentrations in liquid using aqueous/gaseous partition coefficients at 25°C (Firestone et al., 1986) and averaged.

**Treatment Schedule**

All the oocytes exhibiting $I_{\text{Ba}}$ greater than 400 nA underwent control, treatment, and wash protocols. The oocyte was allowed to stabilize in our recording conditions for a period of 6 min. At the end of this stabilization period, the recording solution was superfused for 30 s, followed by the recording of $I_{\text{Ba}}$ at 8 min. Acetyl-$\beta$-methylcholine (MCh) was superfused for 30 s immediately after recording the control current. The effect of MCh was recorded at 10 min, thus exposing the oocytes to MCh for a period of 2 min. Similarly, after the stabilization and control periods, the current was recorded after 2 min of exposure with either halothane (0.59 mM) or isoflurane (0.70 mM), while continuous perfusion of these agents was being used. Following this protocol, basic parameters, such as the dose response, I-V plot, and the control inhibition with MCh and volatile anesthetic, were obtained. The EC$_{50}$ for MCh was found to be 220.4 nM; thus, examination of its second messenger pathway (schedule 1) was carried out using a concentration of 200 nM. However, in studies involving the combination of either volatile anesthetic and MCh (schedules 3A, 3B, and 3C), a higher concentration (500 nM) of MCh, which was equipotent to volatile anesthetic (in causing inhibition of $\alpha$1C channels), was used.

**Schedule 1: Study of the Identification of the Second Messengers Involved in M$_1$ Receptor-Induced Inhibition of L-Type Channels.** The involvement of M$_1$ receptors and the related second messengers (G-proteins, PLC, IP$_3$, and [Ca$^{2+}$]) responsible for the inhibition of L-type channels was studied with the use of blockers of the respective intermediaries. Oocytes were pretreated with atropine (1 $\mu$M) for a period of 5 min before challenging with MCh. Guanosine-5'-O-(2-thiodiphosphate) trilithium (GDP-MCh) was superfused for 30 s immediately after recording the control current. The effect of MCh was recorded at 10 min, thus exposing the oocytes to MCh for a period of 2 min. Similarly, after the stabilization and control periods, the current was recorded after 2 min of exposure with either halothane (0.59 mM) or isoflurane (0.70 mM), while continuous perfusion of these agents was being used. Following this protocol, basic parameters, such as the dose response, I-V plot, and the control inhibition with MCh and volatile anesthetic, were obtained. The EC$_{50}$ for MCh was found to be 220.4 nM; thus, examination of its second messenger pathway (schedule 1) was carried out using a concentration of 200 nM. However, in studies involving the combination of either volatile anesthetic and MCh (schedules 3A, 3B, and 3C), a higher concentration (500 nM) of MCh, which was equipotent to volatile anesthetic (in causing inhibition of $\alpha$1C channels), was used.

**Schedule 2: Examination of the Role of Second Messengers in Volatile Anesthetic-Induced Inhibition of L-Type Channels.** This schedule is similar to that of schedule 1, except that halothane or isoflurane was used in place of MCh. These experiments were conducted in parallel with schedule 1 and after confirmation that the blockers in the concentrations used were inhibiting the action of MCh.

**Schedule 3A: Administration of MCh First, with Subsequent Addition of Volatile Anesthetic (and MCh).** After the completion of control measurements, the oocytes of this group were perfused with MCh, and the response was measured after 2 min. This was followed immediately with the perfusion of either halothane or isoflurane (in the presence of MCh), and the response was recorded at 2 min from the beginning of the perfusion of volatile anesthetic and MCh containing solution.

**Schedule 3B: Administration of Volatile Anesthetic First, with Subsequent Addition of MCh (and Volatile Anesthetic).** This protocol was the reverse of schedule 3A. Briefly, either halothane or isoflurane was perfused first, and $I_{\text{Ba}}$ was recorded after 2 min. This was followed immediately with the continuous perfusion of MCh (in the presence of the respective volatile anesthetic) for 2 min and the recording of $I_{\text{Ba}}$. This was followed by wash protocol.

