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Enflurane Inhibits the Function of Mouse and Human Brain Phosphatidylinositol-Linked Acetylcholine and Serotonin Receptors Expressed in *Xenopus* Oocytes

LIE-HUEY LIN, SHERRY LEONARD, and R. ADRON HARRIS

Department of Pharmacology (L-H.L., S.L., R.A.H.) and Neurosciences Program (L-H.L., R.A.H.), University of Colorado Health Sciences Center, and Veterans Administration Medical Center (S.L., R.A.H.), Denver, Colorado 80262

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SUMMARY

Modulation of the inositol 1,4,5-trisphosphate (IP_3)-mediated signal transduction pathway by the inhalational anesthetic enflurane was studied in *Xenopus* oocytes expressing mouse and human cortical mRNA. We found that enflurane significantly inhibited ion currents activated by m1 muscarinic and 5-hydroxytryptamine (5-HT_{1C}) receptors. This inhibition was dependent upon the concentration of acetylcholine or 5-HT, with large inhibition (80–89%) of low concentrations and small inhibition (8–44%) of high concentrations of acetylcholine and 5-HT. Similar effects were found with either mouse or human receptors. To investigate the mechanism of enflurane action, ion currents induced by intracellular injection of guanosine 5′-(3-*O*-thio)triphosphate and IP_3 were examined. Enflurane strongly suppressed the guanosine 5′-(3-*O*-thio)triphosphate-activated current but not the IP_3-activated current. These results suggest that an inhalational anesthetic can disrupt the function of mouse and human brain phosphatidylinositol-linked receptors by selectively inhibiting the guanine nucleotide-binding protein activity.

Mechanisms of general anesthetics remain obscure, but changes in brain membrane ion permeability that alter neural excitability in the brain form the basis of many theories of anesthesia (1–3). Previous studies on general anesthetics have focused on membrane excitability regulated by ionotropic receptors, such as GABA receptor-gated Cl^- channels (4–6) and NMDA receptor-gated ion channels (7, 8). Little attention has been paid to neural excitability regulated by metabotropic receptors, which activate ion channels via second messengers. Recent studies indicate that metabotropic receptor-mediated second messenger production is modulated by inhalational general anesthetics (9, 10). For instance, muscarinic receptor-mediated inhibition of cAMP production is decreased by several inhalational anesthetics in cardiac membranes (9), and arginine vasopressin-induced inositol phosphate formation and intracellular Ca^{2+} release are suppressed by halothane (an inhalational anesthetic agent) in vascular smooth muscle (10). Thus, it is likely that metabotropic receptor-regulated ion channel activity is inhibited by anesthetic agents, and the present studies were designed to test this hypothesis. Furthermore, because there are multiple steps from ligand binding to channel opening, it is important to elucidate the steps that are sensitive to anesthetic action.

We studied these questions using the *Xenopus* oocyte preparation because some signal transduction pathways, especially PI signaling, are well characterized in oocytes expressing mammalian brain mRNA (11). The best known examples of PI-linked metabotropic receptors are the m1 muscarinic and serotonin 5-HT_{1C} receptors (12). The m1 and 5-HT_{1C} receptors are believed to be linked to a common signal transduction pathway, because of the cross-desensitization between ACh- and 5-HT-activated currents (12, 13). This pathway includes the following steps: activation of G proteins, enhancement of the catalytic activity of phospholipase C, increase of IP_3 production, release of intracellular Ca^{2+}, and activation of Ca^{2+}-dependent Cl^- channels (12). The pathway described above is based on several lines of evidence, as follows. 1) ACh- and 5-HT-activated currents are mimicked by intracellular injections of GTPγS, IP_3, or Ca^{2+} (14–16), and all these currents show a reversal potential close to the Cl^- equilibrium potential in oocytes (14, 17, 18). 2) ACh- and 5-HT-activated currents are blocked by desensitization produced by preceding injections of GTPγS or IP_3 (12, 19). 3) Pertussis toxin and intracellular

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ABBREVIATIONS: GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; PI, phosphatidylinositol; ACh, acetylcholine; 5-HT, 5-hydroxytryptamine (serotonin); G protein, guanine nucleotide-binding protein; IP_3, inositol 1,4,5-trisphosphate; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Gpp(NH)p, 5′-guanylylimidodiphosphate; MBS, modified Barth's saline; MAC, minimum alveolar concentration; AMPA, α-amino-3-hydroxyisoxazolepropionic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

EGTA eliminate ACh- and 5-HT-activated currents (19, 20). This well characterized IP_3-mediated signal transduction pathway provides a system for study of anesthetic actions.

We chose to study enflurane because it is one of the most commonly used inhalational general anesthetics. We found that enflurane significantly affected ionotropic receptor activity in *Xenopus* oocytes (21, 22), thus allowing comparison of its effects on metabotropic receptors with those on ionotropic receptors. We expressed mRNA isolated from cerebral cortex of mouse and human because cerebral cortex is generally believed to play an important role in the production of general anesthesia (3).

