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Inhibitory effects of anesthetics and ethanol on muscarinic receptors expressed in *Xenopus* oocytes

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Abstract

Anesthetics (and ethanol) are known to produce amnesia as well as immobilization. Recent identification of a nonimmobilizing (nonanesthetic) agent (F6 or 1,2-dichlorohexafluorocyclobutane) that impairs learning and memory suggests that distinct mechanisms may be responsible for these two actions of anesthetic agents. Muscarinic receptors are believed to play a role in memory and learning, and we asked if a specific subtype of these receptors is affected by anesthetics as well as the new nonanesthetic. We investigated the effects of halothane, a novel halogenated anesthetic compound F3 (1-chloro-1,2,2-trifluorocyclobutane) and ethanol on acetylcholine-induced current mediated by a muscarinic m_1 receptor expressed in *Xenopus* oocytes. We also studied the effects of halogenated nonanesthetic compounds, F6 and F8 (2,3-chlorooctafluorobutane) on muscarinic m_1 receptors. Halothane, F3, F6 and ethanol inhibited muscarinic m_1 receptor-induced Ca^{2+}-dependent Cl^- currents at pharmacologically relevant concentrations. F8 had no effect on acetylcholine-induced muscarinic m_1 receptor function. The protein kinase C inhibitor, bisindolylmaleimide I (GF109203X), enhanced the acetylcholine-induced current and the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), inhibited this current. GF109203X abolished the inhibitory effects of halothane, F3 and ethanol on muscarinic m_1 receptors but had no effect on actions of F6. These results demonstrate that anesthetics and a nonanesthetic inhibit the function of muscarinic m_1 receptors and suggest activation of protein kinase C as the mechanism of action of anesthetics and ethanol on these receptors. © 1997 Elsevier Science B.V.

Keywords: Halothane; Ethanol; Muscarinic receptor; Protein kinase C; *Xenopus* oocyte

1. Introduction

Although anesthetics are widely used in order to produce immobility and unconsciousness, subanesthetic doses of these agents may suppress awareness and reduce learning (Nabeshima et al., 1990; Dwyer et al., 1992; Melchior et al., 1993; Gonsowski et al., 1995; Kandel et al., 1996). Cholinergic antagonism interferes with learning behavior, and cholinesterase inhibitors can enhance learning (Fibiger et al., 1991) and inhibition of muscarinic receptors leads to sedation or non-REM (rapid eye movement) sleep (Durieux, 1996). The muscarinic m_1 receptor belongs to the family of G-protein coupled receptors and when activated results in production of inositol trisphosphate and diacylglycerol. Several effects of anesthetics on G-protein coupled receptors have been reported. Halothane and enflurane inhibit function of a muscarinic receptor expressed in *Xenopus* oocytes (Lin et al., 1993; Durieux, 1995). In contrast, halothane does not inhibit function of the angiotensin AT_{1A} receptor that also employs the same phoshatidylinositol signalling system as muscarinic m_1 receptors (Durieux, 1995). The muscarinic m_2 receptor, which activates a K^+ channel, is activated by high concentrations of halothane, but inhibited by isoflurane (Magyar and Szabo, 1996).

There is considerable evidence that protein kinase C plays an important role in the regulation of G-protein coupled receptors (Kato et al., 1988; Moran and Dascal, 1989; Singer et al., 1990; Manzoni et al., 1990; Sakuta et al., 1991; Sanna et al., 1994). We recently reported that halothane, F3 (1-chloro-1,2,2-trifluorocyclobutane), and ethanol all inhibit 5-hydroxytryptamine (5-HT)-induced current via the G-protein coupled 5-hydroxytryptamine 2A receptor ($5\text{-}HT_{2A}$) by a protein kinase C sensitive pathway (Minami et al., 1997). Moreover, in vitro studies demonstrate that halothane affects protein kinase C activity in

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brain (Tsuchiya et al., 1988; Slater et al., 1993; Hemmings and Adamo, 1994; Hemmings et al., 1995) and in PC12 cells (Tas and Koschel, 1991). In addition, muscarinic m_1 and m_3 receptors are phosphorylated by protein kinase C (Haga et al., 1996; Tobin et al., 1996). One goal of the present study was to determine if protein kinase C has a role in the action of structurally diverse anesthetics on muscarinic m_1 receptor function.