**Schedule 3C: Simultaneous Administration of MCh and Volatile Anesthetic.** Following control measurements, the oocytes of this group were perfused with recording solution containing MCh and equilibrated with either halothane or isoflurane. This solution was perfused continuously for a period of 2 min, and $I_{\text{Ba}}$ was recorded at the end of this period, followed by wash protocol.

**Chemicals**

Halothane and isoflurane were purchased from halocarbon Laboratories (River Edge, NJ) and Ohmeda PPD, Inc. (Liberty Corner, NJ), respectively. GDP-}$\beta$-S (RBI, Natick, MA), MCh, heparin (Sigma), and BAPTA tetrasodium (Calbiochem, San Diego, CA) were dissolved in distilled water. Thapsigargin, U73122 (RB1), and atropine (Sigma) were dissolved in dimethyl sulfoxide (0.05%). All these agents, except volatile anesthetics, were prepared as concentrated stock solutions and stored frozen at −20°C. They were diluted to their final concentration in recording solution on the day of the experiment before use. Niflumic acid (Sigma) was added to the recording solution, which was stirred overnight in order for it to dissolve.

**Data Analysis**

Data are shown as mean ± S.E.M., unless otherwise indicated. The peak represented the maximum amplitude of the inward current. The current amplitude at 830 ms (of the total period of 850 ms of depolarization) was arbitrarily defined as the late current, which was used as a measure of the relative degree of channel inactivation. Data were analyzed using either the PCS program (Pancrazio, 1993) or Clampfit, version 6.0.2 (Axon Instruments, Foster City, CA). Ba$^{2+}$ conductance ($G_{\text{Ba}}$) was calculated as $I_{\text{Ba}}/(V - V_{\text{rev}})$ using the reversal potential ($V_{\text{rev}}$) as determined by interpolation over the voltage step at which current changed from inward to outward. The voltage dependence of activation of $G_{\text{Ba}}$ was then described by a Boltzmann equation of the form:

$$G_{\text{Ba}} = \frac{G_{\text{Ba,max}}(1 + \exp(V - V_{\text{rev}})/k_{\text{Ba}})}{1 + \exp(V - V_{\text{rev}})/k_{\text{Ba}}}$$

where $V_{\text{rev}}$ is the voltage of half-maximal activation and $k_{\text{Ba}}$ is the slope factor determined from a least-squares fit (Sigma Plot, SPSS, Inc., Chicago, IL). The inactivating component of $G_{\text{Ba}}$ 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:

$$I_{\text{Ba}} = I_{\text{peak}} \cdot \exp(-t/t_{\text{inact}}) + I_{\text{late}},$$

where $I_{\text{peak}}$ is the inactivating current [$t_{\text{inact}}$, $t_{\text{inact}}$] is the time constant of inactivation and $I_{\text{late}}$ is the residual noninactivating ($[t_{\text{inact}}]/t_{\text{inact}}$) $I_{\text{Ba}}$. Statistical significance was determined using paired or unpaired $t$ test and $p < 0.05$ was considered significant.

**Results**

Approximately 70% of the oocytes injected with cDNA expressed the inward $I_{\text{Ba}}$ upon depolarization. After reaching the peak (between 50 and 70 ms), while still being depolar-
In this stabilization period, the recording solution was superfused for 30 s, to stabilize in our recording conditions for a period of 6 min. At the end of this period, the oocyte was exposed to atropine (1 μM) immediately after recording the control current. The effect of MCh was recorded at 10 min, thus exposing the oocytes to MCh for a period of 2 min. A dose-response curve with MCh. The effect of MCh was recorded as described above. MCh-induced inhibition of peak $I_{Ba}$ was fit to the Hill equation. B, typical inhibition of the channel induced by MCh 200 nM. It required intermittent washing in a span of 15 to 20 min for the recovery shown in Fig. 1B. C, absence of MCh response after the treatment with atropine. The oocytes of this group were exposed to atropine (1 μM) for a period of 5 min. This was followed by the superfusion with MCh and the recording of $I_{Ba}$. D, summary of inhibition of various components of $I_{Ba}$ and the effect of atropine treatment. Numbers in parentheses indicate n.

