Halothane Inhibits Signaling through m1 Muscarinic Receptors Expressed in Xenopus Oocytes

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Background: Interactions between volatile anesthetics and muscarinic acetylcholine receptors have been studied primarily in binding assays or in functional systems derived from tissues or cells, often containing multiple receptor subtypes. Because interactions with muscarinic signaling systems may explain some effects and side effects of anesthetics and form a model for anesthetic-protein interactions in general, the author studied anesthetic inhibition of muscarinic signaling in an isolated system.

Methods: mRNA encoding the m1 muscarinic receptor subtype was prepared in vitro and expressed in Xenopus oocytes. Effects of halothane on methylcholine-induced intracellular Ca²⁺ release was measured. Angiotensin II receptors were expressed to evaluate anesthetic effects on intracellular signaling.

Results: m1 Receptors expressed in oocytes were functional, and could be inhibited by atropine and pirenzepine. Halothane depressed m1 muscarinic signaling in a dose-dependent manner: half-maximal inhibition of 10⁻⁷ M methylcholine was obtained with 0.3 mM halothane. The effect was reversible and could be overcome by high concentrations of muscarinic agonist. Angiotensin II signaling was unaffected by 0.34 mM halothane.

Conclusions: m1 Muscarinic signaling is inhibited by halothane, and lack of halothane effect on angiotensin signaling indicates that the intracellular signaling systems of Xenopus oocytes are unaffected. Therefore, the most likely site of halothane action is the receptor and/or G protein. Oocytes provide a versatile system for detailed investigation into the molecular mechanism of anesthetic-protein interactions. (Key words: Anesthetics, volatile; halothane, cDNA. Molecular biology. Receptors: angiotensin; G protein-coupled; muscarinic acetylcholine. Xenopus laevis oocytes.)

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A substantial body of recent research into the molecular actions of anesthetics focuses on the interactions between anesthetic drugs and membrane signaling proteins. Not only have such interactions been shown to exist, but they may well be of importance in bringing about the anesthetic state. In particular, the class of inotropic membrane receptors (receptor-gated ion channels), including the GABA receptor/Cl⁻ channel complex and the NMDA-gated ion channel, has received much attention. However, the large superfamily of G protein-coupled metabotropic membrane receptors has been studied in much less detail, possibly because of the lack of an assay system as sensitive and versatile as the patch clamp. This is unfortunate, because many members of this group have important signaling functions in physiologic control loops known to be affected profoundly by anesthesia. Examples are the receptors involved in maintenance of cardiovascular homeostasis (such as receptors for angiotensin, adrenergic agents, dopamine, and adenosine), the opioid receptors, and the cannabinoid receptors.

One group of receptors particularly interesting from this viewpoint are the muscarinic acetylcholine receptors, because they play a variety of roles in systems of importance to the anesthesiologist. The effects of muscarinic activation by an anticholinesterase, such as neostigmine, or the actions of a muscarinic antagonist, such as atropine, demonstrate the role of muscarinic signaling on the heart, on pulmonary and gut smooth muscle, and on glandular tissue. The pronounced central effects of a muscarinic antagonist, such as scopolamine, show the role of muscarinic signaling in memory, learning, and the maintenance of consciousness. Understanding the interactions of anesthetics with these receptors is, therefore, important.

