Halothane Regulates G-protein-dependent Phospholipase C Activity in Turkey Erythrocyte Membranes*

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The ability of halothane to stimulate phospholipase C (PLC) was examined in turkey erythrocyte membranes prepared from [3H]inositol-labeled turkey erythrocytes by measuring [3H]inositol phosphate formation ([3H]InsP) in the presence and absence of G-protein activation. In the presence of guanosine 5'-3-O-(thiophosphate) (GTPγS), halothane (0.5–10 mM) caused a dose-dependent activation of PLC. The EC50 value for halothane-induced PLC activation was 2.8 ± 0.3 mM. Halothane (0.1–30 mM) had no effect on PLC activity in the absence of G-protein activation and did not affect Ca2+-dependent PLC activity. The activation of PLC by GTPγS occurred after an initial lag period of 60 s which was followed by a linear increase in [3H]InsP. Halothane dose-dependently decreased the lag period for GTPγS-induced PLC activation (minimal value 15 s) and increased the rate of [3H]InsP formation at all time points following this lag. As a result, halothane shifted the EC50 value for GTPγS-induced PLC activation to the left (4-fold) and increased its maximal response. Halothane also caused a dose-dependent activation of PLC in the presence of AlF4-. Half-maximal stimulation of AlF4-activated PLC occurred with an EC50 value of 2.9 ± 0.4 mM halothane, which is similar to the halothane dose giving half-maximal stimulation of PLC in the presence of GTPγS. At low doses (0.1–0.3 mM) halothane inhibited both isoproterenol- and adenosine 5'-O-(2-thiodiphosphate) (ADPβS)-induced [3H]InsP formation, whereas at higher concentrations it stimulated PLC independent of the presence of these agonists. At concentrations chosen to reflect their different membrane/buffer partition coefficients, both hexanol (5 mM) and benzyl alcohol (20 mM) fluidized turkey erythrocyte membranes to the same degree as halothane (5 mM). However, these agents had no effect on GTPγS- or AlF4- induced PLC activity, indicating that halothane-induced PLC activation was not secondary to changes in bulk lipid fluidity properties. Halothane also stimulated [3H]inositol bisphosphate and [3H]inositol trisphosphate formation in intact erythrocytes. These data demonstrate that the anesthetic halothane can stimulate G-protein-dependent PLC activity and modify the responsiveness of this signaling system to activation by receptor-linked agonists.

Halothane is a volatile anesthetic and as such has a pharmacological potency that is a function of its octanol/water partition coefficient, suggesting a hydrophobic site of action within the cell membrane (1, 2). Although the molecular mechanism(s) underlying anesthesia are still largely unknown, recent evidence favors specific sites of action at the level of protein-protein or lipid-protein interactions (1–3). Most studies with halothane have concentrated on effects related to membrane conductance, where it has been shown to have a variety of actions on ion channel activity and synaptic transmission. For example, at concentrations in the range of 0.1–1 mM, halothane stimulates neuronal K+ (4–6) and Cl− currents (7). By contrast, it inhibits Na+ currents (8, 9) and both receptor-operated and voltage-gated routes of Ca2+ influx (10–12) at concentrations of 0.2–30 mM. In addition, halothane (1–6 mM) increases [Ca2+]i in L6 skeletal muscle cells (13), rat cardiomyocytes (14), rat hepatocytes (15), and brain synaptosomes (16).

The transduction of extracellular signals into intracellular second messengers by G-protein-regulated receptor-effector mechanisms relies on interactions between a number of intrinsic and membrane-associated proteins. As such, these processes are likely to be influenced by the membrane lipid environment and may be susceptible to modification by anesthetics and other agents that perturb membrane structure and protein-lipid interactions. However, relatively little is known about the effects of halothane on signal transduction and second messenger signaling processes. Halothane has been shown to inhibit muscarinic receptor regulation of adenylate cyclase activity in rat heart membranes (17). Whole cell studies have shown that vasopressin-induced InsP3 formation and Ca2+ signaling in vascular smooth muscle cells are inhibited by halothane (18), although halothane had no effect on thyrotropin-releasing hormone-induced InsP3 formation in GH3 pituitary cells (19).

