

Effects of Ethanol and Anesthetics on Type 1 and 5 Metabotropic Glutamate Receptors Expressed in *Xenopus laevis* Oocytes

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ABSTRACT

Previous studies have demonstrated that ethanol and volatile anesthetics inhibit the function of some metabotropic (G protein-coupled) receptors, including the 5-hydroxytryptamine₂ and muscarinic cholinergic receptors. The metabotropic glutamate receptors (mGluRs) show little sequence homology with most other metabotropic receptors and are important modulators of synaptic transmission in the mammalian central nervous system. It was of interest to determine drug actions on these receptors, and we investigated the effects of ethanol, halothane, the anesthetic compound F3 (1-chloro-1,2,2-trifluorocyclobutane), and the nonanesthetics F6 (1,2-dichlorohexafluorocyclobutane) and F8 (2,3-chlorooctafluorobutane) on the function of mGluR1 and mGluR5 expressed in *Xenopus laevis* oocytes. Halothane, F3, and ethanol inhibited mGluR5-induced Ca²⁺-dependent Cl⁻ currents, yet pharmacologically relevant concentrations of these compounds had little effect on the

glutamate-induced currents in the oocytes expressing mGluR1. F6 had inhibitory effects on both receptors, and F8 did not affect either mGluR1 or mGluR5 function. The protein kinase C (PKC) inhibitor GF109203X enhanced the glutamate-induced current, and the PKC activator phorbol-12-myristate-13-acetate inhibited this current in the oocytes expressing mGluR5, but these compounds had little effect on mGluR1 function. GF109203X abolished the inhibitory effects of halothane, F3, and ethanol on mGluR5s. Conversely, the phosphatase inhibitor calyculin A prolonged the action of halothane and ethanol. Furthermore, mutation of a PKC consensus site (Ser890) of mGluR5 abolished the inhibitory effects of halothane, F3, and ethanol. These results suggest that ethanol and volatile anesthetics inhibit mGluR5 because they promote PKC-mediated phosphorylation.

A wide range of organic compounds, including ethanol and volatile anesthetics, produce a plethora of behavioral effects, such as impairment of learning, memory, and motor coordination and, at higher doses, loss of consciousness and surgical immobility. Despite the wide use of these drugs, their mechanism of action remains obscure. There is evidence that certain neurotransmitter receptors, including ligand-gated ion channels and G protein-coupled (metabotropic) receptors, are affected by ethanol and volatile anesthetics (Franks and Lieb, 1994; Harris *et al.*, 1995). In particular, the function of muscarinic cholinergic and 5-HT₂ receptors is inhibited by these compounds (Durieux, 1995; Minami *et al.*, 1997; Sanna

et al., 1994). However, not all metabotropic receptors are affected by anesthetics; halothane does not inhibit the function of the AT_{1A} angiotensin II receptor, a receptor that uses the same phosphatidylinositol signaling system as the m1 muscarinic receptor, which is sensitive to halothane (Durieux, 1995). One goal of the current study was to determine whether the actions of ethanol and volatile anesthetics extend to two mGluRs. The mGluRs are distinct from the other metabotropic receptors in that they are much larger proteins and show little sequence similarity to most members of the G protein-coupled receptor family, although there is appreciable homology with the γ -aminobutyric acid_B receptors (Kaupmann *et al.*, 1997). As discussed below, mGluRs are important modulators of synaptic transmission in the mammalian central nervous system and are believed to play a role in processes such as memory and learning; therefore, it was of

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ABBREVIATIONS: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; F3, 1-chloro-1,2,2-trifluorocyclobutane; F6, 1,2-dichlorohexafluorocyclobutane; F8, 2,3-chlorooctafluorobutane; C_m, membrane capacitance; 5-HT, 5-hydroxytryptamine; MAC, minimum alveolar concentration; LTP, long term potentiation; mGluR, metabotropic glutamate receptor; MBS, modified Barth's solution; PMA, phorbol-12-myristate-13-acetate; EGTA, ethylene glycol bis(β -aminoethyl ether)-N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

interest to determine whether the function of these receptors is affected by ethanol or anesthetics.

Previous studies suggest that ethanol and anesthetics inhibit metabotropic receptor function by increasing PKC-dependent desensitization of the receptor (Minami *et al.*, 1997; Sanna *et al.*, 1994) and it was of interest to determine whether this mechanism could account for the actions of these drugs on mGluRs. PKC phosphorylates mGluR5, and this phosphorylation is important in producing oscillations in intracellular Ca^{2+} signaling (Kawabata *et al.*, 1996). In addition, recent data suggest that specific serine residues in mGluR5 are important for desensitization of this receptor, a process that is mediated by PKC (Gereau and Heinemann, in press). Thus, mutation of these sites provides a test of the hypothesis that ethanol and anesthetics inhibit receptor function because they enhance this endogenous PKC-mediated desensitization.

Several new halocarbon compounds may also provide insight regarding the role of metabotropic receptors in anesthetic action. Although most halogenated hydrocarbons produce anesthesia, and it is generally assumed that potency of general anesthetics is determined solely by lipid solubility, recent studies show a more subtle structure-activity relationship for anesthesia (Koblin *et al.*, 1994). In particular, a novel halogenated compound (F3) is an anesthetic, whereas its congener (F6) does not produce anesthesia, and another halocarbon, F8, also is nonanesthetic, despite their high lipid solubilities (Koblin *et al.*, 1994). Although F6 does not produce anesthesia (or, more accurately, surgical immobility), it does interfere with learning and memory, and this property is shared with traditional anesthetic compounds (Gonsowski *et al.*, 1995; Kandel *et al.*, 1996). Thus, different sites of action may be responsible for the immobilizing and amnestic actions of anesthetics; the former would be sensitive to F3 but not F6, whereas the latter would be affected by both compounds. Indeed, several ligand-gated ion channels are affected by F3 but not F6, whereas a metabotropic receptor (5-HT_{2A}) is sensitive to both (Harris *et al.*, 1995; Minami *et al.*, 1997). One goal of the current study was to determine whether mGluRs are affected by both anesthetic and nonanesthetic halocarbons.