Because anesthetics produce a complex spectrum of actions, including amnesia, immobility and cardiovascular effects (Marchall and Longnecker, 1995), it is of interest to try to relate an action of anesthetics on a specific receptor to one of these in vivo effects. This is, of course, a formidable task, but recent studies describing structurally related compounds that display specificity for aspects of anesthetic action provide an approach to this question. In particular, two halogenated cyclobutanes, F3 (1-chloro-1,2,2-trifluorocyclobutane) and F6 (1,2-dichlorohexafluorocyclobutane), would be predicted to produce anesthesia based on their lipid solubility and the Meyer–Overton relationship. However, only F3 produces anesthesia (immobility) in rats (Koblin et al., 1994), but F6 can disrupt learning and memory although it is nonanesthetic (Kandel et al., 1996). Thus, a receptor or signalling system responsible for anesthesia should be affected by F3 but not F6 (assuming that concentrations achieved in vivo are tested), and this was found to be the case for $GABA_A$ and glycine receptors (Mihic et al., 1994; Mascia et al., 1996). However, there must also be other actions of anesthetics (e.g., F3) that are also produced by F6 and are responsible for disrupting learning and memory. One goal of the present study was to ask if the muscarinic m_1 receptor is sensitive to both F3 and F6.

The *Xenopus* oocyte expression system has been used to study a multiplicity of brain receptors with pharmacological properties that mimic those of native brain receptors (Snutch, 1988; Harris et al., 1995) and muscarinic m_1 receptor signalling resulting in activation of Ca^{2+}-dependent Cl^- currents was studied by Pritchett et al. (1988). Stimulation of muscarinic m_1 receptors lead to the activation of phospholipase C, a process that is mediated by a G-protein, resulting in the formation of myo-inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 promotes, through its interaction with IP_3 receptors, the release of Ca^{2+} from the endoplasmic reticulum, and this, in turn, triggers the opening of Cl^- channels in oocytes. This system has been well characterized and has proven useful for studying the effects of anesthetics and ethanol on G-protein coupled receptors and was employed for the present studies.

We investigated the effects of halothane, F3 and ethanol on acetylcholine-induced current via muscarinic m_1 receptors expressed in *Xenopus* oocytes. We also studied the effects of the novel halogenated nonanesthetics, F6 and F8 (2,3-chlorooctafluorobutane) on muscarinic m_1 receptors. Moreover, we studied the effects of these compounds in the absence or presence of a selective protein kinase C inhibitor in order to delineate the mechanisms by which these compounds alter acetylcholine-induced current via muscarinic m_1 receptors.

2. Materials and methods

2.1. Materials

Adult *Xenopus laevis* female frogs were purchased from *Xenopus* I (Ann Arbor, MI); acetylcholine, dimethylsulphoxide (DMSO), phorbol-12-myristate-13-acetate (PMA) and atropine were purchased from Sigma (St. Louis, MO); ethanol was from Asper Alcohol and Chemical (Shelbyville, KY); calyculin A was bought from LC Laboratories (Woburn MA); halothane was bought from Halocarbons Laboratories (River Edge, NJ); F3 (1-chloro-1,2,2-triflurocyclobutane); F6 (1,2-dichlorohexaflurocyclobutane) and F8 (2,3-chlorooctafluorobutane) were obtained from PCR (Gainesville, FL); Ultracomp Escherichia coli transformation kit was from Invitrogen (San Diego, CA). The Qiagen (Chatworth, CA) kit was used for purification of plasmid cDNA. The muscarinic m_1 receptor cRNA was prepared using mCAP mRNA capping kit (Stratagene, La Jolla, CA); bisindolylmaleimide I (GF109203X) was bought from Calbiochem (La Jolla, CA); Muscarinic m_1 receptor cDNA was kindly provided by Dr. T.I. Bonner (National Institute of Mental Health, Bethesda, MD).

2.2. Muscarinic m_1 receptor cRNA preparation

The cDNA for the muscarinic m_1 receptor was inserted into the pGEM vector. The receptor cDNA was linearized with Hind III, phenol chloroform extracted and ethanol precipitated with sodium acetate. cRNA was prepared using the Stratagene transcription kit by using T7 RNA polymerase. The cRNA was extracted using phenol-chloroform and precipitated in ethanol and sodium acetate.