In the present study we have examined the effects of halothane on PLC activity in turkey erythrocyte membranes. Harden and co-workers (20, 21) have demonstrated that this preparation provides an excellent homogeneous model system for studying guanine nucleotide- and receptor-mediated PLC activation in an intact membrane. Thus, turkey erythrocyte

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1 The abbreviations used are: G-protein, guanine nucleotide regulatory protein; PLC, inositol lipid-specific phospholipase C; DPH, 1,6-diphenyl-1,3,5-hexatriene; InsP, total inositol phosphates; InsP3, inositol monophosphate; InsP4, inositol bisphosphate; InsP5, inositol trisphosphate; InsP6, inositol tetrakisphosphate; InsP7, inositol pentakisphosphate; GTPγS, guanosine 5'-3-O-(thio)triphosphate; ADPβS, adenosine 5'-O-(2-thiodiphosphate).
membranes possess a G-protein-regulated, polyphosphoinositide-specific PLC that is functionally coupled to a P2y
purinoceptor receptor (22, 23). Recent studies from our group and others have demonstrated that turkey erythrocyte PLC can also be coupled independently to β-adrenergic receptors (24, 25). Turkey erythrocyte PLC has been purified as a 150-kDa protein, which is distinct from PLC-β or -γ, and reconstituted with erythrocyte membranes to confer both G-protein- and receptor-regulated enzyme activity (26, 27). The G-protein involved in the regulation of this 150-kDa PLC has been purified as a 43-kDa protein demonstrating strong reactivity with antisemur against a 12- amino acid sequence from the carboxyl terminus of Gα1 and Gα5 (28).

The data presented here demonstrate that halothane, at concentrations within the anesthetic range, alters the sensitivity of PLC to G-protein-dependent activation and modifies the responsiveness of this signaling system to activation by receptor-linked agonists. This effect seems to be dependent on the expression of an activated G-protein. This work provides the first demonstration of a volatile anesthetic regulating G-protein-dependent PLC activity.

**EXPERIMENTAL PROCEDURES**

*Labeling of Turkey Erythrocytes with [3H]Inositol—Turkey erythrocytes were prepared essentially according to the method of Harden et al. (20), as described previously (24, 29). Briefly, fresh turkey erythrocytes in Alsever’s solution (12 mN NaCl, 0.8 mM glucose, 27 mM tri-sodium citrate, 10% citric acid, pH 6.1) were centrifuged at 1,100 × g for 5 min and the resultant supernatant removed by aspiration before resuspending the packed cells in 4–6 volumes of ice-cold HEPES buffer (1.5 mM HEPES, 150 mM NaCl, pH 7.2). This centrifugation and washing procedure was repeated a further two times before washing the cells in inositol-free Dulbecco’s modified Eagle’s medium. Finally, 5 ml of packed cells were resuspended in medium containing 9 ml of inositol-free Dulbecco’s modified Eagle’s medium, 2 ml of chicken serum, 0.4 mg/ml gentamycin, and 200–400 μCi of [3H]Inositol. The cell suspension was then incubated at 37 °C in a shaking water bath for 16–18 h under a gas phase of O2/CO2 (95:5%).

*Preparation of [3H]Inositol-labeled Turkey Erythrocyte Ghosts—[3H]Inositol-labeled turkey erythrocytes were lysed by lysis buffer (5 mM sodium phosphate, 5 mM MgCl2, 1 mM EGTA, pH 7.4). The lysed cells were then centrifuged at 17,700 × g for 5 min at 4 °C and the supernatant discarded. The remaining unlysed cells were removed by aspiration before resuspending the membranes in lysis buffer. The membranes were further centrifuged at 17,700 × g for 5 min and then three more times at 8,000 × g for 5 min. After each centrifugation step any unlysed cells were removed and the membranes resuspended in the lysing buffer. Microscopic examination of erythrocyte ghosts prepared in this way showed that they were composed of morphologically intact red cell ghosts, all of which were fully permeable to trypan blue and ethidium bromide.