Part of the rationale for studying the mGluRs is their importance in learning and memory. As mentioned above, ethanol, anesthetics, and at least one nonanesthetic interfere with learning and memory (Dwyer *et al.*, 1992; Givens and McMahon, 1997; Gonsowski *et al.*, 1995; Kandel *et al.*, 1996). A neurotransmitter that has a major role in synaptic excitation in the central nervous system and is critical for information storage in memory and learning is glutamate (Hollman and Heinemann, 1994). The ionotropic glutamate receptors are influenced by anesthetics. For example, volatile anesthetics inhibit *N*-methyl-D-aspartate and AMPA receptor function but enhance kainate (GluR6) receptor responses (Harris *et al.*, 1995). However, these actions are not shared by the nonanesthetic F6, even though it interferes with learning and memory (Harris *et al.*, 1995). Thus, the ionotropic glutamate receptors are unlikely to account for all the amnestic actions produced by halocarbons.

The mGluRs form a family of receptors; eight different subtypes have been described (mGluR1–8; Abe *et al.*, 1992; Conn and Pin, 1997). Based on their pharmacology, second messenger coupling, and sequence differences, these recep-

tors can be divided into class I (mGluR1 and mGluR5), class II (mGluR2 and mGluR3), and class III (mGluR4 and mGluR6–8) (Riedel, 1996). The class I receptors are linked with learning and memory (Riedel, 1996); this is based on pharmacological studies showing that an agonist of mGluR1 and mGluR5 enhances memory and that mutant mice lacking mGluR1 show deficits in learning and memory and reduced hippocampal LTP (Aiba *et al.*, 1994; Conquet *et al.*, 1994; Riedel, 1996). Mice lacking the mGluR1 also display poor motor coordination. More recently, it was reported that mice lacking mGluR5 show impaired learning and reduced CA1 LTP but normal CA3 LTP (Lu *et al.*, 1997). In view of the effects of ethanol and anesthetics on learning and memory as well as motor function, it is of interest to consider the effects of these drugs on class I mGluRs.

There are few studies of the effects of alcohols or anesthetic agents on mGluRs. Ethanol inhibits quisqualate-induced burst activity in rat cultured cerebellar Purkinje neurons (mediated by mGluRs) (Netzeband and Gruol, 1995) and quisqualate-induced currents in oocytes expressing mRNA from rat cerebellum (Sanna *et al.*, 1994) but does not affect glutamate-stimulated phospholipase C activity of brain astrocytes (Smith, 1994). These results suggest that some, but not all, subtypes of mGluRs are inhibited by ethanol and encouraged us to test the effects of ethanol and anesthetics on specific subtypes of mGluRs expressed in *Xenopus laevis* oocytes.

The *X. laevis* oocyte expression system has been used to express a multiplicity of brain receptors from cDNAs or cRNAs with pharmacological properties that mimic those of native brain receptors (Harris *et al.*, 1995; Snutch, 1988). Activation of mGluR1 or mGluR5 receptors results in activation of phospholipase C, mobilization of calcium stores, and activation of an endogenous Ca^{2+} -dependent Cl^- current in oocytes (Abe *et al.*, 1992; Masu *et al.*, 1991). This system has been well characterized for the study of the effects of anesthetics and ethanol on G protein-coupled receptors.

We used the oocyte expression system to study the effects of ethanol, halothane, F3, F6, and F8 on glutamate-induced current via mGluR1 and mGluR5 (class I). Moreover, we analyzed the effects of these compounds in the presence of a PKC inhibitor and a protein phosphatase inhibitor and studied receptors with mutations in PKC phosphorylation sites to investigate the role of PKC in the actions of these drugs.

Experimental Procedures

Materials. Adult *X. laevis* female frogs were purchased from Xenopus I (Ann Arbor, MI). Glutamate, dimethylsulfoxide, phorbol-12-myristate-13-acetate, and L-glutamate were purchased from Sigma Chemical (St. Louis, MO). Ethanol was purchased from Aaper Alcohol and Chemical (Shelbyville, KY). Calyculin A was from LC Laboratories (Woburn, MA). Halothane was from Halocarbons Laboratories (River Edge, NJ). F3, F6, and F8 were obtained from PCR Inc. (Gainesville, FL). Ultracomp *Escherichia coli* transformation kit was from InVitrogen (San Diego, CA). A kit from Qiagen (Chatworth, CA) was used for purification of plasmid cDNA. mGluR1 and mGluR5 cRNAs were prepared using mCAP mRNA capping kit (Stratagene, La Jolla, CA). GF109203X and chelerythrine were from Calbiochem (La Jolla, CA). mGluR1 and mGluR5 cDNAs were kindly provided by Dr. S. Nakanishi (Kyoto University, Kyoto, Japan).

Metabotropic glutamate cRNA preparation. The cDNA for mGluR1 was inserted into the pGEM vector, and the cDNA for

mGluR5 was inserted into the pBluescript SK⁻ vector. The mGluR cDNAs were linearized with *NotI*, phenol-chloroform extracted, and ethanol precipitated with sodium acetate and cRNA prepared using the Stratagene transcription kit. These cRNAs were extracted using phenol-chloroform and precipitated with ethanol and sodium acetate. Site-directed mutagenesis was performed according to the procedure for the Quik-Change site-directed mutagenesis kit (Stratagene) (Gereau and Heinemann, in press). These mutants were inserted into the pBluescript SK⁻ vector; the cDNAs were linearized with *XbaI*, phenol-chloroform extracted, and ethanol precipitated with ethanol and sodium acetate.

Whole-cell voltage-clamp of injected oocytes. Isolation and microinjection of *X. laevis* oocytes were performed as described by Sanna et al. (1994). *X. laevis* oocytes were injected with 25–50 ng of cRNA coding for the mGluRs. Oocytes were placed in a 100- μ l recording chamber and perfused with MBS (containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.91 mM CaCl₂, pH 7.5) at rate of 1.8 ml/min at room temperature. Recording and clamping electrodes (1–5 M Ω) were pulled from 1.2-mm o.d. capillary tubing and filled with 3 M KCl. A recording electrode was impaled into the animal pole; once the resting membrane potential stabilized, a clamping electrode was inserted with the resting membrane potential allowed to restabilize. Warner oocyte clamp OC 725-B (Hampden, CT) was used for voltage-clamping of each oocyte at -70 mV.