**Effect of MCh on L-Type Current.** The $I_{Ba}$ receptor agonist, MCh inhibited the peak and late $I_{Ba}$ through L-type channels in a dose-dependent manner as shown in Fig. 1A. The averaged peak and late $I_{Ba}$ under control condition were $-1867 \pm 152$ nA (range: $-429$ to $-7677$ nA) and $-969 \pm 70$ nA (range: $-85$ to $-3550$ nA), respectively (n = 96). I-V plots showed that, in general, upon activation, the inward $I_{Ba}$ through this channel appeared at 30 mV, peaked around 0 and 10 mV, and reversed between 50 and 70 mV. Compared with the other members of HVGCCs, we often encountered the phenomenon of current rundown through these channels. To rule out the presence of rundown the oocyte was repeatedly depolarized approximately once a minute during the 6-min stabilization period, and the resultant $I_{Ba}$ was analyzed. The oocyte was not used if the $I_{Ba}$ was found to be decreasing progressively with every depolarizing episode.

**Effect of MCh on L-Type Current.** The $I_{Ba}$ receptor agonist, MCh inhibited the peak and late $I_{Ba}$ through L-type channels in a dose-dependent manner as shown in Fig. 1A. The percentage inhibition of peak was fitted to the Hill equation, and the Hill coefficient was calculated to be 1.28, suggesting a single binding site. As the calculated EC$_{50}$ was 220.4 nM, 200 nM MCh was used for the reminder of the study, a concentration that led to the significant inhibition ($<0.001$, compared with control) of both the peak and late $I_{Ba}$. Typical inhibition with 200 nM MCh is shown in Fig. 1B.

Of the two components of $I_{Ba}$, the late current appeared to be more sensitive to MCh, compared with the peak $I_{Ba}$. Although this inhibition was reversible with washing, the recovery was incomplete. It required intermittent washing in a span of 15 to 20 min for the recovery shown in Fig. 1B. Obviously, this inhibition was mediated through muscarinic M$_1$ receptors because the effect of MCh was absent after the pretreatment of oocytes with atropine (Fig. 1C). Analysis of the various components of inactivation showed that the inactivating $I_{Ba}$, $\tau$, and the noninactivating $I_{Ba}$ were all decreased significantly by MCh. The observation of the decreased $\tau$ suggests that the inactivation was enhanced by MCh, which is consistent with the fact that the late current was more sensitive to the action of MCh (Fig. 1D).

**Effect of Volatile Anesthetics on L-Type Current.** Perfusion of recording solution equilibrated with either halothane (0.59 mM) or isoflurane (0.70 mM) led to the inhibition of both the peak and late components of $I_{Ba}$ through L-type channels (Fig. 2A). This effect of volatile anesthetics was readily reversible with washing, a property typical of anesthetics. This inhibition appears to be dependent on voltage, although it is not evident from the I-V plot data (Fig. 2B). However, the voltage dependence is obvious from the plotting of the conductance in control and treatment groups. After the exposure to either anesthetic, $V_n$ as well as $k_n$, were decreased significantly as seen from their shift toward more depolarizing potentials (Fig. 2C). Furthermore, analysis of the various components of $I_{Ba}$ showed that as was the case with MCh, the late $I_{Ba}$ appeared to be more sensitive to volatile anesthetics than the peak current. All the components of inactivation, such as the inactivating current, $\tau$, and noninactivating current, were all decreased significantly (Fig. 2D). The decrease in $\tau$ supports our earlier observation that the anesthetics enhance the inactivation of the HVGCCs (Kamatchi et al., 1999).