*Assay of Phospholipase C Activity—Assays were initiated by adding membrane aliquots (150 μl, 0.7–0.3 mg of protein) to tubes containing 450 μl of assay buffer consisting of 1 mM MgSO4, 115 mM KCl, 5 mM KH2PO4, 1 mM EGTA, 1 mM CaEGTA, and 10 mM HEPES, pH 7.0, at 37 °C. Under these conditions the free Ca2+ concentration was 300 nM as measured with fura-2. All incubations were performed in the absence of ATP or an ATP-regenerating system to eliminate the agonist effects of adenosine nucleotides acting at purinergic receptors and to prevent ATP from acting as a substrate for CAMP formation (24). Reactions were terminated by the addition of perchloric acid (4% final concentration) and samples stored on ice for 20 min. The perchloric acid precipitates were sedimented by centrifugation and the resulting supernatants neutralized by addition of 2 M Tris base. Total inositol phosphates were then determined by counting 600 μl of the neutralized supernatants in 10 ml of scintillation fluid. We have demonstrated previously that [3H]InsP2 and [3H]InsP3 are the only products of guanine nucleotide- and receptor-stimulated PLC in ATP-depleted turkey erythrocyte membranes (24, 29). For measurements of inositol phosphates in intact cells, turkey erythrocytes (150 μl, 6–9 mg of protein) were incubated in Dulbecco’s modified Eagle’s medium (450 μl) and the reactions terminated by the addition of perchloric acid. The perchloric acid supernatants from these samples were neutralized by addition of a freshly prepared 1:1 (v/v) mixture of Freon/tri-octylamine, and the inositol phosphates were then separated by anion exchange chromatography on columns of Dowex 1 anion exchange resin (200–400 mesh, formate form) (24, 29).

*Fluorescence Anisotropy Measurements—Turkey erythrocyte membranes were labeled with DPH and fluorescence anisotropy (steady state) measurements were performed as described previously (30, 31) using an SLM 48000 spectrofluorometer (SLM Instruments, Champagne Il) in the T-format mode. Excitation was at 390 nm and emission was observed at 430 nm. Measurements of DPH fluorescence lifetimes were obtained with a Liconix 4210 NB helium-cadmium laser as the excitation source using the phase modulation technique, as described previously (32, 33).

*Analysis of Data—The concentrations of agonist producing half-maximal stimulation (EC50) were obtained by computer-assisted curve fitting (ALLFIT) (34). Fluorescence lifetime data analysis was performed using GLOBALS UNLIMITED software (Laboratory of Fluorescence Dynamics, Department of Physics, University of Illinois, Urbana) as described by (35) and the data fitted to minimal values of the reduced χ2 parameter. The experimental error used in these analyses was taken as the standard deviation of averaged values for phase and modulation at each frequency (>0.002 and 0.2 in the modulation and phase, respectively).

*Mateials—Freshly drawn turkey erythrocytes were obtained from Cocalico Biologicals Inc. myo-[12-3H]Insitol (15 Ci/mmol) was obtained from Du Pont-New England Nuclear. GTPγ-S was obtained from Boehringer Mannheim and halothane from Aldrich. Chicken serum and Dulbecco’s modified Eagle’s medium were from Life Technologies, Inc. DPH and fura-2 were obtained from Molecular Probes. All other drugs and chemicals were obtained from Sigma or Fisher.

**RESULTS**

*Effect of Halothane on PLC—The ability of halothane to stimulate PLC in turkey erythrocyte membranes was determined by examining its effects on [3H]InsP formation in the presence and absence of G-protein activation. Fig. 1 shows that in the presence of the nonhydrolyzable guanine nucleotide GTPγ-S, halothane induced a dose-dependent activation of PLC. Significant increases in [3H]InsP formation were observed within the anesthetic range of between 0.5 and 1.0 mM halothane (2, 12) and maximal responses obtained with 10 mM halothane. The EC50 for halothane-induced [3H]InsP formation under these assay conditions was 2.8 ± 0.3 mM. At maximal halothane concentrations, the increase in [3H]InsP formation was 110–120% above that elicited by GTPγ-S alone. This is similar in magnitude to the activation of PLC in

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**Fig. 1. Halothane-induced [3H]inositol phosphate formation.** Incubations were initiated by the addition of turkey erythrocyte membranes (150 μl) to assay buffer containing 30 μM GTPγ-S and the indicated concentrations of halothane (final volume, 600 μl in a 1.6-ml sealed tube). Reactions were allowed to proceed for 5 min, and then total inositol phosphates ([3H]InsP) were extracted and measured as described under "Experimental Procedures." In these experiments 30 μM GTPγ-S alone caused a stimulation that was 91 ± 11% of the basal value (881 ± 189 cpm). Results represent the mean ± S.E. of data obtained from five to eight separate experiments.
response to a maximal concentration of the β-adrenergic receptor agonist isoproterenol and about half of the maximal response produced by the P2x-purine receptor agonist ADP/PS in erythrocyte membranes (see Fig. 7 and Ref. 24).