2.3. Whole cell voltage clamp of injected oocytes

Isolation and microinjection of *Xenopus* oocytes were performed as described by Sanna et al. (1994). *Xenopus* oocytes were injected with 30–50 ng of cRNA coding for the muscarinic m_1 receptor. Oocytes were placed in a 100 μl-recording chamber and perfused with MBS (modified Barth's saline) containing (mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; HEPES, 10: $MgSO_4$, 0.82; $Ca(NO_3)_2$, 0.33; $CaCl_2$, 0.91, pH 7.5 at rate of 1.8 ml/min at room temperature. Recording and clamping electrodes (1–5 megaohm) were pulled from 1.2 mm outside diameter capillary tubing and filled with 3 M KCl. A recording electrode was impaled into the animal pole, and once the resting membrane potential stabilized, a clamping elec-

trode was inserted with the resting membrane potential allowed to restabilize. Warner oocyte clamp OC 725-B (Hampden, CT) was used for voltage-clamping each oocyte at −70 mV. We used the peak of the a transient inward current component of the acetylcholine-induced currents for analysis, because this component was dependent on acetylcholine concentrations and quite reproducible, as noted by Dascal et al. (1984). The anesthetic (halothane and F3), nonanesthetics (F6 and F8) and ethanol were pre-applied for 2 min to allow for complete equilibration in the bath. The volatile solutions were freshly prepared immediately before use. The concentrations in the figures represent bath concentrations. We used the previously published values to calculate the final concentration of volatile compounds in the recording chamber (Mihic et al., 1994; Dildy-Mayfield et al., 1996).

To determine whether protein kinase C plays a role in anesthetic, nonanesthetic and ethanol modulation of acetylcholine-induced current *via* the muscarinic m_1 receptors, oocytes were exposed to bisindolylmaleimide I (GF109203X) (200 nM) (Toullec et al., 1991) in incubation media (MBS containing 10 mg of streptomycin and 10 000 U of penicillin G + 50 mg of gentamycin/l + 0.5 mM theophylline + 2 mM sodium pyruvate) for 120 min. Acetylcholine was tested at 5, 20, 40, 60 and 120 min during GF109203X, treatment. Acetylcholine was applied at 20, 40, 80 and 120 min in oocytes incubated without GF109203X as a control. For studying the effects of PMA, we measured the 1 μM acetylcholine-induced currents as a control and perfused with MBS for 20 min. We then treated the oocytes with the 50 nM PMA for 5 min and tested by 1 μM acetylcholine. In other experiments, we injected oocytes with the phosphatase inhibitor, calyculin A (Ishihara et al., 1989) as described in the results section.

To determine if drug treatments altered the membrane surface we measured the membrane capacitance of the oocytes (Vasilets et al., 1990). Capacitive transients elicited by the voltage jumps from the holding potential of −30 to −60 mM were measured in each experiment. Three voltage pulses of 30 mV were applied from the resting potential and the signal, averaged from the three pulses, was integrated using the Strathclyde Electrophysiology Software program, Whole Cell Electrophysiology Program V1.2e, and membrane capacitance (C_m) was determined from the slope of the regression of the area versus voltage relationships.

2.4. Statistical analysis

Results are expressed as percentages of control responses due to variability in oocyte expression. The control responses were measured before and after each drug application, to take into account possible shifts in the control currents as recording proceeded. However, in the experiments testing the effects of GF109203X or PMA, we used the acetylcholine-induced currents before GF109203X or PMA application as a control, because the effects of these drugs are not readily reversible. The '*n*' values refer to the number of oocytes studied. Each experiment was carried out with oocytes from at least two different frogs. Statistical analyses were performed using either a *t*-test or a one-way ANOVA (analysis of variance). Curve fitting and estimation of IC_{50} values for concentration-response curves were performed using Graphpad Inplot Software (San Diego, CA).