**Pharmacology of the Inhibition of L-Type Current by MCh and Volatile Anesthetics (Schedules 1 and 2).** After the treatment of the oocytes with GDP-β-S or U73122, the blockers of G-protein and PLC, respectively, MCh failed to inhibit the $I_{Ba}$ through L-type channels significantly. Contrarily, the inhibitory effect of either volatile anesthetic was not affected following the pretreatment of oocytes with GDP-β-S or U73122. It is striking as the percentage inhibition of both the peak and late $I_{Ba}$ were intact and comparable to the control values after the exposure of oocytes to these blockers (Fig. 3). Another link in the MCh-induced pathway is the intracellular IP3 receptors. Because the inhibitory effect of MCh was absent after the administration of heparin, it may be suggested that M$_1$ receptor activation follows the second messenger pathway involving IP3 receptors. However, any such role for IP3 receptors in the action of volatile anesthetics may be ruled out, because the effect of these agents was still intact after the blockade of IP3 receptors (Fig. 4). Similarly, the effects of MCh and volatile anesthetics were examined after the depletion or buffering of $[Ca^{2+}]_i$, another intracellular second messenger. This was achieved by the treatment of the oocytes with thapsigargin or BAPTA, as described under Materials and Methods. The results indicate that the effect of MCh was totally or near totally blocked, whereas the inhibitory effect of volatile anesthetics was still intact (Fig. 5). Summarizing these effects, it may be suggested that whereas MCh requires the intracellular second messengers...
for its effect, volatile anesthetics may not depend on these intermediaries to inhibit the $I_{Ba}$ through L-type channels.

**Effect of Administration of MCh First, with Subsequent Addition of Volatile Anesthetic (and MCh) (Schedule 3A).** Administration of MCh (500 nM) alone produced $42 \pm 4$ and $54 \pm 4\%$ inhibition of peak and late $I_{Ba}$, respectively, as shown in Fig. 6 (combined values for Fig. 6, A and B; $n = 18$). When it was followed immediately by either volatile anesthetic (in the presence of MCh), the inhibition was doubled, i.e., $81 \pm 1$ and $88 \pm 1\%$ ($n = 18$) of peak and late $I_{Ba}$, respectively. Although the oocytes were exposed to MCh for a total period of 4 min in this experiment, the response with MCh was stable without any evidence of desensitization. This is evident from the control experiments in which 4-min exposure to MCh produced an inhibition of $46 \pm 5$ and $58 \pm 8\%$ ($n = 8$) of peak and late current, respectively. This is consistent with the observation that the inhibition produced by MCh was stable for a prolonged period and that its recovery was incomplete.

**Effect of the Administration of Volatile Anesthetic First, with Subsequent Addition of MCh (and Volatile Anesthetic) (Schedule 3B).** Administration of either volatile anesthetic alone led to nearly $50$ and $60\%$ inhibition of peak and late $I_{Ba}$, respectively, as shown in Fig. 7. Inset, averaged V$n$ and k$n$ for the control and volatile anesthetic. D, summary of the effects of halothane or isoflurane (shown in Fig. 2B) on the various components of $I_{Ba}$. $a p < 0.001$, $b p < 0.01$, $c p < 0.05$, compared with control; paired $t$ test; $n = 7$ or 8.