In the absence of GTPyS, halothane (0.1–30 mM) failed to stimulate [3H]InsP formation (not shown). The effect of halothane on PLC in the absence of G-protein activation was further investigated by examining whether halothane could enhance Ca2+-stimulated PLC activity in erythrocyte membranes. As can be seen from Fig. 2, increasing Ca2+ from 0.07–3 μM resulted in a 2-fold increase in [3H]InsP formation. Higher concentrations of Ca2+ had inhibitory effects on PLC activity (not shown). These effects of Ca2+ on turkey erythrocyte PLC activity are very similar to those described previously by Harden et al. (21). At concentrations of halothane which induced substantial increases in GTPyS-dependent PLC activity, halothane did not cause any further increased [3H]InsP formation in the presence of Ca2+ (Table I). These data demonstrate that halothane does not enhance either basal or Ca2+-stimulated PLC activity in turkey erythrocyte membranes and suggest that G-protein activation may be a prerequisite for the expression of the effect of halothane.

Effects of Halothane on the Kinetics of G-protein-dependent PLC Activation—Previous studies in turkey erythrocyte membranes have demonstrated that the stimulatory effects of receptor agonists on PLC activity reside in their ability to modify the kinetics of G-protein-dependent PLC activation (22, 24, 29). In erythrocyte membranes the activation of both adenylate cyclase and PLC by guanine nucleotides is preceded by an initial lag period of 1–2 min duration, which can be decreased, but not abolished, in the presence of β-adrenergic and P2x-purinergic receptor agonists (22, 24, 36, 37). It has been suggested that this rate-limiting step for G-protein-dependent PLC activation reflects the time required to exchange GTP for GDP on the α-regulatory subunit of Gα (22). Consistent with these findings, we observed an initial lag period of about 60 s in the presence of 30 μM GTPyS before a steady-state rate of [3H]InsP formation was achieved (Fig. 3). The addition of halothane decreased the lag period for GTPyS-induced [3H]InsP formation in a concentration-dependent manner and enhanced the rate of [3H]InsP formation at all time points following this lag period. In common with receptor agonists, halothane did not completely abolish the lag period but decreased it to a minimal value of about 15 s. The basal rate of [3H]InsP formation was unchanged throughout this time course and was unaffected by halothane treatment (data not shown).

Effect of Halothane on GTPyS Dose-Response Curves—To determine whether the effects of halothane on the kinetics of GTPyS-induced PLC activation translate into changes in the sensitivity to GTPyS for PLC activation, the effects of halothane on GTPyS-dose response curves were examined. As shown in Fig. 4, halothane stimulated PLC by increasing the sensitivity and maximal extent of activation by GTPyS. In the absence of halothane, GTPyS caused a dose-dependent increase in PLC activity with an EC50 of 26 ± 7 μM. In the presence of 5 mM halothane the maximal response to GTPyS increased by 77% and the EC50 shifted by 4-fold to a value of 6 ± 0.9 μM. These results are very similar to those obtained by Harden et al. (21). At concentrations of halothane which induced substantial increases in GTPyS-dependent PLC activity, halothane did not cause any further increased [3H]InsP formation in the presence of Ca2+ (Table I). These results are very similar to those obtained with GTPyS the basal activity was 576 ± 49 cpm, for ALF experiments it was 932 ± 135 cpm, and for Ca2+ activation experiments it was 599 ± 42 cpm (measured with no added Ca2+).

**TABLE I**

| Condition | Inositol phosphates | % of control |
|-----------|---------------------|--------------|
| GTPyS     | [3H]InsP formation  |
| ALF       | [3H]InsP formation  |
| Ca2+      | [3H]InsP formation  |

- No addition: 642 ± 53; 1,023 ± 87; 187 ± 20
- 5 mM halothane: 1,042 ± 125; 1,541 ± 204; 178 ± 33
- 5 mM hexanol: 723 ± 82; 1,127 ± 117; 200 ± 25
- 20 mM benzyl alcohol: 556 ± 66; 1,087 ± 143; 187 ± 7

*Statistically significant from response obtained with no additions using Student's t test (p > 0.05).
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FIG. 4. Effect of halothane on dose-response curves for GTPγS-induced [3H]inositol phosphate formation. Turkey erythrocyte membranes were incubated with the indicated concentrations of GTPγS for 5 min. Dose-response curves are shown for GTPγS alone (□) and GTPγS in the presence of 5 mM halothane (■). Total inositol phosphates were extracted and measured as described under "Experimental Procedures." Results are expressed as a percentage of the control activity (619 ± 84 cpm) in the absence of GTPγS. Each value represents the mean ± S.E. of data from five or six separate experiments. Points with no error bars have an S.E. within the symbol.