3. Results

Anesthetic modulation of receptor function often depends upon the degree of receptor activation (Harris et al., 1995), and it was necessary to determine the acetylcholine concentration-response relationship under our experimental conditions before testing anesthetics (Fig. 1). Nonlinear regression analysis of these curves yielded an EC_{50} for acetylcholine of 0.9 μM and a Hill coefficient of 1.1. Maximal currents were observed at 100 μM. The current induced by 1 μM acetylcholine was completely abolished by atropine (10 μM). The effects of anesthetics on acetylcholine-induced currents were next examined using an acetylcholine concentration of 1 μM. Halothane, F3 and ethanol resulted in inhibitory effects on currents activated by acetylcholine (Fig. 2A and C). Halothane inhibited the action of acetylcholine to 61 ± 6%, 46 ± 13% and 24 ± 9% of control at 0.25, 1 and 2 mM of halothane, respectively. The concentration of halothane producing half-maximal inhibition (IC_{50}) of acetylcholine-induced currents was 0.5 ± 0.1 mM. F3 inhibited the currents activated by 1 μM acetylcholine to 50 ± 7% and 31 ± 5% of control at 0.4 and 0.8 mM, respectively and the IC_{50} of F3 was 0.3 ± 0.1 mM. Ethanol inhibited the currents activated by acetylcholine to 75 ± 7%, 59 ± 6% and 42 ± 9% of control at 50, 100 and 200 mM, respectively and the IC_{50} of

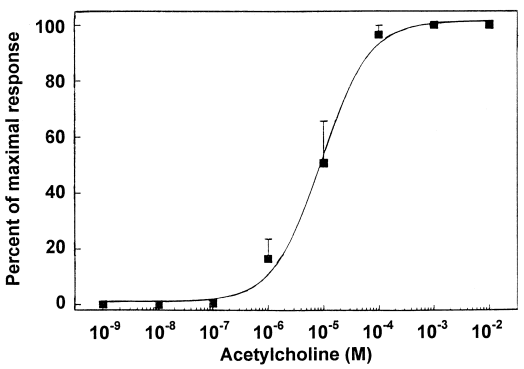

Fig. 1. Concentration-response curve for acetylcholine (100 pM–1 mM) activation of a Ca^{2+}-dependent Cl^- current in *Xenopus* oocytes expressing muscarinic m_1 receptor cRNA. Oocytes were voltage-clamped at −70 mV; acetylcholine was applied for 20 s and the peak current was measured. Values are mean ± SEM from 10 oocytes. In some cases, the error bars are smaller than the symbols.