Experimental Procedures

Materials. Drugs and chemicals used and their sources were as follows: enflurane from Anaquest Co. (Madison, WI), spiperone from Research Biochemicals (Wayland, MA), IP_3 from Calbiochem (San Diego, CA), and GTPγS from Boehringer Mannheim (Indianapolis, IN). The other drugs and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of mRNA. Adult (6–8 weeks of age) male BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were sacrificed by decapitation. The cerebral cortex was dissected and immediately frozen in liquid nitrogen. Polyadenylated RNA was isolated using the Fast Track mRNA isolation kit purchased from Invitrogen (San Diego, CA). The frozen tissue from nine or 10 mice was ground using a mortar and pestle and was homogenized in lysis buffer using a Polytron high speed homogenizer. The lysate was then incubated at 45–50° for 90 min to allow proteins to be digested. After incubation, the NaCl concentration was adjusted to 0.5 M by adding NaCl to the lysate. The lysate was mixed with oligo(dT)-cellulose for 50 min, using a rocker. After the mixing step, the mRNA-oligo(dT) hybrid pellet was washed three or four times and the mRNA was eluted with elution buffer. The mRNA was then purified by phenol/chloroform extraction and was precipitated with 0.15 volumes of 2 M sodium acetate and 2 volumes of absolute ethanol. The mRNA preparation was stored at −20° (21).

The postmortem human cortical tissue was obtained from The Schizophrenia Research Center of The Denver Veterans Affairs Medical Center. The brain was removed from the body 16 hr after the subject died (67-year-old man; death was due to myocardial infarction) and was stored at −70° for 15 months before use. Polyadenylated RNA was isolated using the same procedures used for mouse tissue.

Oocyte preparation and RNA injection. *Xenopus laevis* frogs were kept in aquarium tanks at 19–21° with a 12/12-hr light/dark cycle, and oocytes were obtained as described previously (21). Oocytes of stages V and VI were obtained by manually dissecting the inner ovarian epithelium layer and theca layer in isolation medium (108 mM NaCl, 2 mM KCl, 1 mM EDTA. 10 mM HEPES, pH 7.9). The follicular cell layer of oocytes was removed by treatment with Sigma type IA collagenase (0.5 mg/ml) in collagenase buffer (83 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) for 10 min. Denuded oocytes were then transferred to MBS [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.9 mM $CaCl_2$, 0.8 mM $MgSO_4$, 10 mM HEPES, adjusted to pH 7.5 with 10 N NaOH]. The oocytes were injected with 150 ng (mouse) or 200 ng (human) of mRNA (in 50 nl of diethylpyrocarbonate-treated water). The oocytes were incubated at 19–21° for 2–3 days in sterile MBS supplemented with 2 mM sodium pyruvate, 10,000 units/liter penicillin, 10 mg/liter streptomycin, 50 mg/liter gentamycin, and 0.5 mM theophylline.

Electrophysiological recording. A rectangular recording chamber (100 μl) was used for containing oocytes and perfusing drugs (21). During recording, oocytes were perfused (rate, 1.7 ml/min) with MBS, and recording (10–30 MΩ) and voltage-clamping (1–5 MΩ) electrodes were placed in the animal hemisphere of the oocytes. The oocytes were then clamped at −70 mV using an Axoclamp IIA amplifier (Axon Instruments, Inc., Burlingame, CA). Currents measured were plotted in real time using a strip-chart recorder (Cole-Parmer Instrument Co., Chicago, IL). The recordings were performed at room temperature (20–22°).

Drugs were prepared in MBS. The control ACh- or 5-HT-activated currents were obtained by perfusion of ACh or 5-HT for 30 sec (10 sec were required for equilibration in the recording chamber). Effects of various drugs (enflurane, atropine, pirenzepine, and spiperone) on control ACh- or 5-HT-activated currents were obtained by preincubation for 60 sec before a coapplication of drugs and ACh/5-HT for 30 sec. At least 30 min were allowed for washout between successive drug applications. Enflurane solutions were prepared immediately before use, by dissolving the liquid stock in MBS, and were sealed in air-tight vials. Before perfusion, the vial caps were replaced by ones with a hole to allow passage of the tubing. Because spiperone was not water soluble, it was dissolved in 0.02% dimethylsulfoxide. This concentration of dimethylsulfoxide was tested in oocytes and found to induce no observable currents.

Intracellular injections of EGTA, IP_3, and GTPγS were conducted using a digital microdispenser (Drummond Scientific, Broomall, PA). EGTA (50 mM, pH 7.0–7.5 titrated with KOH), GTPγS (2–10 mM), and IP_3 (0.5 μM) were dissolved in distilled water and loaded into an injection pipette (10-μm tip size) connected to the digital microdispenser. To ensure that the pipettes were not clogged, they were examined under a microscope. A few droplets of the solution were pushed out of the pipettes just before the oocyte impalement. The pipette was placed with equal spacing relative to the recording and clamping electrodes in the animal hemisphere of the oocytes. A volume of 10 nl was then injected into the oocytes, which are approximately 1 μl in size. An injection of EGTA alone did not induce any observable currents, and recording began 10 min after EGTA injection. GTPγS and IP_3 were prepared immediately before the injections. Currents were observed immediately after GTPγS or IP_3 injections. Effects of enflurane on GTPγS- and IP_3-induced currents were obtained with a preincubation of enflurane for 60 sec before injections, and enflurane was continuously perfused until the currents returned to base line. After recording, injection pipettes were again examined under the microscope, and data were not used if the pipette was clogged.

Quantification of anesthetic concentrations in the recording chamber. Bath concentrations of enflurane were quantified using a Varian 3300 gas chromatograph (Varian Associates, Inc., Walnut Creek, CA). The standard samples were prepared by dissolving the liquid stock directly in MBS. The bath samples were collected from the recording chamber after the drug reached equilibrium (i.e., 15 sec after drug perfusion). The standard and bath (100-μl) samples were bottled in separate septum-capped vials and were extracted with an equal volume of heptane containing 0.01% hexane as the internal standard. The vials were then chilled on ice. The heptane phase (2 μl) of each sample was drawn using a Hamilton microsyringe and was injected into the injector (250°), which was connected to a 30-meter fused silica capillary column (100°) (DB-5; J & W Scientific, Folsom, CA). The column in turn led to an electron capture detector (250°). The retention time of enflurane was 5–5.1 min, whereas that of hexane was 5.5 min.