FIG. 5. Dose-response for halothane-induced [3H]inositol phosphate formation in the presence of AlCl₃. Turkey erythrocyte membranes were incubated with 30 mM NaF and 10 μM AlCl₃ (AlCl₃) in the presence of the indicated concentrations of halothane. Reactions were allowed to proceed for 5 min, and then total inositol phosphates were extracted and measured as described under "Experimental Procedures." In these experiments AlCl₃ caused a stimulation that was 651 ± 152% of the basal value (1,150 ± 267 cpm). Results represent the mean ± S.E. of data obtained from four or five separate experiments. Points with no error bars have an S.E. within the symbol.

FIG. 6. Effect of halothane on dose-response curves for AlF₃-induced [3H]inositol phosphate formation. Turkey erythrocyte membranes were incubated with the indicated concentrations of NaF in the presence of 10 μM AlCl₃ for 5 min. Total inositol phosphates were extracted and measured as described under "Experimental Procedures." Dose-response curves are shown for inositol phosphate accumulation in response to NaF (□) and NaF in the presence of 5 mM halothane (■). PLC activity is expressed as a percentage of the basal value (1,060 ± 293 cpm) measured in the absence of fluoride. Results represent the mean ± S.E. of three or four separate experiments. Points with no error bars have an S.E. within the symbol.

with ADPβS and isoproterenol on PLC activation curves in the presence of GTPγS (22, 24) and are consistent with the ability of halothane and receptor agonists to decrease the lag period and increase the rate of GTPγS-dependent PLC activation.

Effects of Halothane on AlF₃-induced PLC Activation—
AlF₃ is a nonspecific activator of G-proteins which has been suggested to interact with the GDP-bound form of the α-regulatory subunit and mimic the γ-phosphate of GTP. Consequently, AlF₃ stimulates PLC by a mechanism that is independent of guanine nucleotide exchange. We therefore examined the ability of halothane to stimulate PLC in the presence of AlF₃ as a means of further assessing the role of guanine nucleotide exchange in the action of halothane. In the presence of 30 mM NaF and 10 μM AlCl₃ (AlF₃) halothane induced a dose-dependent activation of PLC with an observed EC₅₀ value of 2.9 ± 0.4 mM (Fig. 5). At maximal concentrations of halothane, PLC activity was more than 2-fold higher than the activity observed with AlF₃ alone. Both the EC₅₀ value and the magnitude of halothane-induced PLC activation in the presence of AlF₃ are similar to the values obtained for halothane in the presence of GTPγS. Furthermore, halothane also caused a leftward shift in the dose-response curve for AlF₃-induced PLC activation. In the presence of 10 μM AlCl₃, NaF caused a dose-dependent activation of PLC which was evident over the range 0.1-30 mM F⁻ (Fig. 6). At higher concentrations PLC activity was inhibited (data not shown), probably because of nonspecific effects. Halothane significantly enhanced [3H]InsP formation at every concentration of F⁻ tested and caused a substantial shift in the activation curve to give an EC₅₀ value of 1.5 ± 0.2 mM for NaF. This provides the first example in which an activator of the turkey erythrocyte G-protein-dependent PLC is able to enhance PLC activity in the presence of both GTPγS and AlF₃ and indicates that the ability of halothane to activate PLC is independent of the manner in which the G-protein is activated.

Effect of Halothane on Agonist-induced PLC Activation—
Previous studies from this laboratory have shown that the effects of two agonists (ADPβS and isoproterenol) are nonadditive at maximal doses (24). Presumably these agonists share a common mechanism of PLC activation. Therefore, it was of interest to examine the additivity of halothane action with ADPβS and isoproterenol in the presence of GTPγS. Fig. 7 shows the effect of a range of halothane concentrations on the PLC response to maximal levels of ADPβS and isoproterenol. At low doses (0.1-0.3 mM), where it had negligible effects on PLC activity in the absence of agonist, halothane inhibited both isoproterenol- and ADPβS-induced [3H]InsP formation by 61 and 28%, respectively (Fig. 7). This somewhat unexpected result is in marked contrast to the lack of any inhibitory effects of halothane in the absence of agonist. At higher concentrations (0.5-30 mM), halothane caused an activation of PLC above the partially inhibited agonist response, which was of a similar magnitude and dose response to the activation caused by halothane in the absence of agonist (see
Fig. 7. Effect of halothane on isoproterenol- and ADPβS-stimulated [3H]inositol phosphate formation in turkey erythrocyte membranes. Turkey erythrocyte membranes were incubated with 30 μM GTPγS + 10 μM isoproterenol (left) or 30 μM GTPγS + 10 μM ADPβS (right) in the presence of the indicated concentrations of halothane. Incubations were allowed to proceed for 5 min before total inositol phosphates were extracted and measured as described under "Experimental Procedures." In these experiments 30 μM GTPγS alone produced a stimulation that was 1,120 ± 105% of the basal value (688 ± 105 cpm). Results represent the mean ± S.E. of data obtained from two to four separate experiments.