For the glutamate concentration-response curves, we tested each oocyte with all glutamate concentrations for the concentration-response curves. We waited 20 min between applications at concentrations of ≤ 100 μ M and ≥ 60 min for application of concentration of > 100 μ M. The lowest concentrations were tested first. The anesthetics (halothane and F3), nonanesthetics (F6 and F8), and ethanol were preapplied for 2 min to allow complete equilibration in the bath. Solutions of volatile compounds were prepared immediately before use. The anesthetic, nonanesthetic, and ethanol concentrations in the figures represent bath concentrations, measured as described previously (Mihic et al., 1994; Minami et al., 1997).

Oocytes were exposed to GF109203X (200 nM) (Toullec et al., 1991) in incubation media (MBS containing 10 mg of streptomycin and 10,000 units of penicillin G, 50 mg of gentamycin/liter, 0.5 mM theophylline, 2 mM sodium pyruvate) for 120 min. Glutamate was tested at 5, 20, 40, 60, and 120 min during GF109203X treatment and in oocytes incubated without GF109203X as a control. Oocytes were exposed to chelerythrine (200 μ M) (Ko et al., 1990) in incubation media for 60 min. Glutamate was tested at 5, 20, 40, 60, and 120 min during GF109203X treatment and in oocytes incubated without GF109203X as a control. The effects of PMA were determined in oocytes treated with 50 nM PMA for 5 min and stimulated by glutamate after removal of PMA. In other experiments, we injected oocytes with the phosphatase inhibitor calyculin A (Ishihara et al., 1989), as described in Results.

C_m measurements. Capacitative transients elicited by the voltage jumps from the holding potential of -30 to -60 mV were measured (Vasilets et al., 1990). Three voltage pulses of 30 mV were applied from the resting potential. The signal, which was averaged from the three pulses, was integrated using the Strathclyde Electrophysiology Software program (courtesy of John Dempster, Glasgow, UK), Whole Cell Electrophysiology Program V1.2e, and C_m was determined from the slope of the regression of the area-versus-voltage relationships.

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 to study the effects of GF109203X or PMA, we used the glutamate-induced currents before GF109203X or PMA application as a control. Each experiment was carried out with oocytes from at least two different frogs. Statistical analyses were performed using either a *t* test or one-way ANOVA.

Curve fitting and estimation of EC₅₀ values for concentration-response curves were performed using Inplot (GraphPAD Software, San Diego, CA).

Results

Effects of ethanol, anesthetics, and nonanesthetics on currents activated by glutamate in oocytes expressing mGluRs. Glutamate concentration-response curves were determined in *X. laevis* oocytes expressing mGluR1 and mGluR5 (Fig. 1). Nonlinear regression analysis of these curves of mGluR1 yielded an EC₅₀ value for glutamate of 1 mM and a Hill coefficient of 1.1. Maximal currents were observed at 10 mM. *X. laevis* oocytes expressing mGluR5 yielded an EC₅₀ value for glutamate of 0.4 mM and a Hill coefficient of 1.4.

The effects of halothane, F3, F6, F8, and ethanol on glutamate-activated currents were determined in oocytes expressing mGluR1 and mGluR5 (Fig. 1B,C).

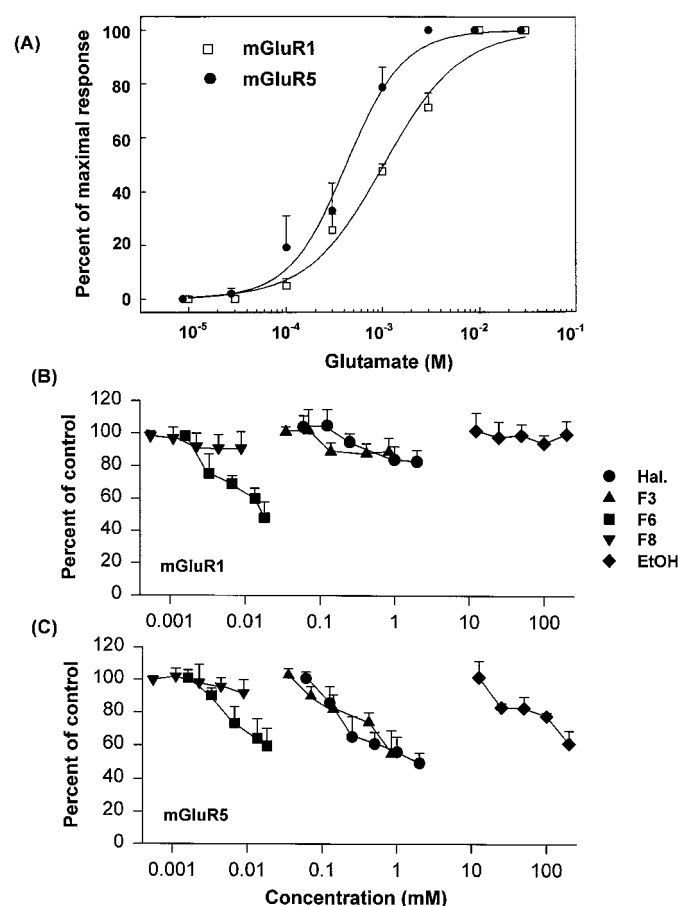


Fig. 1. A, Concentration-response curves for glutamate-activated Ca²⁺-dependent Cl⁻ current in *X. laevis* oocytes expressing mGluR1 and mGluR5. Oocytes were voltage-clamped at -70 mV; glutamate was applied for 20 sec, and the peak current was measured. Nonlinear regression analysis of the concentration-response curves of mGluR1 yielded an EC₅₀ value for glutamate of 1 mM and a Hill coefficient of 1.1. Maximal currents were observed at 10 mM. Concentration-response curves of mGluR5 yielded an EC₅₀ value for glutamate of 0.4 mM and a Hill coefficient of 1.4. Maximal currents were observed at 3 mM. Values are mean \pm standard error from six to eight oocytes. B and C, Effects of anesthetics [halothane (Hal.) and F3], nonanesthetics (F6 and F8), and ethanol (EtOH) on the current evoked by 1 mM glutamate in oocytes expressing mGluR1 (B) and on the current evoked by 100 μ M glutamate in oocytes expressing mGluR5 (C). Halothane (0.06–2 mM), F3 (0.05–0.8 mM), F6 (0.06–17.8 μ M), F8 (0.06–8.8 μ M), and ethanol (12.5–200 mM) were preapplied for 2 min before being coapplied with glutamate for 20 sec. Values are mean \pm standard error of 9–14 oocytes.