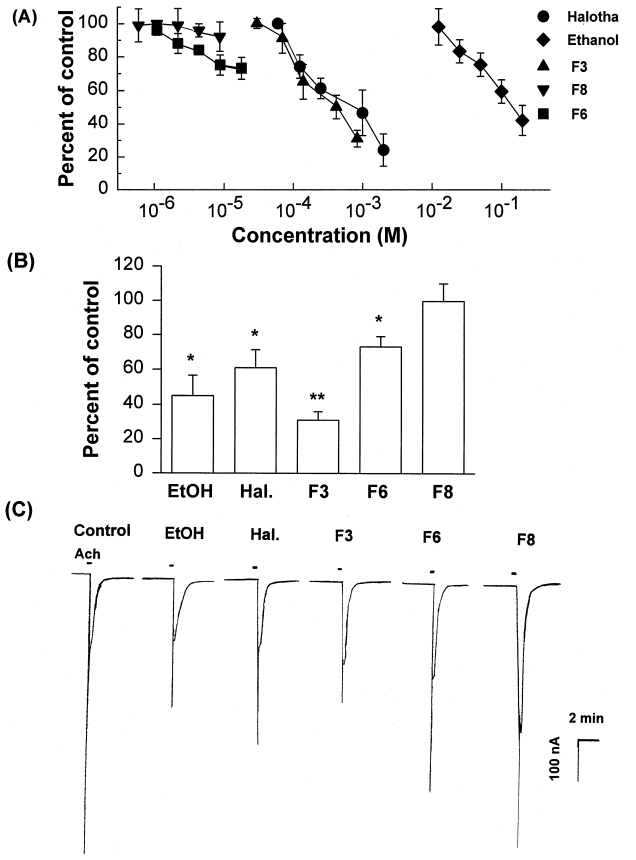

Fig. 2. (A) Effects of volatile anesthetics (halothane and F3), nonanesthetics (F6 and F8) and ethanol on the current evoked by 1 μM acetylcholine in oocytes expressing muscarinic m_1 receptor cRNA. Halothane (0.06–2 mM), F3 (0.05–0.8 mM), F6 (1.1–17.8 μM), F8 (0.6–8.8 μM) and ethanol (12.5–200 mM) were pre-applied for 2 min before being co-applied with acetylcholine for 20 sec. Data represented the mean ± SEM of 10–13 oocytes. (B) Effects of concentrations of halothane, F3, F6, F8 and ethanol corresponding to MAC or predicted MAC on currents evoked by 1 μM acetylcholine in oocytes expressing muscarinic m_1 receptors. The concentrations used were: halothane, 0.25 mM; F3, 0.8 mM; ethanol, 190 mM; F6, 18 μM; F8, 9 μM (see Harris et al., 1995). Data represent the mean ± SEM of 5 oocytes. Significant action of GF109203X indicated by: $^{*}P < 0.05$ and $^{**}P < 0.01$ (paired t-test). (C) Tracings were obtained from a single oocyte and showed the effects of halothane, ethanol, F3, F6 and F8 corresponding to MAC or predicted MAC on currents evoked by 1 μM acetylcholine in oocytes expressing muscarinic m_1 receptors.

ethanol was 78 ± 15 mM. For comparison, the minimal anesthetic concentrations (MAC) of halothane, F3 and ethanol are about 0.25, 0.8 and 190 mM (see Harris et al., 1995); thus 50% inhibition of acetylcholine action required about 2 MAC for halothane, 0.4 MAC for F3 and 0.3 MAC for ethanol. Thus, F3 and ethanol had effects on muscarinic m_1 receptors at subanesthetic concentrations. The nonanesthetic F8 did not alter currents elicited by acetylcholine, but the other nonanesthetic, F6 had weak inhibitory effects (Fig. 2A and C). We compared the effects of halothane, F3, F6, F8 and ethanol at concentrations corresponding to MAC, or predicted MAC in the case of F6 and F8, on acetylcholine-induced currents (Fig. 2B and C). At these 'equieffective' concentrations, all agents inhibited acetylcholine action except for the nonanesthetic F8 which had no effect.

Next, we investigated whether the inhibitory effects of halothane, F3, F6 and ethanol on acetylcholine-induced current could be modulated by protein kinase C. These compounds were studied using *Xenopus* oocytes that were pretreated with the protein kinase C inhibitor, GF109203X (200 nM) (Toullec et al., 1991). GF109203X produced a 7 fold enhancement of the initial current activated by 1 μM acetylcholine (Fig. 3A), but did not alter the EC_{50} for