The concentration of each standard sample was converted into a ratio by dividing the peak height of each standard sample by that of hexane. The standard/hexane ratios were plotted against the standard concentrations to generate the standard curve. The curve was then fitted to a linear function using least-squares regression; the goodness of fit (i.e., r^2) was 0.991–0.998. The concentrations of bath samples were then determined by an interpolation of the bath/hexane ratios. In this study, the bath concentration of enflurane was controlled at 1.8 mM, which was equivalent to 1.7 MAC in mice and 2.0 MAC in humans. The MAC values [1.95% and 1.68% for mice (23) and humans (24), respectively] were first converted to the concentrations (0.77 mM and 0.66 mM for mice and humans, respectively) in the air using Henry's

Law; these in turn were converted to the concentrations (1.04 mM and 0.90 mM for mice and humans, respectively) in the physiological solution using Krebs' solution/air partition coefficients (25) and temperature coefficients of solubility (26). The corresponding vial concentration for this bath concentration was 3 mM.

Results

Inhibition by enflurane of currents activated by ACh and 5-HT receptors. ACh activated inward oscillating currents with a slow onset (5–10 sec). The current amplitude was dependent on the ACh concentration; 10 μM ACh induced the maximal current and 100 nM ACh induced a small current (30–50 nA) (Fig. 1A). The currents activated by 100 nM ACh were blocked in the presence of enflurane; however, enflurane produced no effect with maximal ACh concentrations (Fig. 1B).

In agreement with others (19), we found that the first ACh response was substantially larger than the following responses even after a 1–2-hr wash; however, subsequent ACh responses were relatively consistent. Therefore, the first ACh response was excluded. It should be pointed out that ACh responses sometimes decreased gradually in the course of recording. To take into account the declining base line over time, ACh responses were measured before and after each enflurane treatment and were averaged as control responses. The bath concentration of enflurane in the present study was 1.8 mM (see Experimental Procedures), which is within the anesthetic concentration range.

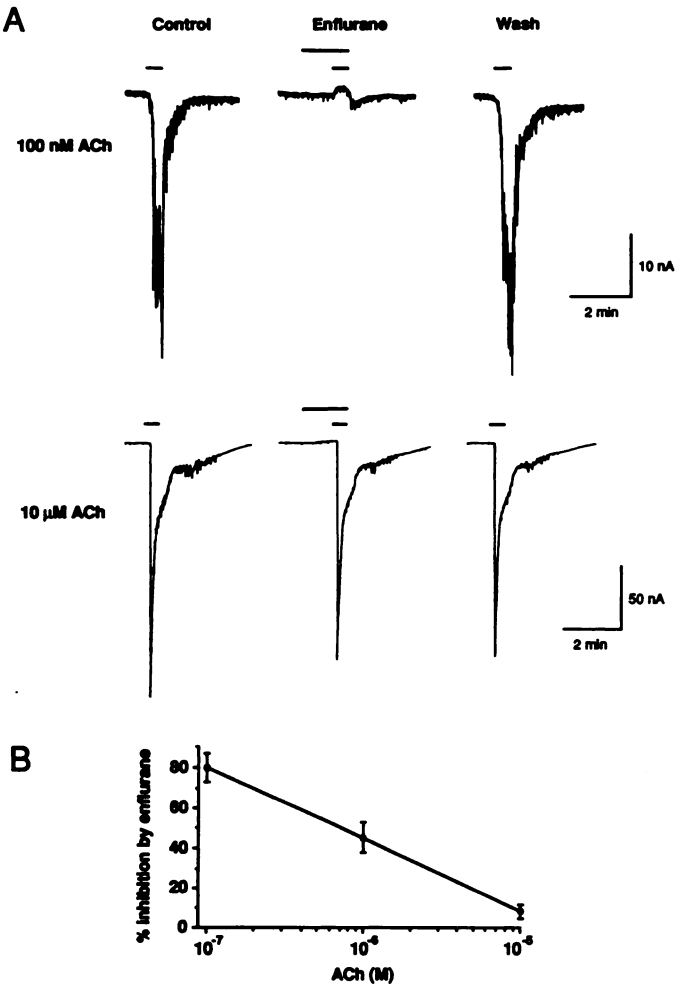

Fig. 1. Inhibition of ACh-activated currents by enflurane is dependent upon the concentration of ACh. A, *Traces* represent minimal (*upper*) and maximal (*lower*) ACh-induced currents (*left*), currents in the presence of enflurane (*middle*), and currents after wash (*right*). *Horizontal bars above the traces*, drug applications; ACh was applied for 30 sec and enflurane was applied for 60 sec before a 30-sec coapplication with ACh. At least 30 min of washout were allowed between successive drug applications. The same methods were used in Figs. 2–7. B, Average percentage of inhibition by enflurane of the currents activated by different concentrations of ACh. Each *data point* represents mean ± standard error; $n = 4$ oocytes.

5-HT, similarly to ACh, induced delayed inward oscillating currents with clear desensitization (27). The effects of enflurane on 5-HT actions were similar to those for ACh. We found that enflurane completely inhibited the current induced by 10 nM 5-HT, and the inhibition remained at about 50% for the maximal current (which was induced by 100 nM 5-HT) (Fig. 2).