mate-induced currents were examined in oocytes expressing mGluR1 and mGluR5 (Fig. 1, B and C). Ethanol and F3 had little effect on currents activated by 1 mM glutamate in the oocytes expressing mGluR1, and halothane produced inhibition only at high concentrations (Fig. 1B). In contrast, halothane, F3, and ethanol inhibited the currents evoked by 100 μ M glutamate in oocytes expressing mGluR5 (Figs. 1C and 2). The nonanesthetic F8 did not alter currents elicited by glutamate on either mGluR1 or mGluR5. However, the other nonanesthetic, F6, inhibited the function of both receptors. Drug effects are compared at equieffective concentrations that correspond to the MAC (anesthetic EC_{50}) in Fig. 3. In the case of the nonanesthetic, the concentration used corresponds to the MAC predicted from the gas/oil solubility coefficient (Koblin *et al.*, 1994; Mihic *et al.*, 1994). At these concentrations, mGluR1 was inhibited only by F6, whereas mGluR5 was inhibited to a similar extent by all the compounds with the exception of the nonanesthetic F8, which was inactive (Fig. 3).

Effects of a PKC inhibitors on mGluR function. Because PKC plays an important role in regulating some G protein-coupled receptors (Kato *et al.*, 1988; Manzoni *et al.*, 1990; Moran and Dascal, 1989; Sanna *et al.*, 1994) and has been shown to regulate mGluR function (Catania *et al.*, 1991; Schoepp and Johnson, 1988), we studied glutamate responses in *X. laevis* oocytes that were pretreated with the PKC inhibitor GF109203X (200 nM) (Toullec *et al.*, 1991). GF109203X had no effects on currents activated by glutamate in the oocytes expressing mGluR1 but markedly ($213 \pm 55\%$) enhanced glutamate-induced currents in oocytes expressing mGluR5 (Fig. 4, A and B) without altering the EC_{50} value for glutamate (0.2 mM) or the Hill coefficient (1.5). We

also studied the effects of the PKC activator PMA on glutamate-induced currents in oocytes expressing mGluR5. We measured the 100 μ M glutamate-induced currents as a control and perfused the oocytes with MBS for 20 min. We then treated the oocytes with 50 and 10 nM PMA for 5 min and tested them with 100 μ M glutamate. The 5-min application of the PKC activator PMA inhibited 100 μ M glutamate-evoked currents to $6 \pm 4\%$ (six oocytes) and $11 \pm 4\%$ (six oocytes) of control response at 50 and 10 nM PMA, respectively. However, PMA (50 nM for 5 min) had no effect on mGluR1 function (control, 759 ± 157 nA, 11 oocytes; PMA treatment, 747 ± 224 nA, 10 oocytes). Enhancement by GF109203X was observed with maximal (3 mM) and submaximal (300 μ M) concentrations of glutamate in oocytes expressing mGluR5. Treatment with GF109203X enhanced the 3 mM glutamate-induced currents to $245 \pm 41\%$ of initial currents and 300 μ M glutamate-induced currents to $192 \pm 39\%$ of initial currents.

Recently, several investigators reported that activation of PKC reduces the density of membrane receptors and transporters because it promotes the internalization of surface membrane (detected by changes in C_m) (Qian *et al.*, 1997; Vasilets *et al.*, 1990). We investigated whether the marked increase in receptor function produced by GF109203X was due to increased membrane surface by measuring changes in cell capacitive transients.

Treatment of oocytes with GF109203X induced no significant change in C_m (during the treatment of GF109203X, $C_m = 101 \pm 7$, 111 ± 8 , 95 ± 6 , and 92 ± 6 nF at 0, 30, 60, and 120 min, respectively). We also investigated the effects of glutamate (100 μ M), halothane (0.25 mM), and ethanol (200 mM) on C_m . These compounds had no significant effects (Table 1).

Next, we investigated the effects of GF109203X on the inhibition of mGluR5 function produced by anesthetics, the nonanesthetic, and ethanol. The inhibitory effects of halothane, F3, and ethanol on glutamate-induced currents were blocked in oocytes treated with PKC inhibitor (Fig. 4). Moreover, we studied the effects of the another PKC inhibitor, chelerythrine (20 μ M) (Ko *et al.*, 1990) on the inhibition of mGluR5 function produced by halothane (0.25 mM). Halothane did not inhibit glutamate-induced currents in oocytes treated with chelerythrine (20 μ M) for 1 hr. However, GF109203X did not affect F6 modulation of mGluR1 and mGluR5.

These results suggested that anesthetics may inhibit mGluR5 responses by activation of PKC. If this were the case, then it might be possible to "lock in" the increased phosphorylation with an inhibitor of protein phosphatases and then 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 100 μ M glutamate was measured, and 20 min later we injected calyculin A (or water). We waited 5 min after injection and applied buffer (control), halothane (0.25 mM), or ethanol (200 mM) for 2 min. After the application of these compounds, oocytes were washed with buffer for 3 min, and the response to glutamate was measured. Calyculin A did not significantly affect the action of glutamate when the oocytes were not exposed to anesthetics (Fig. 5). However, when calyculin A was followed by halothane or ethanol, the glutamate-induced currents were in-

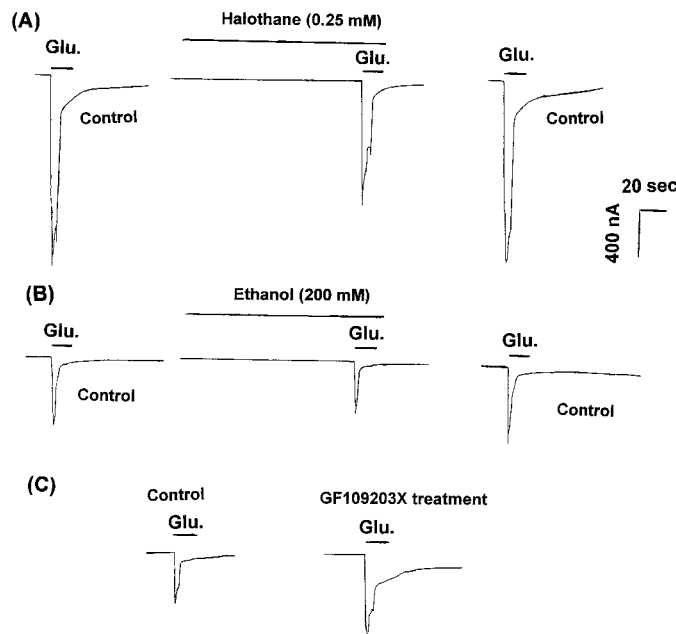


Fig. 2. A and B, Tracings were obtained from a single oocyte and show the effects of halothane (0.25 mM) and ethanol (200 mM) on currents evoked by 100 μ M glutamate (Glu.) in oocytes expressing mGluR5s. The control responses were measured 20 min before and 20 min after each drug application. C, Tracings were obtained from a single oocyte and show the effect of GF109203X on currents evoked by 100 μ M glutamate in oocytes expressing mGluR5s. Oocytes expressing mGluR5s were incubated with GF109203X (200 nM) for 2 hr.