Most of the studies reported thus far have not investigated the signaling function of the receptors, but, instead, describe effects of volatile anesthetics on agonist and antagonist binding. Briefly, many volatile anes-
Anesthetics inhibit binding of antagonist without affecting agonist binding, and alter the effect of guanine nucleotides on agonist binding. These studies, although providing useful information, do not address the interaction of anesthetics with muscarinic signaling per se. In addition, they are hampered by the fact that most cells and tissues contain several subtypes of muscarinic receptors, possibly affected differentially by anesthetics, which makes interpretation difficult. A similar limitation applies to the studies of signaling effects reported thus far. The five subtypes of muscarinic receptor known at present have been cloned and are, therefore, available for study in isolated systems, such as *Xenopus laevis* oocytes. *Xenopus* oocytes provide a well-characterized system for the study of G protein-coupled receptors under controlled conditions. RNA, either extracted from tissues or encoding a cloned receptor, can be injected into the cells, and will be translated faithfully into protein and inserted into the oocyte membrane. The cells are virtually devoid of endogenous metabolotropic receptors (with the exception of a receptor for lysophosphatidate), yet contain the appropriate intracellular pathways to link activation of the receptor, through phospholipase C-mediated inositoltrisphosphate generation, to intracellular Ca\(^{2+}\) release (Fig. 1). In addition, they have an endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) channel that can be used as a convenient method to report changes in intracellular Ca\(^{2+}\). The system has been used to study isolated muscarinic receptors, beginning with the original cloning of the muscarinic receptor in 1986, and has been particularly useful to elucidate the sequence elements coupling the various receptor subtypes to G proteins. Therefore, the *Xenopus* oocyte model was chosen to study the interactions between volatile anesthetics and the functional activity of muscarinic receptors. I selected halothane as a clinically used and thoroughly studied anesthetic drug, and the m1 subtype, the most prominent one in cortical brain tissue, as a representative muscarinic receptor. Changes in intracellular Ca\(^{2+}\) were used as an endpoint. I attempted to answer the following specific questions:

1. Does halothane, in clinically relevant concentrations, affect the increases in intracellular Ca\(^{2+}\) resulting from m1 muscarinic receptor activation?
2. If so, is this effect localized to the receptor and/or G protein, or are intracellular pathways affected by the anesthetic as well?

Materials and Methods

The study protocol was approved by the Animal Research Committee at the University of Virginia.

Animals

Female *Xenopus laevis* toads were obtained from Xenopus I (Ann Arbor, MI), housed in an established frog colony, and fed regular frog brittle twice weekly. Frogs were operated for oocyte harvesting once every 2 months at most, and killed by decapitation after six operations. For removal of oocytes, a frog was immersed in ice until unresponsive to a painful stimulus (toe pinching). A 1-cm-long incision was made in a lower abdominal quadrant and a lobule of ovarian tissue, containing approximately 200 oocytes, was removed and placed in modified Barth's solution (containing, in mm: NaCl 88, KCl 1, NaHCO\(_3\) 2.4, CaCl\(_2\) 0.41, MgSO\(_4\) 0.82, Ca(NO\(_3\))\(_2\) 0.3, gentamicin 0.1, and HEPES 15, pH adjusted to 7.6). 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 ovarian tissue was washed immediately and copiously with modified Barth's solution and dissected.
into small clusters of 20–50 oocytes. Mature oocytes (Dumont stage V and VI) were isolated manually and cultured in modified Barth’s solution at 18°C. The follicular cell layer was removed manually from each oocyte using microforceps.

**mRNA Synthesis and Injection**

The rat m1 muscarinic acetylcholine receptor complementary DNA (cDNA) was obtained from Dr. T. I. Bonner (National Institute of Mental Health, Bethesda, MD). It consists of a 2.8-kilobasepair fragment in a commercial vector (pGEM1; Promega, Madison, WI). The construct was linearized by digestion with the nuclease *Hind* III and mRNA was prepared by transcription *in vitro* using the bacteriophage RNA polymerase T7. A capping analog (7$’$GpppG) was included in the reaction to generate capped transcripts, as these are translated more efficiently in the oocyte. The resulting mRNA was quantified by spectrometry and diluted in 100 mM KCl to a concentration of 0.1 mg/ml.

The rat AT$_1$A angiotensin II receptor clone was obtained from Dr. K. R. Lynch (University of Virginia, Charlottesville, VA) as a cDNA of 1.2 kilobasepair in the CDM8 vector (Invitrogen, San Diego, CA). The construct was linearized with the nuclease *Xho* I and transcribed in the presence of capping analog by T7 polymerase.

Oocytes were injected with 50 ml of mRNA (5 ng) in 100 mM KCl, using an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA). The adequacy of injection was confirmed by noting the slight increase in cell size during injection. The cells were then cultured for 3 days before study.