We also carried out similar experiments in oocytes expressing human cortical mRNA. In this experiment, only ACh, and not 5-HT, induced significant currents (>20 nA). Also, only high (1–10 μM), and not low (100 nM), concentrations of ACh induced observable currents. The average size of currents induced by 10 μM ACh in oocytes expressing human mRNA was 1700 ± 350 nA (mean ± standard error, $n = 6$ oocytes), in comparison with 370 ± 70 nA ($n = 4$ oocytes) in those expressing mouse mRNA. Despite these differences, enflurane produced similar inhibition of currents induced by 10 μM (10 ± 2% and 8 ± 3%, $n = 3$ or 4 oocytes) and 1 μM (50 ± 6% and 45 ± 7%, $n = 4$ or 5 oocytes) ACh in experiments with human and mouse mRNA, respectively.

Pharmacological characterization of ACh and 5-HT receptors. The predominant subtypes of ACh and 5-HT receptors expressed from rat brain mRNA in oocytes are m1 and 5-HT_{1C} receptors, respectively (11, 27). To assess whether the same subtypes of ACh and 5-HT receptors were expressed from mouse cortical mRNA, several pharmacological characterizations were carried out. Atropine, a selective antagonist of the muscarinic receptor, completely abolished ACh-activated currents in all oocytes tested (Fig. 3A) and the suppression was reversible after washing for 1 hr, indicating that the response was of a muscarinic nature. There are at least five subtypes of muscarinic receptors, PI linked (m1, m3, and m5) and adenylate cyclase linked (m2 and m4) (28). To assess which pathway mediated the observed ACh-activated currents, we examined the response to ACh before and after intracellular injection of the Ca^{2+} chelator EGTA. The fast transient component of ACh-activated current was completely abolished by EGTA (Fig. 3B), suggesting that the response to ACh was primarily mediated by intracellular Ca^{2+}. Thus, the ACh receptors were predominantly linked to IP_3 formation/Ca^{2+} mobilization. Note that the blockade by EGTA was not reversible after a 2–3-hr wash. In addition, the inhibition was not due to stress from injection, because the ACh-activated current remained intact after an injection of the same volume of water.

We used a selective m1 antagonist, pirenzepine, to determine whether the ACh receptors were of the m1 subtype. Pirenzepine at a concentration close to the IC_{50} (89 nM) (29) for the m1 receptor produced significant inhibition (50 ± 3%, $n = 5$ oocytes) of currents induced by 1 μM ACh in oocytes expressing mouse mRNA (Fig. 3C). The inhibition was reversible after a 30-min wash. In addition, pirenzepine produced even greater

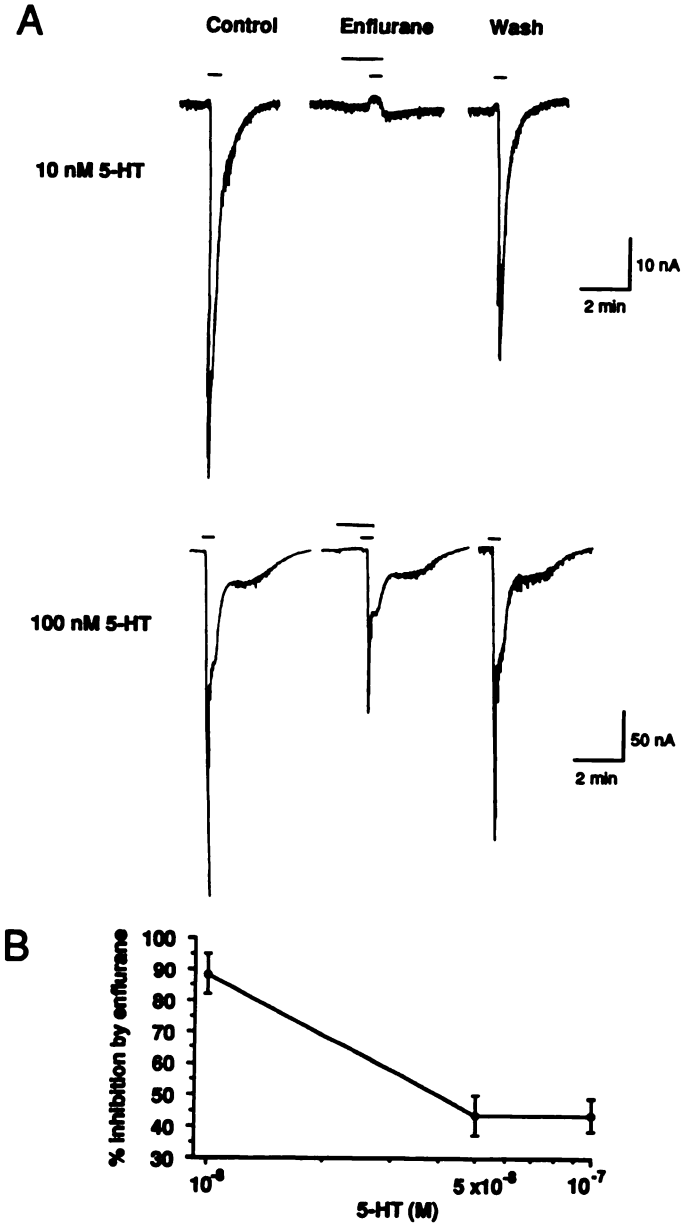

Fig. 2. Inhibition of 5-HT-activated currents by enflurane is dependent upon the concentration of 5-HT. A, *Traces* represent minimal (*upper*) and maximal (*lower*) currents induced by 5-HT (control). B, Average percentage of inhibition by enflurane of the current activated by different concentrations of 5-HT. Each *data point* represents the mean ± standard error, $n = 4$ or 5 oocytes.