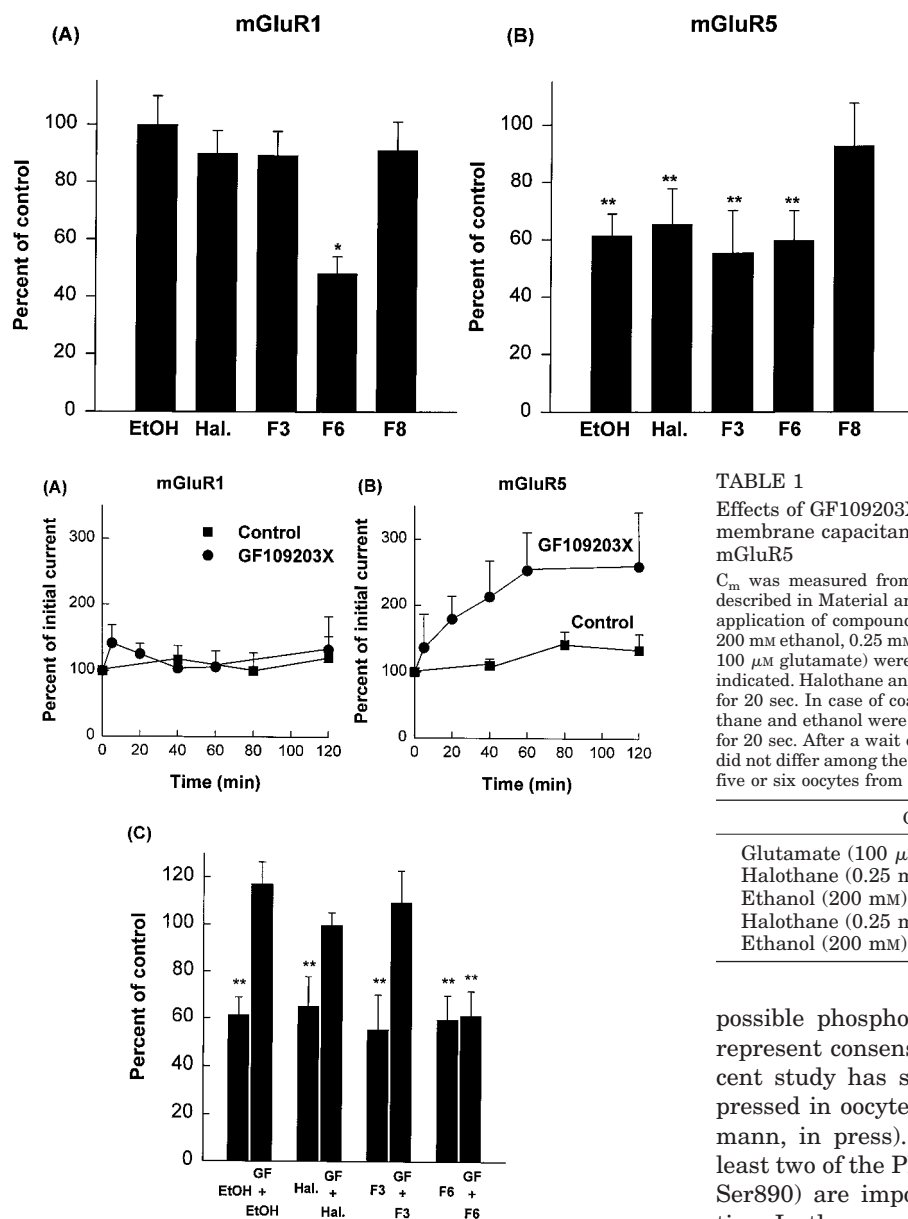


Fig. 3. A, Effects of anesthetic (MAC) concentrations of halothane, F3, F6, F8, and ethanol on currents evoked by 1 mM glutamate in oocytes expressing mGluR1s. The MAC concentrations of ethanol (EtOH), halothane (Hal.), and F3 are 190, 0.25, and 0.8 mM, respectively. The predicted MAC concentrations of F6 and F8 are 17.8 and 8.8 μ M, respectively. Values are mean \pm standard error of five oocytes. *, $p < 0.05$ versus control response with 1 mM glutamate (paired t test). B, Effects of anesthetic (MAC) concentrations of halothane, F3, F6, F8, and ethanol on currents evoked by 100 μ M glutamate in oocytes expressing mGluR5. Values are mean \pm standard error of five or six oocytes. **, $p < 0.01$ versus control response obtained with 100 μ M glutamate (paired t test).

TABLE 1

Effects of GF109203X, halothane, ethanol, and glutamate on membrane capacitance (C_m) in oocytes injected with cRNA for the mGluR5

C_m was measured from the capacitive transients elicited by the voltage change described in Material and Methods. The control C_m was measured 5 min before the application of compounds. The compounds (100 μ M glutamate, 0.25 mM halothane, 200 mM ethanol, 0.25 mM halothane plus 100 μ M glutamate, and 200 mM ethanol plus 100 μ M glutamate) were applied either separately or coadministered to oocytes as indicated. Halothane and ethanol were applied for 2 min, and glutamate was applied for 20 sec. In case of coapplication with glutamate and halothane or ethanol, halothane and ethanol were preapplied for 2 min before being coapplied with glutamate for 20 sec. After a wait of 5 or 20 min, the C_m was measured again. The control C_m did not differ among the groups: 101 ± 7 nF. Values are mean \pm standard error from five or six oocytes from two batches of oocytes.