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**Fig. 2.** Effects of halothane or isoflurane on L-type channels in *Xenopus* oocytes coexpressed with M1 receptors. $I_{Ba}$ was recorded as described under Materials and Methods by holding the oocytes at $-80$ mV. A, inhibition of $I_{Ba}$ by volatile anesthetics. Recording solution equilibrated with the respective volatile anesthetic was perfused continuously for a period of 2 to 4 min, followed by the recording of $I_{Ba}$. The wash response was obtained after intermittent washing during a period of 2 to 4 min. B, I-V plot for the peak $I_{Ba}$ for control, treatment, and wash. Oocytes were depolarized (from the holding potential) to step potentials starting from $-50$ to $100$ mV in $10$-mV increments for a duration of $450$ ms; $n = 7$. C, conductance plot for the peak $I_{Ba}$. The plot shows the averaged conductance for peak $I_{Ba}$ shown in the I-V plots in Fig. 2B. $G_{Ba}$ was calculated as, $G_{Ba} = I_{Ba}/(V - V_{rev})$ 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 shown under the data analysis. D, summary of the effects of halothane or isoflurane (shown in Fig. 2B) on the various components of $I_{Ba}$.
age-gated L-type channels were readily inhibited by the application of volatile anesthetic.

**Effect of Simultaneous Administration of MCh and Either Volatile Anesthetic (Schedule 3C).** Exposure of the oocytes simultaneously to the two inhibitors, MCh (500 nM) and halothane or isoflurane, led to the inhibition of $I_{\text{Ba}}$, which was approximately equal to the individual effects of MCh and volatile anesthetic, added together (Fig. 8). To be precise, MCh produced an inhibition of $42 \pm 4$ and $54 \pm 4\%$ of peak and late $I_{\text{Ba}}$, respectively (combined MCh values for Fig. 6, A and B; $n = 18$). Similarly, the inhibition produced by volatile anesthetics was $53 \pm 2$ and $63 \pm 2\%$ of peak and late $I_{\text{Ba}}$, respectively (combined halothane and isoflurane values as shown in Fig. 7, A and B; $n = 23$). The combined administration of either volatile anesthetic and MCh led to the inhibition of $79 \pm 2$ and $88 \pm 2\%$ (the combination of MCh and halothane and MCh and isoflurane as shown in Fig. 8, A and B; $n = 16$) of peak and late currents, respectively. This indicates that volatile anesthetic effect on the modulation of L-type channels by M$_1$ receptors may be minimal, whereas its direct effect is predominant.

**Discussion**

L-type HVGCCs appear to be the prime source of calcium entry into cells present in the skeletal muscle, cardiovascular tissue, and also contribute to calcium regulation in neurons of various brain regions. The “L” stands for the “long-lasting” characteristics of this current as these channels inactivate very slowly, compared with the other members of HVGCCs. An L-type channel with near-native properties has been demonstrated with the combined expression of α1C, β, and α2δ.

Fig. 3. Effect of blockade of G-proteins or PLC with GDP-β-S or U73122 on the modulation of L-type channels by MCh or volatile anesthetics in *Xenopus* oocytes coexpressed with M$_1$ receptors. A, oocytes belonging to this group were injected with 10 mM GDP-β-S (50.6 nl) at least 30 to 60 min before their transfer to the recording chamber. They were held at −80 mV and allowed to equilibrate for about 8 min in the recording chamber before recording the control $I_{\text{Ba}}$. The testing with MCh followed this. Following the confirmation of the efficacy of GDP-β-S, this batch of oocytes was used to test volatile anesthetics. B, these oocytes were incubated with U73122 (2 μM) at room temperature for 40 min to 1 h before the recording of current, as previously described. Following confirmation of the blockade of the MCh effect, these oocytes were used for testing volatile anesthetics. C, summary of the responses obtained after the blockade of G-protein or PLC. Numbers in parentheses indicate $n$. *$p < 0.001$, compared with the control; paired $t$ test.