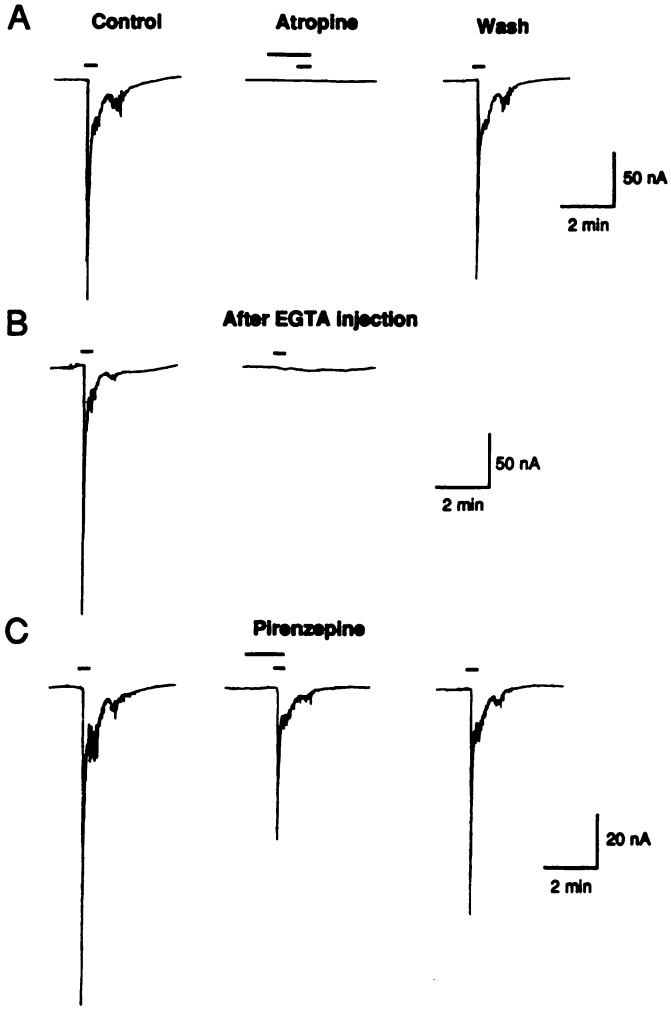

Fig. 3. Pharmacological characterization of ACh-activated currents, indicating a predominance of m1 muscarinic receptor-gated responses. A, Atropine (1 μM) reversibly blocked the ACh (1 μM)-activated current. B, Intracellular EGTA (0.5 nmol) injection completely abolished the transient current activated by ACh (1 μM). The concentration of EGTA was estimated to be approximately 500 μM, based on an assumption that the oocyte size is about 1 μl. C, Pirenzepine (100 nM) reversibly inhibited the ACh (1 μM)-activated current. Results are from one oocyte for each condition; similar results were obtained from two to four other oocytes.

inhibition (84 ± 16%, $n = 3$ oocytes) of currents induced by 1 μM ACh in oocytes expressing human mRNA. Because the other subtypes (m2-m5) of muscarinic receptors have much lower affinity (561–1540 nM) (29) for pirenzepine, these results suggest that ACh receptors expressed by mouse and human mRNAs were primarily of the m1 subtype.

There are at least six subtypes of 5-HT receptors, PI linked (5-HT_{1C} and 5-HT_2), cAMP linked (5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D}), and ionotropic (5-HT_3) (30). Our assessment revealed that 5-HT-activated currents were abolished after intracellular EGTA injection (Fig. 4A). This suggests that the 5-HT receptors were linked to PI turnover and Ca^{2+} mobilization. We used spiperone to distinguish the 5-HT_{1C} from the 5-HT_2 receptors, because the affinity of spiperone for the former is in the micromolar range and for the latter is in the nanomolar range (27). Spiperone, at a concentration as high as 5 μM, inhibited 5-HT-induced currents by only 11 ± 9% ($n = 4$ oocytes) (Fig. 4B). This suggests that the 5-HT receptors expressed in oocytes were primarily of the 5-HT_{1C} subtype. This conclusion is also supported by the finding that 1 μM 5-HT did not induce larger currents than did 100 nM 5-HT, because the affinities for 5-HT_{1C} and 5-HT_2 receptors are in the nanomolar and micromolar ranges, respectively (27).

Evidence that enflurane significantly inhibits GTPγS-activated currents but not IP_3-activated currents. To assess the target site for enflurane action, effects of enflurane on GTPγS- and IP_3-induced currents were examined. Intracellular injection of GTPγS, a nonhydrolyzable analog of GTP, produced a long lasting inward current that was sometimes followed by a delayed outward current (Fig. 5A, *left*), in agreement with the findings of Kaneko *et al.* (16). In a few cases, a

fast transient inward current before the long lasting inward current was observed. GTPγS also induced oscillations, which occurred 5–10 min after the onset of the outward current. Furthermore, the GTPγS-activated current was not reversible even after a 1–2-hr wash. Therefore, each oocyte received only one GTPγS injection. In oocytes constantly perfused with enflurane, GTPγS induced either no currents or currents with much smaller amplitude (Fig. 5A, *right*), depending on GTPγS concentrations (Fig. 6).

If enflurane was applied after the GTPγS-activated current was induced, then enflurane produced little inhibition (Fig. 5B). This suggests that enflurane inhibition occurs at an early stage of the PI pathway, such that the processes subsequent to G protein activation are little affected. This hypothesis was supported by effects of enflurane on the current induced by intracellular injection of IP_3. IP_3 typically induced currents with three distinctive components, a fast transient inward current (D1), a delayed inward current (D2), and a long lasting outward current (Fig. 7, *upper*), as described by Dascal *et al.* (17). Oscillations often occurred at the rising phase of D1, the peak of D2, and part of the outward current. Each oocyte received only one injection of IP_3, because IP_3-induced currents were not reversed even after a 1–2-hr wash. We found that enflurane produced little effect on any of the components of the currents induced by IP_3 (Fig. 7). It should be noted that the IP_3 dose (5 fmol) used in this experiment activates only a small fractional response (31).