Compound	Percent of control
Glutamate (100 μ M)	103 \pm 13
Halothane (0.25 mM)	108 \pm 8
Ethanol (200 mM)	95 \pm 11
Halothane (0.25 mM) + glutamate (100 μ M)	106 \pm 11
Ethanol (200 mM) + glutamate (100 μ M)	91 \pm 7

possible phosphorylation sites in mGluR5, some of which represent consensus phosphorylation sites for PKC. One recent study has shown that desensitization of mGluR5 expressed in oocytes is mediated by PKC (Gereau and Heinemann, in press). Furthermore, this study showed that at least two of the PKC consensus sites in mGluR5 (Ser613 and Ser890) are important for this PKC-mediated desensitization. In the current study, these two sites were mutated on mGluR5, and the glutamate concentration-response curves were determined in *X. laevis* oocytes expressing mGluR5(S890G) and mGluR5(S613G) receptors (Fig. 7). Analysis of (S613G) yielded an EC_{50} value for glutamate of 2 mM and a Hill coefficient of 1.6, and mGluR5(S890G) yielded an EC_{50} value for glutamate of 1.5 mM and a Hill coefficient of 1.6. The magnitude of the current produced by maximally effective concentrations of glutamate was greater for the S890G mutation than for the wild-type or S613G mutation. The glutamate (100 μ M)-induced currents on the wild-type, mGluR5(S613G), and mGluR5(S890G) were 512 ± 129 nA (27 oocytes), 1470 ± 303 nA (17 oocytes, $p < 0.05$), and 2116 ± 432 nA (18 oocytes, $p < 0.01$) (unpaired t test and Dunnett's correction), respectively.

We compared the effects of halothane, F3, F6, F8, and ethanol at MAC (or predicted MAC in the case of F6 and F8) on glutamate-induced currents in oocytes expressing mGluR5, mGluR5(S613G), or mGluR5(S890G) (Fig. 7). Halothane, F3, and ethanol had no significant effect on the function of mGluR5(S890G) (Figs. 8 and 9) but inhibited

Fig. 4. A, Oocytes expressing mGluR1s were incubated with GF109203X (200 nM) for 2 hr. Glutamate (1 mM) was applied at 5, 20, 40, 60, and 120 min during the treatment with GF109203X or at 20, 40, 80, and 120 min in oocytes not treated with GF109203X (control). Values are mean \pm standard error of nine oocytes. B, Oocytes expressing mGluR5s were incubated with GF109203X (200 nM) for 2 hr. Glutamate (100 μ M) was applied at 5, 20, 40, 60, and 120 min in oocytes treated with GF109203X or at 20, 40, 80, and 120 min in oocytes not treated with GF109203X (control). Values are mean \pm standard error of 14–20 oocytes. C, GF109203X (GF) blocked the action of anesthetics [halothane (Hal.) and ethanol (EtOH)] but not the nonanesthetic F6. Inhibition of glutamate-induced responses was measured in oocytes expressing mGluR5s. Oocytes were incubated with 200 nM GF109203X for 2 hr. Halothane (0.25–2 mM) or F3 (0.2–0.8 mM) was preapplied for 2 min before being coapplied with glutamate (100 μ M) for 20 sec. The anesthetics, nonanesthetics, and ethanol (EtOH) were preapplied for 2 min before being coapplied with glutamate (100 μ M) for 20 sec. Values are mean \pm standard error of five separate determinations. **, $p < 0.01$ versus control response obtained with 100 μ M glutamate (paired t test).

hibited even though the anesthetics were removed by washing for 3 min (Fig. 6).

Effects of ethanol, anesthetics, and nonanesthetics on mutant mGluR5s. Abe et al. (1992) suggested several

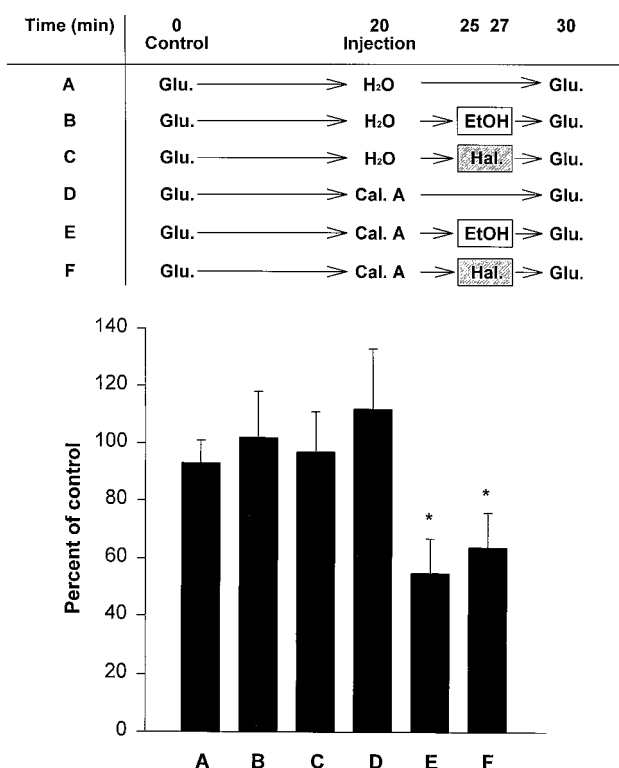


Fig. 5. A, Effects of halothane and ethanol (EtOH) on mGluR5 function in oocytes treated with the protein phosphatase inhibitor calyculin A (Cal A). The control current produced by glutamate (Glu.) (100 μ M) was measured 20 min before injection of 30 nl of calyculin A (2 μ M) or 30 nl of water. At 5 min after injection, halothane (0.25 mM) or ethanol (200 mM) was applied for 2 min. After the application of these compounds, oocytes were washed with buffer for 3 min, and the current was remeasured. Bars, effects of injection with water on control responses (A), effects of ethanol (200 mM) on oocytes injected with water (B), effects of halothane (0.25 mM) on oocytes injected with water (C), effects of injection with calyculin A (D), effects of ethanol (200 mM) on oocytes injected with calyculin A (E), and effects of halothane (0.25 mM) on oocytes injected with calyculin A (F). Values are mean \pm standard error of five or six oocytes. Statistical analyses were performed using a paired *t* test. *, *p* < 0.05 versus control response.

mGluR5(S613G). However, for ethanol and F3, the S613G mutant was less affected than the wild-type receptor. The inhibition produced by F6 was similar for wild-type and both mutant receptors (Fig. 8).