Fig. 4. Effect of heparin (molecular weight ~3000; 2 mM; 50.6 nl) on the modulation of L-type channels by MCh or volatile anesthetics in *Xenopus* oocytes coexpressed with M$_1$ receptors. Oocytes belonging to this group were injected with heparin 30 to 60 min before current recording. This was followed by their transfer to the recording chamber, held at −80 mV for 8 min before recording the control $I_{\text{Ba}}$. Testing with MCh followed this. Following the confirmation of the efficacy of heparin treatment, this batch of oocytes was used to test volatile anesthetics. Numbers in parentheses indicate $n$. *$p < 0.001$, compared with the control; paired $t$ test.
subunits in artificial systems, compatible with biochemical and immunoprecipitation studies that have shown these channels as heterologmeric complexes composed of these subunits (Krizanova, 1996). The critical conductance characteristic of L-type channels are derived from the \( \alpha_{1C} \) subunit, which constitutes the channel pore and harbors the binding site for its selective antagonist (and agonists), the dihydropyridine group of compounds. Another distinctive feature of the \( \alpha_{1C} \) subunit is the presence of the \( \text{Ca}^{2+} \) sensor in its cytoplasmic C terminus, which modulates the \( \text{Ca}^{2+} \)-induced feedback inhibition (Zhou et al., 1997; Zuhlke and Reuter, 1998). The \( \alpha_{1C} \) subunit appears to be the target for the modulation not only by \( \text{Ca}^{2+} \) entering through the pore, but also by processes known to release \( \left[\text{Ca}^{2+}\right]_{\text{i}} \). For example, the activation of coexpressed AT \(_{1A} \) receptors, which is known to follow a second messenger pathway involving G-proteins, PLC, IP3, and \( \left[\text{Ca}^{2+}\right]_{\text{i}} \), resulted in the inhibition of L-type channels (Oz et al., 1998). Such a pathway may be involved in MCh-induced decrease in peak \( I_{\text{Ba}} \) and the associated acceleration of inactivation of L-type channels (Fig. 1), based on the known involvement of the above second messengers with \( \left[\text{Ca}^{2+}\right]_{\text{i}} \). Numbers in parentheses indicate \( n \). \(* p < 0.001\), compared with the control; paired \( t \) test.

(Figs. 3–5). Although the stimulation of M\(_1\) receptors is also known to activate PKC, via diacylglycerol, this mechanism did not appear to play a role because exposure of these oocytes to PMA failed to alter the L-type \( I_{\text{Ba}} \), significantly (G. Kamatchi, S. Tiwari, C. Chen, M. Durieux, and C. Lynch, unpublished observations).

Halothane or isoflurane also inhibited peak \( I_{\text{Ba}} \) in addition

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**Fig. 5.** Effect of depletion or buffering of \( \left[\text{Ca}^{2+}\right]_{\text{i}} \), with thapsigargin or BAPTA on the modulation of L-type channels by MCh or volatile anesthetics in Xenopus oocytes coexpressed with M\(_1\) receptors. A, oocytes belonging to this group were incubated overnight with thapsigargin (1 \( \mu M \)). These oocytes were transferred to the recording chamber, held at \(-80 \text{ mV}\) for 8 min before recording the control \( I_{\text{Ba}} \). Testing with MCh followed this. Following the confirmation of the efficacy of thapsigargin, this batch of oocytes was used to test volatile anesthetics. B, these oocytes were injected with BAPTA tetrasodium solution (40 mM; 41.4 nl) 1 to 3 h before clamping. Control and testing with MCh followed this. Following the confirmation of the efficacy of BAPTA, this batch of oocytes was used to test volatile anesthetics. C, summary of the responses obtained after depletion or buffering of \( \left[\text{Ca}^{2+}\right]_{\text{i}} \). Numbers in parentheses indicate \( n \). \(* p < 0.001\), compared with the control; paired \( t \) test.