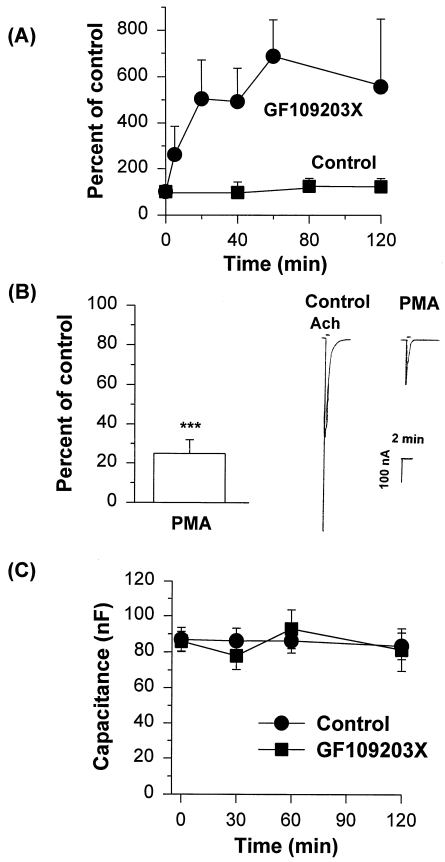

Fig. 3. (A) The protein kinase C inhibitor, GF109203X, enhanced acetylcholine-induced current in oocytes expressing muscarinic m_1 receptors. Oocytes were incubated with GF109203X (200 nM) for 2 h. Acetylcholine (1 μM) was tested at 5, 20, 40, 60 and 120 min during the application of GF109203X. Acetylcholine (1 μM) was applied at 20, 40, 80 and 120 min in oocytes incubated without GF109203X as a control. Control data represent the mean ± SEM of 10 oocytes. Data from GF109203X treated oocytes represent the mean ± SEM from 10 oocytes. (B) Phorbol 12-myristate 13-acetate (PMA) inhibited acetylcholine-induced current in oocytes expressing muscarinic m_1 receptors. We measured the 1 μM acetylcholine-induced currents as a control and perfused with MBS for 20 min and then treated the oocytes with the 50 nM PMA for 5 min and tested again 1 μM acetylcholine. Data represent the mean ± SEM of 9 oocytes. Significant inhibition, $^{***}P < 0.001$ (paired *t*-test). Tracings were obtained from a single oocyte and show the effects of PMA (50 nM) on currents evoked by 1 μM acetylcholine in oocytes expressing muscarinic m_1 receptors. (C) Effects of GF109203X on membrane capacitance (C_m) in oocytes expressing the muscarinic m_1 receptor. Oocytes were incubated with GF109203X (200 nM) for 2 h. C_m was measured at 30, 60 and 120 min during the application of GF109203X. Data represent the mean ± SEM of 6 oocytes.

Table 1
Effects of halothane, ethanol and acetylcholine on membrane capacitance (C_m) in oocytes injected with cRNA for the muscarinic m_1 receptor.

Compounds	Response (percent of control)
Acetylcholine (1 μM)	122 ± 17
Acetylcholine (1 mM)	109 ± 13
Halothane (0.25 mM)	99 ± 5
Ethanol (200 mM)	93 ± 5
Halothane (0.25 mM) + acetylcholine (1 μM)	106 ± 23
Ethanol (200 mM) + acetylcholine (1 μM)	93 ± 3

C_m was measured from the capacitive transients elicited by a voltage change described in Section 2. The control C_m was measured 5 min before application of compounds. Halothane and ethanol were applied for 2 min and acetylcholine was applied for 20 s. For co-application with acetylcholine, halothane and ethanol were pre-applied for 2 min before being co-applied with acetylcholine for 20 s. After 5 or 20 min of washout, a control C_m was measured again. The control C_m did not differ among the groups: 98.3 ± 3 nF. Data are mean ± SEM from 4–6 oocytes from 2 batches of oocytes.

acetylcholine (1 μM) or the Hill coefficient (1.1). We also found that the application of the protein kinase C activator, PMA (50 nM, 5 min), inhibited 1 μM acetylcholine-evoked currents (Fig. 3B).

Recently, several investigators reported that activation of protein kinase C reduces the density of membrane receptors and transporters because it promotes the internalization of surface membrane (detected by changes in membrane capacitance)(Vasilets et al., 1990; Anderson and Horne, 1992; Kitayama et al., 1994; Miller and Hoffman, 1994; Qian et al., 1997). To determine if the marked increase in receptor function produced by GF109203X was due to increased membrane surface we measured changes in cell capacitive transients. Treatment of oocytes with GF109203X did not change membrane capacitance (Fig. 3C). We also investigated the effects of acetylcholine (1 μM), halothane (0.25 mM) and ethanol (200 mM) on C_m and there were no significant effects of these compounds (Table 1).

Next, we investigated the effects of GF109203X on the inhibition of muscarinic m_1 receptor function produced by anesthetics, the nonanesthetic and ethanol. As shown in Fig. 4A, the inhibitory effects of halothane, F3 and ethanol on acetylcholine-induced currents were blocked in oocytes treated with the PKC inhibitor (Fig. 4B). However, we were concerned that the marked potentiation of acetylcholine action by GF109203X might have somehow obscured the action of the anesthetics. To test this, we used a low concentration of acetylcholine to produce currents in the presence of GF109203X that were similar to those observed in the absence of GF109203X in previous experiments. Halothane, F3 and ethanol inhibited the 100 nM acetylcholine-evoked currents to 31 ± 4%, 32 ± 8% and 46 ± 10% of control, respectively. We found that GF109203X abolished the inhibitory effects of halothane, F3 and ethanol using this low concentration (100 nM) of acetylcholine (Fig. 4C). However, GF109203X did not affect F6 modulation of muscarinic m_1 receptors (Fig. 4B).