Discussion

Our results demonstrate that ethanol, halothane, and F3 inhibited mGluR5 but had little effect on mGluR1 function. The amino acid sequence of mGluR5 is homologous to that of the other members of the mGluR family and most closely related to that of mGluR1 (60% sequence identity) (Abe *et al.*, 1992). The question arises as to how these anesthetics and ethanol inhibit mGluR5 but not mGluR1 function. Several mechanisms of anesthetic action on G protein-coupled receptors have been suggested. Durieux (1995) proposed that the site of interaction between halothane and muscarinic receptors could be a hydrophobic domain in the receptor protein. However, we suggested that anesthetics and ethanol do not act directly on metabotropic receptors but rather enhance receptor phosphorylation by PKC and thereby inhibit receptor function (Minami *et al.*, 1997; Sanna *et al.*, 1994). The current study provides substantial evidence to support this

hypothesis; the findings can be summarized as follows: 1) The function of the mGluR5 is reduced by an activator of PKC and enhanced by a selective PKC inhibitor, indicating that receptor function may be modulated in both directions by changes in PKC activity. 2) A selective PKC inhibitor completely abolished ethanol-, halothane-, and F3-induced inhibition of mGluR5 function. (It should be noted that the effects of anesthetics and ethanol in the absence or presence of PKC inhibitor were tested at the same EC value; thus, the reduction in the anesthetic effects in the presence of the PKC inhibitors is not due to an inhibitor-induced shift in the glutamate concentration-response curve.) 3) A protein phosphatase inhibitor was able to sustain the actions of ethanol and anesthetics even after they were removed from the bath, suggesting that these drugs do not act directly on the receptor (and do not act on these protein phosphatases). 4) Mutation of a putative PKC phosphorylation site (Ser890) enhanced receptor function and prevented the action of ethanol and anesthetics. It is important to note that mGluR1 does not have a sequence corresponding to Ser890 and its function was not affected by an activator or inhibitor of PKC or by ethanol or anesthetics. However, both mGluR1 and mGluR5 contain identical PKC consensus sequences at Ser613, and we found that this amino acid is not critical for modulation of receptor function by PKC activators/inhibitors but may influence the action of some anesthetics. Although Ser890 is clearly the dominant site, Ser613 may play some role in mediating the effects of ethanol and anesthetics. Thus, our data suggest that the sensitivity of mGluR5 to ethanol and anesthetics could be due to a consensus PKC phosphorylation site at Ser890. Taken together, our results provide evidence that ethanol and anesthetics indirectly inhibit metabotropic receptor function by activation of PKC. It should be noted that the studies of effects of anesthetics on PKC activity *in vitro* have had mixed results. For example, halothane stimulates PKC activity in brain synaptosomes (Hemmings and Adamo, 1996, 1997), brain cytosol (Tsuchiya *et al.*, 1988), and PC12 cells (Tas and Koeschel, 1991). Studies of purified PKC are less consistent, and both inhibition and enhancement of PKC activity have been obtained with halothane and ethanol (Hemmings *et al.*, 1995; Hemmings and Adamo, 1994; Slater *et al.*, 1993, 1997). Further studies are needed to determine whether ethanol and anesthetics increase the phosphorylation of mGluR5 and whether Ser890 or Ser613 is indeed phosphorylated by PKC. Moreover, additional experiments will be necessary to investigate whether anesthetics modulate PKC access to its phosphorylation site in the receptor. It is possible that the Ser890 site is important for the coupling of the receptor with a specific G protein. It also is possible that the Ser890 site is important for the coupling of the receptor with a specific G protein. Another possibility is raised by the recent report that the protein "Homer" regulates the metabotropic glutamate signaling by an interaction with the carboxyl-terminal domain of mGluR1 and mGluR5 (Brakeman *et al.*, 1997). This alternative mechanism proposes that activation of PKC by alcohols and anesthetics inhibits receptor function by phosphorylation of receptor-associated proteins rather than the receptor. Further studies are required to distinguish among these hypotheses.

It is of interest to consider the functional importance of mGluR5 and possible consequences of inhibition of receptor function by ethanol and anesthetics. *In situ* hybridization

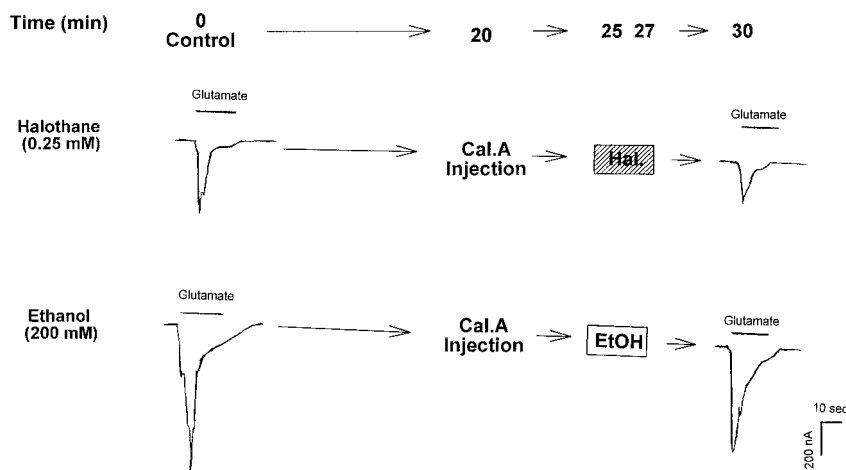
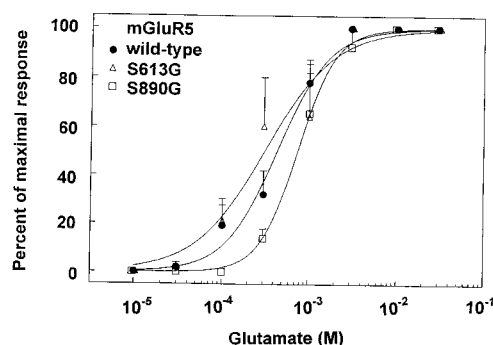


Fig. 6. Tracings were obtained from a single oocyte and showed the effects of ethanol (EtOH) (200 mM) and halothane (Hal.) (0.25 mM) on oocytes injected with calyculin A (CalA). The control current produced by glutamate (100 μ M) was measured 20 min before the injection of 30 nl of calyculin A (2 μ M). Five minutes after injection, halothane (0.25 mM) or ethanol (200 mM) was applied for 2 min. After the application of these compounds, oocytes were washed with buffer for 3 min, and the current was remeasured.