**Fig. 6.** Effect of administration of MCh (500 nM) first, with subsequent addition of halothane or isoflurane in Xenopus oocytes coexpressed with M\(_1\) receptors and L-type channels (schedule 3A). Following the measurement of control \( I_{\text{Ba}} \), these oocytes were exposed to MCh for a period of 2 min, as described under Materials and Methods. Immediately after recording the response with MCh, these oocytes were perfused continuously with the solution equilibrated with halothane or isoflurane (in the presence of MCh). \( I_{\text{Ba}} \) was recorded after 2 min and followed by the wash protocol. The dotted line indicates the nearly 80% inhibition of the peak \( I_{\text{Ba}} \) as shown in Fig. 8. Numbers in parentheses indicate \( n \). \(* p < 0.001\), compared with the control; \( \ast p < 0.001\), compared with MCh; paired \( t \) test.
to accelerating inactivation. Unlike the M₁-mediated MCh effect, volatile anesthetic-induced inhibition was readily reversible with washing (Fig. 2). The inhibitory effect of volatile anesthetic appeared to be dependent on voltage with a small depolarizing shift in $V_n$, the voltage at half-maximal activation, and a decrease in $k_n$, the slope factor of voltage dependence. In addition to inhibition of L-type channels (Pancrazio, 1996), halothane has also been shown to uncouple muscarinic receptors from G-proteins in rat cerebral cortex and brainstem preparations (Aronstam et al., 1986). By interfering with the function of α or βγ subunits of inhibitory G-proteins with the effector, halothane led to the stimulation of adenylyl cyclase activity (Schmidt et al., 1995). However, GDP-β-S caused no change in volatile anesthetic inhibition, making a G-protein-mediated action unlikely (Fig. 3). Fur-

Fig. 7. Effect of administration of halothane or isoflurane first, followed by MCh (500 nM) in *Xenopus* oocytes coexpressed with M₁ receptors and L-type channels (schedule 3B). Following the measurement of control $I_{\text{Ba}}$, these oocytes were exposed to either halothane or isoflurane for a period of 2 min, and the $I_{\text{Ba}}$ was recorded. This was followed immediately with the continuous perfusion of a solution containing MCh that was equilibrated with either halothane or isoflurane. $I_{\text{Ba}}$ was recorded and followed by the wash protocol. The dotted line indicates the nearly 80% inhibition of the peak $I_{\text{Ba}}$ caused by the combined administration of MCh and halothane or isoflurane, as shown in Fig. 8. Numbers in parentheses indicate $n$. *p < 0.001, compared with the control; $^p < 0.001, ^b p < 0.001$, compared with the respective volatile anesthetic; $^* p < 0.001$, compared with MCh (500 nM) response shown in Fig. 6; paired $t$ test.

Fig. 8. Effect of combined administration of MCh (500 nM) and halothane or isoflurane in *Xenopus* oocytes coexpressed with M₁ receptors and L-type channels. Following the measurement of control $I_{\text{Ba}}$, the oocytes were perfused with a solution containing MCh and equilibrated with either halothane or isoflurane for a period of 2 min, and $I_{\text{Ba}}$ was recorded. This was followed by the wash protocol. Numbers in parentheses indicate $n$. *p < 0.001, compared with control; paired $t$ test.
thermore, halothane significantly inhibited arginine vasopressin-induced increase in IP3 formation, suggesting a means by which the anesthetics may alter agonist-induced [Ca2+]i release (Sill et al., 1991). In contrast, anesthetics at relevant concentrations produced no inhibition of agonist-induced accumulation of total IP3 (Bazil and Minneman, 1989). The lack of effect of heparin on volatile anesthetic inhibition suggests that this mechanism is not likely to be involved (Fig. 4). In an another study, halothane or isoflurane inhibited Na+/Ca2+ exchange at concentrations relevant to anesthesia. Although these authors did not correlate this alteration of Ca2+ homeostasis to general anesthesia, it was suggested to underlie the in vivo vasodilator effects of the anesthetic (Haworth and Goknur, 1995). Halothane has also been shown to activate release of Ca2+ from internal stores in a variety of cell types in ryanodine receptors (Lynch and Frazer, 1994; Pajewski et al., 1996), although none of these possible alterations in Ca2+ stores appear to contribute in this case, since BAPTA or thapsigargin did not alter volatile anesthetic inhibition (Fig. 5). Thus, the inhibition of L-type channels by volatile anesthetics after the blockade of these components of this intracellular signaling pathway suggests that the primary action may lie directly on the channel protein or the membrane lipid bilayer.