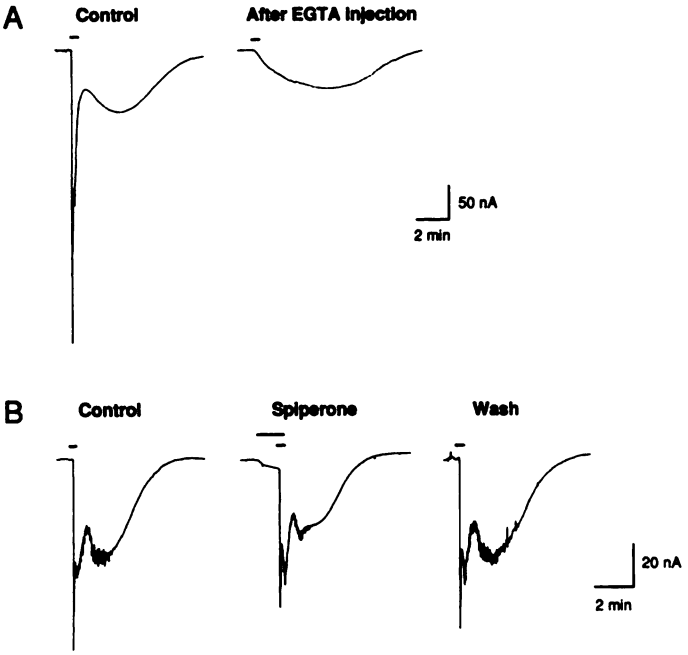

Fig. 4. Pharmacological characterization of 5-HT-activated currents, indicating a predominance of 5-HT_{1C} receptor-gated responses. A, Intracellular EGTA (0.5 nmol) injection completely abolished the transient current activated by 5-HT (50 nM). EGTA application was the same as Fig. 3B. B, Spiperone (5 μM) slightly reduced the 5-HT (50 nM)-activated current. Results are from one oocyte for each condition; similar results were obtained from two or three other oocytes.

Discussion

The present study provides the first electrophysiological evidence that an inhalational anesthetic agent can disrupt the function of mouse and human brain PI-linked metabotropic receptors. Other work suggests that ionotropic receptors, such as $GABA_A$ and glutamate receptors, are primary target sites of action for anesthetics (2, 7, 21). Our findings provide an additional perspective, suggesting that metabotropic receptors may be more sensitive to anesthetics. For example, an anesthetic concentration of enflurane produced only 20–40% inhibition of NMDA, kainate, and AMPA currents (22), whereas the same

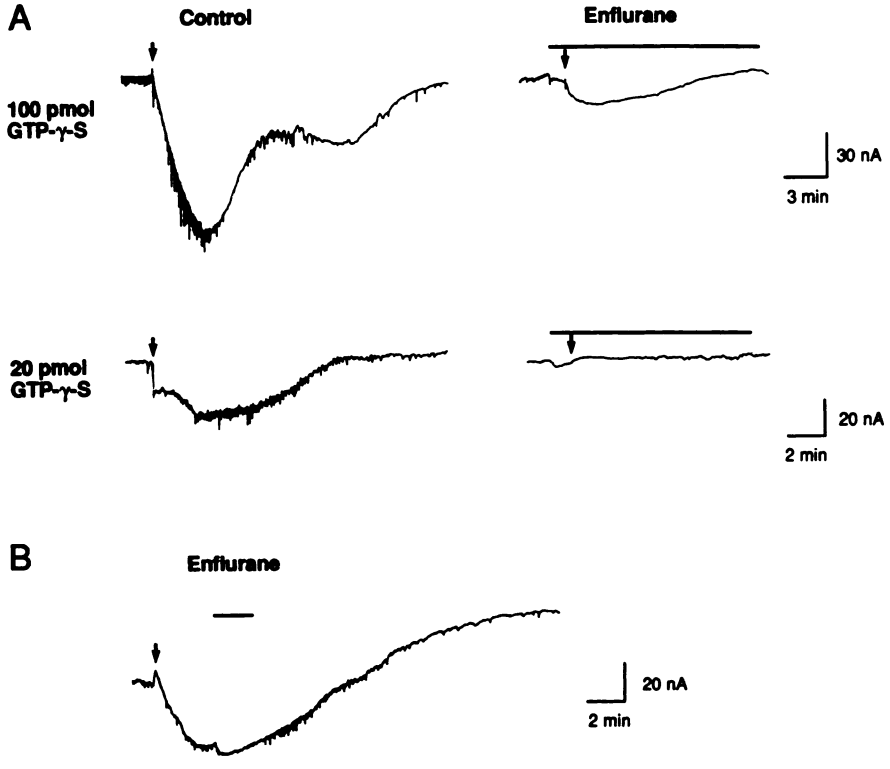

Fig. 5. Enflurane suppresses GTPγS-activated currents. A, Representative *traces* of currents activated by GTPγS in the absence (*left*) and presence (*right*) of enflurane. *Arrows above the traces*, injection of GTPγS. Enflurane was applied 60 sec before GTPγS injection and was continued for 10–30 min (*bar*) until the current returned to the base line. Note that the GTPγS-activated currents were obtained from different oocytes. B, Insignificant effect of enflurane when enflurane was applied after the GTPγS-activated current had reached the maximal amplitude. The amount of GTPγS injected was 50 pmol.

concentration of enflurane almost completely abolished the currents resulting from activation of the m1 and 5-HT_{1C} receptors by low concentrations of agonists. The results are consistent with a previous study in cortical slices showing that inhalational anesthetics were more potent inhibitors of ACh-induced excitation (presumably via muscarinic receptors) than glutamate- or NMDA-induced excitation (32). Together, these results indicate that metabotropic receptors are at least as sensitive to enflurane as are ionotropic receptors.