(A)



(B)

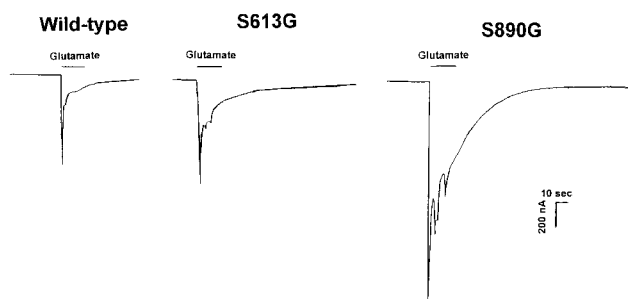


Fig. 7. Responses of mutant receptors to glutamate and anesthetic agents. A, Concentration-response curves for glutamate-activated Ca^{2+} -dependent Cl^- current in *X. laevis* oocyte expressing mGluR5, mGluR5(S613G), or mGluR5(S890G). Oocytes were voltage-clamped at -70 mV; glutamate was applied for 20 sec, and the peak current was measured. Values are mean \pm standard error from five oocytes. B, Representative tracings of currents induced by 100 μ M glutamate in oocytes expressing mGluR5, mGluR5(S613G), or mGluR5(S890G). Glutamate was applied for 20 sec. Calibration bars, 10 sec and 200 nA.

found prominent expression of mGluR5 mRNA in cerebral cortex, nucleus accumbens, striatum, hippocampal CA1–4 and dentate gyrus regions, lateral septum and cerebellar Golgi, and internal granule cells (Abe et al., 1992). This localization raises the possibility of roles in cognition, learning and memory, reinforcement, and motor control. There is recent evidence that inhibition of mGluR1 and/or mGluR5 can impair learning and memory and reduce motor coordina-

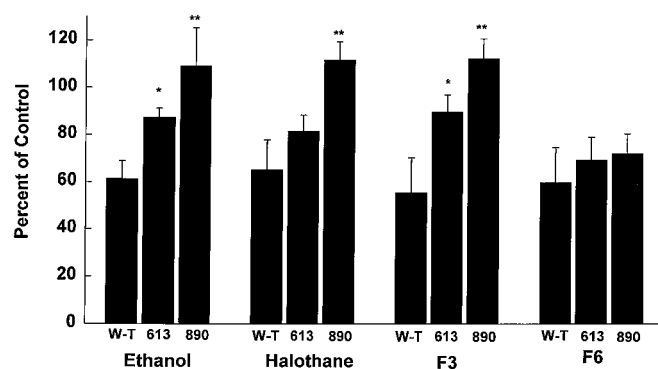


Fig. 8. Effects of ethanol, halothane, F3, or F6 on currents evoked by glutamate in oocytes expressing mGluR5, mGluR5(S613G), or mGluR5(S890G). Anesthetic or predicted anesthetic (MAC) concentrations were used (see Fig. 3); the glutamate concentration was 100 μ M. Values are mean \pm standard error of five or six oocytes. Statistical analyses were performed using unpaired *t* test and Dunnett correction. *, $p < 0.05$ and **, $p < 0.01$ versus wild-type (W-T).

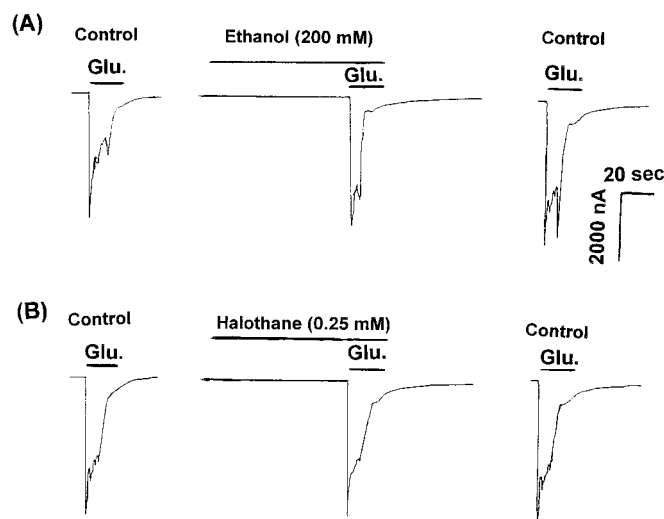


Fig. 9. Tracings obtained from a single oocyte show the effects of ethanol (200 mM) and halothane (0.25 mM) in oocytes expressing mGluR5(S890G). The control responses were measured 20 min before and 20 min after each drug application. Glu., glutamate.

tion and anxiety (Aiba et al., 1994; Chojnacka-Wojcik et al., 1997; Conquet et al., 1994; Lu et al., 1997; Riedel, 1996).

The nonanesthetic F6 inhibited function of both mGluR1 and mGluR5 equally, and this inhibition was not affected by

the PKC inhibitor or by mutation of Ser890. This is consistent with our earlier finding that F6 inhibits the function of 5-HT_{2A} receptors by a mechanism that does not require PKC (Minami *et al.*, 1997). The inhibition of mGluR5 receptor function by both F3 and F6 (albeit by different mechanisms) is of interest in view of the report (Kandel *et al.*, 1996) that F6 suppresses learning at doses of 0.5–1 times the predicted MAC. In earlier studies, concentrations of F6 corresponding to these doses had no effect on several ligand-gated ion channels affected by F3 (Dildy-Mayfield *et al.*, 1996; Mascia *et al.*, 1996; Mihic *et al.*, 1994), making it unlikely that these channels are important for the amnestic actions of F6 (and perhaps of anesthetics). However, the metabotropic receptors may be responsible for some actions, such as amnesia, that are shared by both F3 and F6. Conversely, it is unlikely that inhibition of metabotropic receptor function by anesthetics is responsible for immobility because F6 does not produce this component of anesthesia, yet it inhibits metabotropic receptor function (Minami *et al.*, 1997).

In conclusion, our results show that ethanol and volatile anesthetics inhibited mGluR5 function but had little effect on mGluR1. This receptor specificity is likely due to the presence of a site (Ser890) in mGluR5 that is absent in mGluR1. These findings, together with those of others (Minami *et al.*, 1997; Sanna *et al.*, 1994), suggest that alcohols and anesthetics amplify an endogenous regulatory mechanism that uses PKC phosphorylation to reduce receptor function.

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