**Electrophysiologic Recording**

A single defolliculated oocyte was placed in a perfusable recording chamber (3 ml volume) filled with Tyrode’s solution (containing, in mM: NaCl 150, KCl 5, MgCl$_2$ 1, CaCl$_2$ 2, dextrose 10, and HEPES 10, pH adjusted to 7.4). Microelectrodes were pulled in one stage from capillary glass (BBL with fiber; World Precision Instruments, Sarasota, FL) on a micropipette puller (model 700C; David Kopf Instruments, Tujunga, CA). Tips were broken to a diameter of approximately 10 μm. They were filled with 3 M KCl and tip resistances were usually 1–3 MΩ. The cell was voltage clamped using a two-microelectrode oocyte voltage clamp amplifier (OC725A; Warner Corporation, New Haven, CT), connected to a data acquisition and analysis system running on an IBM-compatible personal computer. The acquisition system consisted of a DAS-8 A/D conversion board (Keithley-Metabyte, Taunton, MA) and analysis was performed with a custom-written program that has been described previously (Oo-Clamp$^{15}$). Holding potential was −70 mV unless indicated otherwise. Only cells that showed a stable holding current of less than 1 μA during a 1-min equilibration period (more than 95% of cells) were included for analysis. Membrane current was sampled at 125 Hz and recorded for 5 s before and 5 s after application of the test compounds. Compounds were delivered in 30-μl aliquots over 1–2 s using a hand-held micropipettor positioned approximately 5 mm from the oocyte. Responses were quantified by integrating the current trace by quadrature and are reported as microCoulombs (μC), because this reflects CI$^-$ flux better than does peak current.$^{11,12,16}$ All experiments were performed at room temperature.

For intracellular injections of EGTA, a third microelectrode, connected to an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA), was inserted into the oocyte. Cells were injected under voltage clamp and the adequacy of injection was verified by observing the small increase in cell size on injection.

**Anesthetic Administration**

To determine the effect of halothane, output from a halothane vaporizer was bubbled through a reservoir filled with 40 ml of Tyrode’s solution. Air at a flowrate of 500 ml/min was used as the carrier gas, and at least 10 min were allowed for equilibration. The solution from the reservoir was then perfused through the recording chamber at a rate of approximately 10 ml/min, and measurements were obtained after 10 bath volumes had been exchanged. To quantify the concentrations of halothane 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 a halothane standard. Results were converted to concentrations in liquid using aqueous/gaseous partition coefficients at 25°C.$^{17}$

**Data Analysis**

Responses are reported as mean ± SEM. Because variability between batches of oocytes is common, responses were, at times, normalized to controls from the same batch to allow inclusion of data from multiple frogs into the same comparison. Differences between
treatment groups were analyzed using Student’s t test; multiple responses in single oocytes were analyzed using paired t test. If multiple comparisons were performed, ANOVA was used, followed by t test appropriately corrected for multiple comparisons. P < 0.05 was considered significant.

Materials
Molecular biology reagents were obtained from Promega (Madison, WI). All other chemicals were obtained from Sigma (St. Louis, MO).

Results

Expression of Functional m1 Receptors in Oocytes

Although application of acetyl-β-methylcholine (MCh) to un.injected oocytes was without effect (data not shown), application of MCh to oocytes injected with 5 ng m1 receptor mRNA resulted in a transient inward current (Fig. 2A). The current developed after a latency of approximately 1 s and consisted of a fast inward component, followed by a relaxation over several seconds, on which small fluctuations were superimposed. This is the typical response pattern for G protein-coupled receptors expressed in Xenopus oocytes. Charge movements in response to 10^{-7} M MCh were 3.52 ± 0.58 μC (n = 15), respectively. The responses did not desensitize (Fig. 2B). To confirm that the responses were, indeed, caused by muscarinic signaling, the ability of muscarinic antagonists to inhibit the currents was evaluated (Fig. 2C). Responses to MCh could be blocked completely by prior application to the recording chamber of atropine (1 μM; n = 3) or the specific m1 receptor antagonist pirenzepine (1 μM; n = 3), confirming the involvement of a muscarinic pathway.