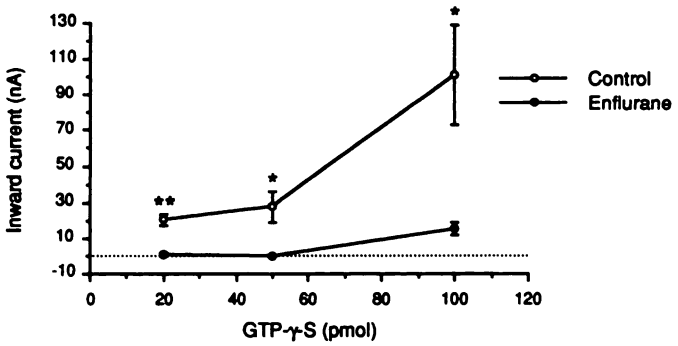

Fig. 6. Enflurane suppresses GTPγS-activated currents, with dependence on GTPγS concentrations. The currents activated by various concentrations of GTPγS were significantly suppressed. *, $p < 0.01$; **, $p < 0.001$. Each *data point* represents mean ± standard error, $n = 10–13$ different oocytes.

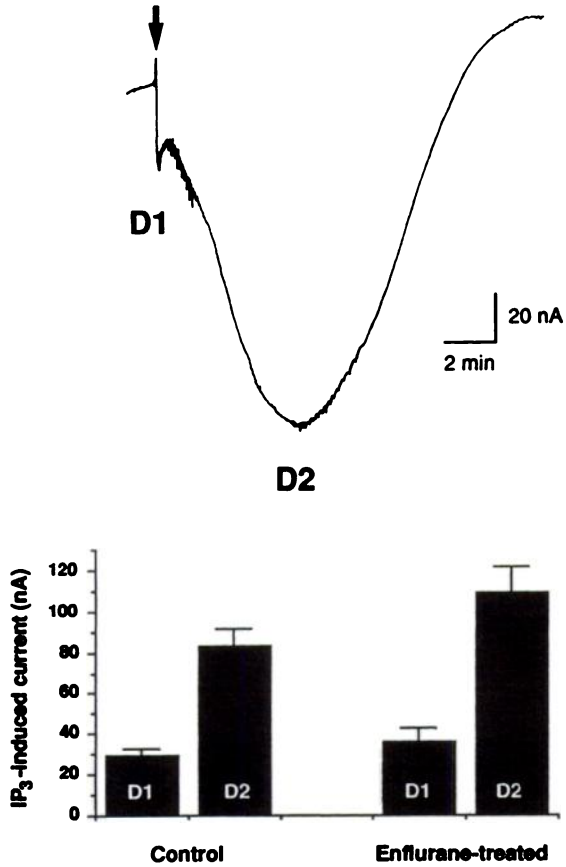

Fig. 7. Enflurane does not inhibit IP_3-activated currents. A representative *trace* of the IP_3-activated current in the absence of enflurane is shown. *Arrow*, injection of 5 fmol of IP_3 into the oocytes. *D1* and *D2*, major components of the IP_3-activated current. Control oocytes received only IP_3 injection, whereas the enflurane-treated oocytes were preincubated with enflurane for 60 sec before IP_3 injections and were continuously perfused with enflurane until the currents returned to the base line. Enflurane treatment produced no significant changes in the D1 component ($t = 0.90$, $p = 0.38$) or the D2 component ($t = 1.74$, $p = 0.09$) of the IP_3-activated current. Data represent mean ± standard error, $n = 15$ (control) or 12 (enflurane-treated) oocytes.

Muscarinic neurotransmission may amplify sensory input signals (33) and enhance memory function (34), and 5-HT modulates sleep (35) and pain perception (36). Thus, a suppression of ACh and 5-HT receptor-gated ion channel activity should lead to loss of sensory awareness, pain perception, and memory, which are three major effects of anesthetic agents (37). This background, together with our results, provides evidence that metabotropic receptors may play a role in the production of anesthesia.

At which step(s) does enflurane inhibit ACh- and 5-HT-mediated responses? Our results suggest that enflurane inhibits m1 and 5-HT_{1C} receptor-activated Cl^- currents by affecting the intermediate steps of the PI signaling pathway, rather than receptors or ion channels. Two pieces of evidence support the conclusion that the activity of Ca^{2+}-dependent Cl^- channels is not affected by enflurane treatment. First, enflurane did not inhibit the IP_3-activated current. This rules out the possibility that IP_3 receptors, Ca^{2+} release machinery, or Ca^{2+}-dependent Cl^- channels are the targets of enflurane. Second, the effect of enflurane on GTPγS-activated current was abolished if enflurane was applied after the current was initiated, suggesting that the later stages of the PI pathway are not affected. It is also unlikely that enflurane has a direct effect on the m1 and 5-HT_{1C} receptors, because inhalational anesthetic agents do not alter agonist binding affinity for muscarinic receptors (38, 39). Finally, we found that enflurane markedly suppressed GTPγS-induced currents. Together, these results suggest that enflurane affects steps (e.g., G proteins or phospholipase C) after the m1 or 5-HT_{1C} receptors but before IP_3 receptors and thereby inhibits ACh- and 5-HT-induced currents.