Our efforts to examine the relative sensitivity of the voltage-gated L-type channel and G-protein-coupled M1 receptors to volatile anesthetics revealed interesting results. M1 receptor-induced inhibition of L-type channels by preadministered MCh, was still present (Fig. 6) when volatile anesthetic was administered subsequently. This is evident as the addition of the inhibition (of peak I_max) produced by these two agents per se was approximately equal to the inhibition produced by the combined administration of MCh and either volatile anesthetic as shown in Fig. 8 (see the dotted line in Fig. 6C). Hence, it is evident that neither the existing effect of MCh nor the effect produced by the combined administration of MCh was affected by volatile anesthetics as seen in Figs. 6 and 8. This may be explained on the basis of the membrane action of the volatile anesthetics. Even though MCh acts on M1 receptors on the membrane, its ultimate effect is intracellularly mediated through the release of [Ca2+]i, which is known to act at the Ca2+ sensor of the a1C subunit of the calcium channel (Zhou et al., 1997; Zuhlke and Reuter, 1998) possibly by long-term modification of the channels by calcium-dependent kinase. This failure to interfere with the intracellular effect of MCh by simultaneous or subsequent volatile anesthetic application combined with their ongoing calcium channel inhibition even after the blockade of intracellular second messengers suggests that volatile anesthetics may be acting at a site distant from that altered by M1 activation. However, the volatile anesthetics could probably interfere with the M1 pathway when they preceded MCh application, clearly interfering with this G-protein-mediated signaling mechanism (Fig. 7).

An interesting contrast can be made with our previous work examining volatile anesthetic and M1 receptor or PMA-induced modulation of R-type HVGCCs. In that case, either MCh or PMA increased currents by a PKC-dependent pathway. Although prior or even simultaneous application of volatile anesthetics blocked PMA-induced PKC effects on R-type HVGCC currents, M1 activation still resulted in enhancement of currents (Kamatchi et al., 2000). Thus, although M1 signaling via PKC was resistant to interference by volatile anesthetics, the apparently calcium-mediated effect on L-type channels by MCh was altered by volatile anesthetics, resulting in the attenuation of the inhibitory effect. That is, although the volatile anesthetics were inhibitory, they prevented the inhibitory action of M1 activation if present before the receptor activation (Fig. 7).

Inhibition of calcium channels may well be relevant to the state of anesthesia, since nonspecific HVGCC blockade by Cd2+, as well as L-type channel blocker by verapamil, have been found to increase the anesthetic potencies of ethanol, pentobarbitone, or ketamine in mice (Dolin and Little, 1986; Shen and Pappano, 1995). Funnel-web spider toxin that blocks P-type and other HVGCCs can cause lethargy and stupor in mice (Llinás et al., 1989; Norris et al., 1996), whereas blockade of N-type channels by spinally administrated α-conotoxin MVIIA (SNX-111, ziconotide) has distinct antinociceptive actions (Bowersox et al., 1996). Similarly, muscarinic inhibition in the central nervous system can result in sedative actions, effects that could augment other volatile anesthetic actions (Durieux, 1996). Although the inhibition of L-type channels by M1 receptor activation appears to be relevant in heart where the parasympathetic tone exerts an inhibitory effect on the heart rate, conduction, and contraction, the relative absence of M1 receptors (and the presence of M2 receptors) in the heart precludes such an assumption. However, such an effect is possible in the central nervous system, especially hippocampus, where both M1 receptors and L-type channels are present in higher levels (Stea et al., 1995; Levey, 1996). Presumably, the actions of volatile anesthetic on HVGCCs and other sites (e.g., GABA_A channels) assume predominance over the varied muscarinic actions on HVGCCs and other ion channels and signaling pathways.

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