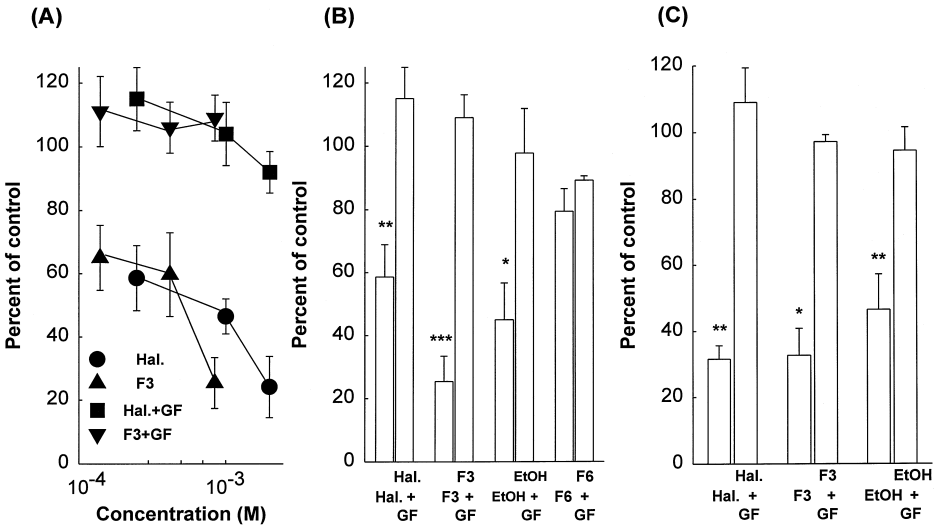

Fig. 4. GF109203X blocked the actions of ethanol, halothane and F3 but not those of the nonanesthetic F6. (A) Oocytes were incubated with 200 nM GF109203X for 2 h. Drugs were pre-applied for 2 min before being co-applied with acetylcholine (1 μM) for 20 s. Data represent the mean ± SEM of 5 separate determinations. (B) Comparison of effects of GF109203X on the inhibitory effects of concentrations of anesthetics corresponding to MAC or predicted MAC for the nonanesthetics. Oocytes were incubated with 200 nM GF109203X for 2 h. Halothane (0.25 mM), F3 (0.8 mM), F6 (17.8 μM) or ethanol (190 mM) were pre-applied for 2 min before being co-applied with acetylcholine (1 μM) for 20 s. Data represent the mean ± SEM of 5 separate determinations. A paired *t*-test was used for statistical analysis. Significant inhibition, $^{*}P < 0.05$ and $^{**}P < 0.01$. (C) GF109203X blocked the inhibitory effects of halothane, F3 and ethanol on currents produced by a low concentration of acetylcholine. Oocytes were incubated with 200 nM GF109203X for 2 h. Halothane (0.25 mM), F3 (0.8 mM) or ethanol (190 mM) were pre-applied for 2 min before being co-applied with acetylcholine (100 nM) for 20 s. Data represent the mean ± SEM of 5 separate determinations. A paired *t*-test was used for statistical analysis. Significant action of GF109203X indicated by: $^{*}P < 0.05$ and $^{**}P < 0.01$.

Table 2
Effects of halothane and ethanol on acetylcholine-induced current in oocytes treated with calyculin A

Compounds	Response (percent of control)
H_2O injection, Ethanol (200 mM)	104 ± 11%
H_2O injection, Halothane (0.25 mM)	105 ± 15%
Calyculin A injection, Ethanol (200 mM)	56 ± 12%*
Calyculin A injection, Halothane (0.25 mM)	52 ± 2%**

The control current produced by acetylcholine (1 μM) was measured 20 min before injection of 30 nl of calyculin A (2 μM) or 30 nl of H_2O. Five min after injection, halothane (0.25 mM) or ethanol (200 mM) was applied for 2 min. After applications of these compounds, oocytes were washed by MBS for 3 min and the current evoked by acetylcholine measured again. Injection of H_2O (99 ± 12%) or calyculin A (98 ± 6%) did not affect acetylcholine-induced current when tested in the absence of anesthetics. Data represent the mean ± SEM of 4–7 oocytes. Statistical analyses were performed using a paired *t*-test. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to oocytes injected with H_2O rather than calyculin A.