TABLE II  
Fluorescence anisotropy measurements in turkey erythrocyte membranes

| Condition          | Fluorescence anisotropy | Δr       |
|--------------------|------------------------|----------|
| No addition        | 0.202 ± 0.001          |          |
| 5 mM halothane     | 0.193 ± 0.001          | -0.009   |
| 5 mM hexanol       | 0.189 ± 0.002          | -0.013   |
| 20 mM benzyl alcohol | 0.197 ± 0.002      | -0.005   |

Although it is not possible to separate completely the inhibitory and stimulatory components of halothane action, it is apparent that the stimulation of PLC by halothane is fully additive with the agonist responses.

Relationship between Membrane Lipid-disordering and G-protein-dependent PLC Activity—Since halothane fluidizes the bulk lipid component of membranes, it is possible that PLC activation by halothane is a secondary consequence of a nonspecific effect on membrane properties. To investigate the relationship between lipid disordering and PLC activation, the effects of halothane (5 mM) on fluidity and PLC activity were compared with those of hexanol (5 mM) and benzyl alcohol (20 mM), two other well known membrane fluidizing agents (2, 38). The membrane/buffer partition coefficients of these agents differ, so that concentrations were chosen to achieve approximately equal membrane lipid disordering effects (2, 38). All three agents disordered (or fluidized) turkey erythrocyte membrane lipids as reflected by decreased fluorescence anisotropy values of DPH (Table II). Unlike hexanol and benzyl alcohol, halothane is a fluorescence quenching agent, as shown by a reduction in the major lifetime component of DPH from 9.1 to 8.5 ns (from a biexponential analysis) for 5 mM halothane. This causes an apparent increase in the steady-state anisotropy value, since this parameter is sensitive to the lifetime of the fluorophore (39). As a result, the magnitude of the decrease in anisotropy caused by the halothane-induced increase in fluidity reflects a slight underestimation of the true fluidity change. Based on time-resolved anisotropy measurements with lipid vesicles and using the equation relating steady-state anisotropy to its time-resolved components (39), it was calculated that the quenching effect of halothane caused the anisotropy change resulting from membrane fluidization to be underestimated by 0.003. Thus, correction of the Δr parameter for halothane in Table II in this way would yield a value of −0.012, essentially the same as that for hexanol.

Despite the fact that all three agents fluidized turkey erythrocyte membranes, neither hexanol nor benzyl alcohol mimicked the effects of halothane to activate G-protein-dependent PLC activity. As shown in Table I, under conditions in which halothane stimulated PLC activity in the presence of GTPγS or ADPβS, hexanol and benzyl alcohol had no significant effect on PLC activity. These agents also had no effect on PLC activity in the absence of G-protein activation (not shown) or on Ca2+-stimulated PLC activity (Table I). These data indicate that the ability of halothane to stimulate G-protein-dependent PLC activity cannot be explained simply by its ability to increase membrane fluidity and argue in favor of a more specific locus of halothane action.

Effect of Halothane on PLC Activity in Intact Turkey Erythrocytes—The data of Table III provide important confirmation of the ability of halothane to stimulate PLC in intact turkey erythrocytes. In intact erythrocytes prelabeled with [3H]inositol, halothane (5 mM) stimulated the formation of both [3H]InsP2 and [3H]InsP3. The response to halothane was comparable with that of isoproterenol and about 30% of the maximal response produced by ADPβS (Table III). Thus, these data demonstrate that the ability of halothane to stimulate PLC is not solely a property of the membrane preparation. As described previously for receptor agonists in intact erythrocytes (24), no significant increases in [3H]InsP2 were observed. Although halothane did appear to enhance [3H]InsP3 formation, these changes were difficult to resolve accurately because of the high levels of [3H]InsP3 in turkey erythrocytes.