The Induced Responses are I_{Cl(Ca)}

The oocyte membrane contains Ca^{2+}-activated Cl^- channels, and release of intracellular Ca^{2+} by activation of expressed receptors therefore results in a Ca^{2+}-activated Cl^- current (I_{Cl(Ca)}). To determine if the responses observed were I_{Cl(Ca)}, I performed the following experiments.

When oocytes were injected with 50 nl of 100 mm EGTA (estimated intracellular concentration 5–10 mm) before recording, MCh was no longer able to induce electrophysiologic responses (Fig. 3A; n = 3). To confirm that the unresponsiveness was not caused by the manipulation involved in the injection, some oocytes were injected with 50 nl of sterile water. In these oocytes, normal responses were obtained (8.83 ± 4.40 μC with 10^{-6} M MCh; n = 3). Therefore, the response is dependent on intracellular Ca^{2+}. I also determined the role of extracellular Ca^{2+} by eliciting responses to MCh in nominally Ca^{2+}-free bath solution. Responses to 10^{-6} M MCh were 10.0 ± 0.6 μC (n = 3), indicating that influx of extracellular Ca^{2+} plays, at most, a minor role in the response.

To establish the ionic nature of the current, I measured its reversal potential by inducing responses at different holding potentials (Fig. 3B). The reversal po-
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After establishing that functionally coupled m1 receptors could be expressed in oocytes, I studied the effect of halothane on this signaling pathway. Halothane depressed muscarinic signaling in a dose-dependent manner (Fig. 4A). At a halothane concentration of 0.34 mm, the responses were decreased by 34% of control. Curve fitting using the Hill equation showed an EC50 for halothane of 0.3 mm.

To confirm that the effect of halothane was reversible, responses to MCh were tested three times: before exposure to halothane, after exposure to 0.34 mm halothane, and after a wash with anesthetic-free solution. A relatively high concentration of anesthetic was chosen because it was considered more likely to induce irreversible effects. However, no significant differences were found between the first and third responses to MCh (Fig. 4B). Therefore, the inhibitory effects of halothane are reversible.

I also tested the dependence of the inhibitory effect of halothane on the MCh concentration (Fig. 4C). The degree of inhibition obtained by halothane decreased as the MCh concentration was increased; with 10^{-3} M MCh and higher, no significant depression of the currents occurred.

Halothane Inhibition Occurs at the Receptor or G Protein Level

The signaling pathway for G protein-coupled receptors in oocytes is a complex one (Fig. 1) and it is, therefore, conceivable that anesthetic interactions with this pathway could take place at several levels. Some potential interactions, particularly effects on the Xenopus-specific Ca^{2+}-activated Cl^- channel, would be of little clinical relevance. It was, therefore, of importance to determine where halothane interferes with muscarinic signaling in this system. To determine whether downstream signaling systems were affected, I expressed the AT1a angiotensin II receptor, a G protein-coupled receptor that has not been shown to be affected by volatile anesthetics. The AT1a receptor, when expressed in oocytes, also induces I_{Ca(L)} through a signaling pathway that, apart from receptor and (possibly) G protein, is considered to be the same as that for muscarinic signaling. Thus, lack of anesthetic inhibition of angiotensin signaling would indicate that, when inhibiting muscarinic signaling, halothane affects the receptor, the G protein, or the interaction between the two.

Angiotensin II (All, 10^{-7} M) induced no responses in uninjected oocytes (data not shown), but induced currents in AT1a-injected oocytes that were indistinguishable from those induced by MCh in m1-injected oocytes (Fig. 5A). Average charge movements were 9.78 ± 2.18 µC. These currents were inhibited by the nonpeptide angiotensin receptor antagonist losartan (data not shown), indicating that they were caused by angiotensin signaling. The presence of 0.34 mm halothane did not interfere with angiotensin signaling (Fig. 5B). If anything, the trend was for the responses to increase. Thus, the intracellular signaling cascade from G protein to the Ca^{2+}-activated Cl^- channel appears unaffected by halothane. Because this segment of the signaling pathway is the same for muscarinic and angiotensin signaling, halothane apparently inhibits...
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muscarinic signaling by interfering either with receptor function or receptor-G protein coupling.