The question is raised here of whether a G protein or a G protein-coupled enzyme (i.e., phospholipase C) is the target of enflurane. Recent studies indicate that general anesthetic agents may interact with G proteins. For example, the ability of Gpp(NH)p, a nonhydrolyzable GTP analog, to decrease the high affinity binding of muscarinic receptors is eliminated by various inhalational anesthetic agents (39, 40). These results suggest that either guanine nucleotide binding to G proteins or receptor-G protein coupling is disrupted in the presence of inhalational anesthetics. In addition, the ability of GTPγS to activate Ca^{2+}-dependent Cl^- channels is markedly decreased in the presence of enflurane. Because receptor-G protein coupling is not included in the pathway of GTPγS action, we speculate that the G proteins that couple m1 and 5-HT_{1C} receptors to phospholipase C may represent important targets for anesthetic action. However, we cannot exclude an action of enflurane on phospholipase C.

Can the action of enflurane on G proteins account for the differential inhibition by enflurane of the effects of low and high concentrations of ACh and 5-HT? We found that enflurane inhibition of GTPγS-activated currents depended somewhat on GTPγS concentrations; enflurane inhibition was less robust with high concentrations of GTPγS. The competitive nature of the interaction between G proteins and inhalational anesthetic agents has been reported by Anthony *et al.* (39), who found that the inhibition by inhalational anesthetic agents of the Gpp(NH)p-induced decrease of muscarinic receptor binding

affinity was overcome by higher concentrations (30–100 fold) of Gpp(NH)p. If enflurane inhibits ACh- and 5-HT-induced currents by interfering with the binding of endogenous GTP to G proteins, then it is reasonable to think that the inhibition can be overcome by higher concentrations of ACh and 5-HT, which also cause more endogenous GTP to bind to G proteins.

Although native oocytes contain endogenous G proteins, it has been demonstrated that exogenous G proteins are indeed expressed from rat brain mRNA, in that currents induced by GTPγS in mRNA-injected oocytes are much larger than those in uninjected oocytes (16). In addition, the GTPγS-induced currents increase in parallel with an increase of proteins that can be ADP-ribosylated by pertussis toxin (16). These findings point out that exogenous G proteins are expressed from brain mRNA, suggesting that enflurane may affect G proteins in the brain in a similar way. In oocytes, m1 and 5-HT_{1C} receptors activate pertussis toxin-sensitive G_i and G_o proteins, respectively (41), which in turn activate phospholipase C. It is likely that enflurane in the brain, similarly to its effects in *Xenopus* oocytes, may exert inhibitory actions on G_i and G_o proteins. In the brain, the G_i protein is primarily coupled to adenylate cyclase. However, divergent coupling is often found for G_i and G_o proteins; both proteins are also coupled to phospholipase C (42). Thus, it is likely that enflurane may disrupt PI signal transduction in the brain.

Before this study, human mRNAs prepared from fresh adult brain tissue (43) or postmortem fetal brain tissue had been expressed in oocytes (44). However, this study is the first to demonstrate that mRNA prepared from postmortem adult brain tissue can be expressed in *Xenopus* oocytes. This finding opens the possibility of studying pathological human receptors in the future and relating the findings to clinical diseases.

We found that enflurane affected human and mouse m1 receptors in very similar ways; the percentages of inhibition of currents induced by 1 μM ACh and 10 μM ACh were identical. This implies that it is experimentally valuable to use animal preparations to conduct neuropharmacological research of anesthetics. In this study, only 1–10 μM, and not 100 nM, ACh induced observable currents in oocytes expressing human mRNA. The lack of 100 nM ACh-induced currents was not due to poor expression of human mRNA, because ACh-activated currents gated by human m1 receptors were actually bigger than those gated by mouse m1 receptors. Although good expression of human m1 receptors was obtained with human postmortem mRNA, responses to 5-HT were small, compared with those of the mouse receptors. mRNA degradation in postmortem human brain is not well characterized but is likely to involve different rates and mechanisms for mRNA species (45). Our results suggest either that expression of 5-HT receptors is lower in human than in mouse brain or that there is differential loss of 5-HT mRNA in postmortem tissue. Expression and regulation of the human m1 receptors in oocytes injected with the RNA, as well as an agarose gel of RNA isolated from this tissue (data not shown), indicate that the sample was not generally degraded. Further investigation will be necessary to determine the levels and stability of the 5-HT message in postmortem human brain.

In summary, we demonstrated that enflurane disrupts the function of PI-linked m1 and 5-HT_{1C} receptors and that this action is likely due to an action on G proteins. A selective inhibition by enflurane of G proteins, but not the Ca^{2+}-dependent Cl^- channels that are also embedded in cytoplasmic membranes, suggests that enflurane may act directly on G proteins to modulate the function. On the other hand, we cannot exclude the possibility that perturbation of the lipid bilayer by enflurane (46), although not sufficient to interfere with the function of the Ca^{2+}-dependent Cl^- channels, may be sufficient to change the mobility of G proteins and then change the interaction between G proteins and phospholipase C or receptors. The lack of enflurane effect on the Ca^{2+}-dependent Cl^- channels is surprising, because $GABA_A$ receptor-gated Cl^- channels, which have the same ion permeability as the Ca^{2+}-dependent Cl^- channels, have been shown to be potentiated by numerous anesthetic agents (5, 21). Thus, even ion channels that are permeable to the same ion (i.e., Cl^-), are not equally sensitive to anesthetic agents. Together, these results indicate that the action of anesthetic agents, even those with simple chemical structures such as enflurane, can be in fact quite selective.

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Send reprint requests to: R. Adron Harris, Department of Pharmacology, Box C-236, University of Colorado Health Sciences Center, 4200 E. 9th Avenue, Denver, CO 80262.