These results suggest that anesthetics may inhibit muscarinic m_1 receptor responses by activation of protein kinase C (see Section 4). If this were the case, then it might be possible to 'lock in' the increased phosphorylation with an inhibitor of protein phosphatases, remove the anesthetic and maintain the inhibition of receptor function. We used oocytes treated with the phosphatase inhibitor calyculin A (Ishihara et al., 1989) to test this idea. The experimental design was as follows: The control current produced by acetylcholine (1 μM) was measured and 20 min later we injected calyculin A (or water). We waited 5 min after injection and buffer (control), halothane (0.25 mM) or ethanol (200 mM) was applied for 2 min. After application of these compounds, oocytes were washed with buffer for 3 min and the response to acetylcholine was measured. Calyculin A did not affect the action of acetylcholine when the oocytes were not exposed to anesthetics (Table 2). However, when calyculin A was followed by halothane or ethanol, the acetylcholine-induced currents were inhibited even though the anesthetics were removed by washing for 3 min (Table 2).

4. Discussion

Our results demonstrate that anesthetics such as ethanol, halothane and F3 inhibit muscarinic m_1 receptor function and our findings with halothane are consistent with an earlier study by Durieux (1995). The question arises as to how these anesthetics inhibit muscarinic m_1 receptor function. Durieux (1995) proposed that the site of interaction between halothane and muscarinic m_1 would be a hydrophobic domain in the receptor protein. However, another possibility is that anesthetics activate protein kinase C and thereby inhibit function of metabotropic receptors, including muscarinic m_1 receptor (Sanna et al., 1994; Minami et al., 1997). This hypothesis is consistent with the present findings that a selective protein kinase C inhibitor, GF109203X, completely abolished ethanol-, halothane- and F3-induced inhibition on muscarinic m_1 receptor function. Another possibility is that the anesthetics enhance protein phosphorylation by inhibiting protein phosphatases. However, we found that a phosphatase inhibitor was able to maintain the action of the anesthetics, which supports an action on protein kinase C rather than phosphatases.

In support of this hypothesis, several investigators have suggested that halothane activates protein kinase C activity. Halothane stimulates protein kinase C activity in synaptosomes (Hemmings and Adamo, 1996), brain cytosol (Tsuchiya et al., 1988) and in PC12 cells (Tas and Koschel, 1991). Other investigators have suggested that ethanol activates protein kinase C activity in vivo by promoting its translocation from the cytosol to the membrane fraction (Skwish and Shain, 1990; DePetrillo and Swift, 1992; Tuominen et al., 1992). However, it should be noted that effects of anesthetics on protein kinase C has not been measured in *Xenopus* oocytes, nor have effects of anesthetics been measured at the level of receptor protein phosphorylation. It would also be of interest to determine if mutation of phosphorylation sites in metabotropic receptors would abolish the actions of anesthetics, as predicted by our hypothesis. Such studies are currently underway in this laboratory.

Kandel et al. (1996) reported that the nonanesthetics F6 suppresses learning at doses of 0.5–1 × the predicted MAC. However, at concentrations corresponding to these doses, F6 has no effect on several ligand-gated ion channels that are affected by F3 (Mihic et al., 1994; Dildy-Mayfield et al., 1996; Mascia et al., 1996), making it unlikely that these channels are important for the amnesic actions of F6 (and perhaps of anesthetics). In contrast, F6 inhibited muscarinic m_1 receptors function in the present study. Although the inhibition was modest (about 20%), it occurred with concentrations corresponding to 0.3–1 × the predicted MAC. It is of interest to note that the protein kinase inhibitor did not abolish the inhibitory effects of F6 on muscarinic m_1 receptors. These results suggest that the inhibitory action of F6 does not involve protein kinase C and is different from that of F3, halothane and ethanol. F6 also inhibits 5-hydroxytryptamine 2A receptor ($5\text{-}HT_{2A}$) function and this also does not involve protein kinase C modulation (Minami et al., 1997).

In conclusion, our results suggest that halothane, F3 and ethanol inhibit muscarinic m_1 receptor function by enhancing protein kinase C activity. The finding that the nonanesthetic F6 produced inhibitory effects on muscarinic m_1 receptors suggests that this receptor may be important in effects of these compounds on memory and learning rather than the immobilizing action of anesthetics.

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