Discussion

I have demonstrated that halothane, at clinically relevant concentrations, inhibits muscarinic signaling, and the lack of effect on angiotensin signaling indicates that the interaction probably takes place at the receptor or G protein level.

I chose *Xenopus* oocytes as my expression system because they have been shown to generate and traffic receptors well, and have an appropriate, albeit complex (Fig. 1), intracellular signaling system that allows convenient quantitation of responses. Using mRNA extracted from tissue, oocytes have been used to study the effects of barbiturates on Ca\(^{2+}\) channels,\(^6\) enflurane on NMDA channels,\(^9\) enflurane\(^8\) and methoxyflurane\(^21\) on serotonin receptors, and enflurane on muscarinic receptors.\(^8\) The use of cDNA-derived RNA has advantages over extracted RNA, because it allows the study of receptor subtypes in isolation and, through mutagenesis, makes further molecular characterization of the interaction between anesthetic and receptor possible. However, potential problems with the technique should be considered when interpreting the data. First, there have been reports of endogenous muscarinic responses in *Xenopus* oocytes.\(^22,23\) In this and previous studies using this model,\(^12\) I have not seen responses in uninjected oocytes. Other investigators similarly have not reported endogenous muscarinic activity.\(^8,13\)

Apparently, these responses are frog dependent, and many frogs do not express endogenous receptors. Second, the possibility exists that intracellular pathways or the Ca\(^{2+}\)-activated Cl\(^-\) channel may be affected by anesthetics, confounding the results. However, my results with expressed angiotensin receptors make this unlikely. Others have found that Ca\(^{2+}\)-activated Cl\(^-\) currents induced by inositoltrisphosphate\(^8,21\) or by direct injection of Ca\(^{2+}\)\(^22\) are also unaffected by volatile anesthetics. Third, my experiments were performed at room temperature, whereas the expressed receptor is derived from a homeothermic animal. Although in theory this may influence its behavior, I felt it more important to keep the cell membrane at its normal state, because changes in lipid bilayer structure with abnormally high temperature would be likely to affect membrane interaction, both with the anesthetic and with the receptor. Anesthetic concentrations were corrected for temperature-dependent solubility. Finally, the introduction of a single clone into a cell always carries the possibility that an essential or modulating cofactor may be lacking. Although, with multisubunit channels and inotropic receptors, this is a very significant prob-

![Graph A](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931309/)

![Graph B](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931309/)

![Graph C](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931309/)

Fig. 4. Halothane inhibits muscarinic signaling. All measurements were performed in oocytes voltage clamped at \(-70\) mV. (A) Halothane has a dose-dependent inhibitory effect on Ca\(^{2+}\)-activated Cl\(^-\) currents induced by \(10^{-7}\) M MCh in oocytes expressing the m1 muscarinic receptor. Number of oocytes used for each data point are indicated. *P < 0.01. (B) Inhibition by halothane is reversible. Three consecutive measurements were taken in single oocytes, and data are presented as percent of the control response. CTRL = control response; HAL = response in the presence of 0.34 mM halothane; REC = response after recovery. *P < 0.05. (C) Inhibition by 0.34 mM halothane is dependent on MCh concentration. White bars are responses in the absence, black bars in the presence of halothane. *P < 0.01 for \(10^{-7}\) M MCh, *P < 0.05 for \(10^{-6}\) M MCh.
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has high pirenzepine affinity and is, therefore, believed to correspond to the pharmacologic M₁ type. Studies of the tissue distribution of mRNAs have shown that most tissues contain multiple subtypes. The heart, primarily expressing m₂, appears to be an exception.

Previous investigations of the interactions between anesthetics and muscarinic receptors have focused primarily on binding studies. Aronstam et al. showed that halothane increased the binding of tritiated N-methylscopolamine to muscarinic receptors in rat cerebral cortex and brainstem membranes. This increased antagonist affinity was caused by a decrease in the dissociation constant. Although the affinity of the agonist carbachol was unaltered by the presence of 2% halothane, the anesthetic eliminated the ability of guanine nucleotides to lower agonist binding affinity, indicating interference with G protein coupling. Similar findings were later reported for diethylether. With the additional observation that binding of the agonist oxotremorine-M was inhibited modestly by 2% anesthetic. Oxotremorine was found to bind to a subpool of receptors, with both binding affinity and nucleotide sensitivity affected by anesthetics. Chloroform, enflurane, and isoflurane (each at 2%) have been shown to exert similar effects. The results are, therefore, consistent across the various studies. However, the use of brain tissue results in a mixture of muscarinic subtypes being studied, as borne out by the oxotremorine findings.