**Table III**  
[3H]Inositol phosphate formation in intact turkey erythrocytes

Intact [3H]inositol-labeled turkey erythrocytes were incubated in Dulbecco's modified Eagle's medium with the indicated additions for 5 min. Individual inositol phosphates were extracted and separated as described under "Experimental Procedures." The mean values for [3H]InsP2 and [3H]InsP3 formation in control cells with no additions were 97 ± 12 and 107 ± 32 cpm, respectively. Results represent the mean ± S.E. of data from three experiments.

| Condition          | [3H]InsP2 | [3H]InsP3 | % of control |
|--------------------|-----------|-----------|-------------|
| No additions       | 100       | 100       |             |
| 5 mM halothane     | 178 ± 8   | 259 ± 45  |             |
| 10 μM isoproterenol| 226 ± 71  | 253 ± 70  |             |
| 10 μM ADPβS        | 765 ± 68  | 535 ± 64  |             |
formation but can also inhibit receptor-mediated PLC activation with even greater potency. Both of these effects are observed within the anesthetic range of between 0.3 and 1 mM halothane, although the EC_{50} value for halothane action in our studies with erythrocyte ghosts was somewhat higher than its ED_{50} for anesthesia (1, 2). This does not preclude a possible role for PLC in halothane anesthesia, since anesthesia is a complex end point that is unlikely to bear a simple relationship to the magnitude of alterations in the activity of the molecular targets of halothane action. It should also be noted that the effects of halothane on PLC could contribute to side effects not associated with anesthesia.

The stimulatory effect of halothane on PLC appears to be dependent on prior G-protein activation, as shown by the fact that halothane has no effect on either basal or Ca^{2+}-stimulated PLC activity. It has been shown that the sensitivity of endogenous and reconstituted turkey erythrocyte PLC to activation by Ca^{2+} is not modified by G-protein activation (21, 28). Similar results have also been obtained for G_{a} activation of PLC-β1 in bovine liver (40), suggesting that Ca^{2+} activates PLC by increasing the intrinsic activity of the enzyme. The inability of halothane to enhance Ca^{2+}-activated PLC demonstrates that the effect of halothane is dependent on the mechanism by which PLC activation is achieved. This argues against a direct effect of halothane on PLC itself.

Further evidence that halothane interacts with G-protein-dependent PLC is provided by its effects on the kinetics of GTPγS-induced PLC activation. Thus, halothane dose-dependently decreases the lag period for activation of PLC and increases the steady-state rate of [3H]InsP formation. The fact that halothane changes the time course of guanine nucleotide activation of PLC is not consistent with a mechanism whereby halothane affects the catalytic activity of PLC. These results are similar to those obtained with β-adrenergic and P_{2}Y-purinergic receptor agonists in turkey erythrocyte membranes. In addition, as with receptor agonists, halothane increases the sensitivity and maximal responsiveness of PLC to activation by GTPγS.

The effects of halothane on GTPγS-dependent PLC activity are compatible with a mechanism in which halothane stimulates PLC by enhancing the rate of guanine nucleotide exchange on the a-regulatory subunit of G_{a11}, as described previously for receptor agonists (22, 24). In our previous studies with turkey erythrocyte membranes, an important indicator of the dependence on guanine nucleotide exchange as a site for agonist-induced stimulation of PLC was the finding that agonists do not enhance PLC activity in the presence of AlF_{4}. However, in the present study we observed that halothane induced a dose-dependent activation of AlF_{4}-stimulated PLC with an EC_{50} value similar to that obtained for halothane stimulation of PLC in the presence of GTPγS. There are two possible explanations for the finding that halothane activates PLC similarly in the presence of either GTPγS or AlF_{4}. It could act at two distinct sites, one similar to the site affected by agonists (which apparently enhance the rate of guanine nucleotide exchange), and a second site at a step beyond guanine nucleotide binding which would increase the efficacy of the AlF_{4}-activated G-protein to stimulate PLC. Alternatively, halothane could interact at a single step in the PLC activation cascade which is common to both GTPγS- and AlF_{4}-stimulated PLC. In the latter case, potential sites of halothane action would include the dissociation of the heterotrimeric G-protein or to enhance the interaction between the activated G-protein and PLC. Some evidence to suggest that halothane does not act by exactly the same mechanism as agonist-activated receptors comes from our finding that PLC activation by halothane is entirely additive with the activation by maximal doses of either isoproterenol or ADPβS (after allowing for the inhibition occurring at low levels of halothane). By contrast, these agonists do not stimulate PLC in an additive manner when they are added in combination (24).