Functional studies confirm that volatile anesthetics affect muscarinic signaling. In rat heart, diethylether, enflurane, and isoflurane decreased the ability of muscarinic agonists to inhibit forskolin-stimulated cAMP accumulation. Chloroform and halothane, in contrast, decreased basal adenylate cyclase activity. Because heart expresses the m₂ subtype exclusively, these studies have fewer confounding variables than the ones mentioned previously. However, it is impossible to rule out an effect of the anesthetics on the intracellular signaling pathway. This issue has been addressed in more detail for anesthetic effects on inositolphosphate generation induced by muscarinic stimulation. Davidson et al. studied SH-SY5Y neuroblastoma cells and found that halothane (2.5%) inhibits stimulation of phosphoinositide turnover in response to carbachol, but not to bradykinin, indicating the receptor as its site of action. A recent study by Lin et al. expressed mouse brain mRNA in Xenopus oocytes and determined the effects of enflurane (1.8 mM, approximately 2 MAC) on Ca²⁺-activated Cl⁻ currents induced by acetylcho-

Fig. 5. Responses to angiotensin II are not affected by halothane. Arrow indicates the time when the agonist was applied. Oocytes were voltage clamped at −70 mV. (A) Response to 10⁻⁷ M angiotensin II in oocyte injected with 5 ng of AT₁a mRNA 3 days before. Charge movement is −5.0 μC. (B) Responses to 10⁻⁷ M angiotensin II in the absence and presence of 0.34 mM halothane, expressed as percent of the control response.

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line and serotonin.\textsuperscript{8} Enflurane was shown to depress responses to either agonist and to injected guanosine 5\textendash; (3\textendash; O-thio)triphosphate, but not to injected inositoltrisphosphate. Thus, these studies confirm my findings that muscarinic inhibition takes place at either the receptor or the G protein level and that downstream pathways are not affected. Lin \textit{et al.} also showed that inhibition by enflurane was dependent on the acetylcholine dose, with approximately 80\% inhibition noted at $10^{-7}$ M and approximately 10\% inhibition at $10^{-5}$ M acetylcholine. This, again, is in agreement with my findings with halothane. The use of brain mRNA has the disadvantage, however, of expressing several subtypes of receptors (at least four of the five subtypes have been shown to be present in brain).\textsuperscript{9} As the oxotremorine studies described above indicate, different subtypes may show different responses. In addition, the use of a single anesthetic dose is a disadvantage of this study and several others described above.

My results have several implications. The finding that not all G protein-coupled receptors are inhibited equally by anesthetics makes a localized interaction between anesthetics and muscarinic receptors likely. The site of this interaction would presumably be a hydrophobic domain in the protein, although it is not known where this would be localized. The finding that antagonist binding, but not agonist binding, to muscarinic receptors is affected by volatile anesthetics\textsuperscript{25,26,28} has lead to the suggestion that anesthetics interact with proposed hydrophobic accessory domains, contiguous to the acetylcholine binding site in the third transmembrane segment of the receptor.\textsuperscript{6} These sites are important for antagonist, but not for agonist, binding. On the other hand, the apparent effects of anesthetics on G protein coupling would indicate a site of interaction in the G protein-binding domain, primarily localized in the third intracellular loop and carboxyterminus. Because I have shown that the internal signaling pathways of oocytes are unaffected by anesthetic, these cells provide an excellent assay system to investigate these issues. A detailed comparison of responses to anesthetics in the different muscarinic subtypes, followed by the construction of chimeric proteins and site-directed mutagenesis, may make it possible to localize the site of interaction at the molecular level. This would help us to understand the interactions between anesthetics and proteins in general and, in addition, may explain in detail some of the antimuscarinic actions and side effects of volatile anesthetics.

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