Recent studies have described an alternative mechanism to G-protein α-subunit regulation of PLC activity, in which free G-protein βγ-subunits can directly activate the enzyme (41, 42) in a manner that appears to be selective for the PLC-β2 isoform (43, 44). However, the observation that βγ-subunits are much less potent than G_{a11} for PLC activation in both turkey erythrocytes and bovine liver (41, 42) has led to the conclusion that receptor-mediated PLC activation is mediated by α-subunits in these preparations. The effects of halothane on the kinetics of PLC activation make it unlikely that it acts by enhancing the interaction between free βγ and PLC in turkey erythrocytes. Modulation of βγ-subunit association with PLC would not be expected to cause a GTPγS-dependent decrease in the lag period. A role for βγ-subunits in PLC activation is also unlikely in view of the fact that adenosine, which stimulates the dissociation of G_{a} into α and βγ-subunits in turkey erythrocyte membranes, has no effect on PLC activity in this preparation (25). It seems more likely that βγ stimulation of PLC may provide a mechanism whereby pertussis toxin-sensitive G-proteins can stimulate PLC (41-44).

Further evidence for a specific site of halothane action comes from the finding that other agents with similar effects on bulk lipid fluidity properties did not mimic the effects of halothane on PLC activity. Thus, concentrations of hexanol and benzyl alcohol which fluidized turkey erythrocyte membranes to a degree similar to halothane had no effect on GTPγS- or AlF_{4}-induced PLC activation. This suggests that the effects of halothane on G-protein-dependent PLC are not caused by effects on membrane fluidity (i.e. changes in bulk lipid order or accessibility of substrate). However, this does not exclude the possibility that halothane may interact more potently with a limited membrane domain or the lipid interfacial region of a specific signal transduction protein, with this site of halothane action being relatively insensitive to the other membrane-fluidizing agents. Regardless of the physicochemical nature of the site at which halothane modulates PLC activity, it is clear that the result is a specific modification in the signal transduction pathway either at the level of G-protein activation or the interaction of the G-protein with PLC. In view of the fact that halothane is most likely to act at a locus within the hydrophobic region of the membrane, it is tempting to suggest that it increases the probability or efficacy of G-protein/PLC interaction. The catalytic function of PLC itself does not appear to be affected by halothane.

An additional important finding of the present study was that halothane, at concentrations that are well within the anesthetic range (0.1-0.3 mM), significantly inhibited both isoproterenol- and ADPβS-induced [3H]InsP formation. The action of low concentrations of halothane to inhibit agonist-stimulated PLC activity most probably occurs at a distinct site from the stimulatory effect of halothane for a number of reasons. First, halothane appears to be more potent in inhibiting PLC activity at low halothane concentrations than at high concentrations, where the inhibition would obscure any further depression of the receptor-mediated effect. If this were the case, then the PLC stimulation induced by halothane could be the presence of agonists would actually represent a synergistic activation, greater than that observed with halothane in the absence of agonist.
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iting the effects of agonists, since this occurs at concentrations at which halothane does not stimulate GTPγS- or AlF₄⁻-activated PLC. Second, since halothane at these concentrations does not inhibit PLC activity in the presence of GTPγS or AlF₄⁻ (in the absence of agonist), the inhibitory site is presumably specific to the receptor-mediated pathway of PLC activation. One possibility would be that halothane interferes with agonist binding. This seems relatively unlikely in view of the fact that the inhibition by halothane occurs at supramaximal agonist doses. Furthermore, both the β-adrenergic and Pₐₐ-purinergic receptor responses were affected over a similar halothane concentration range. The most obvious site of halothane action would be to modify the interaction between the activated receptor and the G-protein. This could result in a reduced ability of the receptor to stimulate GDP dissociation. Alternatively, halothane might inhibit an additional component involved in the receptor stimulation of PLC activity, which is not shared by GTPγS or AlF₄⁻. Evidence for such an additional mode of receptor action has come from time course studies of the kinetics of PLC activation, which have shown that maximal PLC activity in the presence of GTPγS never reaches that attained in the presence of GTPγS plus receptor agonists, even after stable linear rates have been achieved under both conditions (22, 24, 29). At present the identity of this additional site of receptor action is unknown.

In conclusion, the data presented here demonstrate that halothane can both inhibit and stimulate PLC activity in turkey erythrocyte membranes in a manner that seems to be dependent on the expression of an activated G-protein. Previous studies have described inhibitory and stimulatory effects of halothane on ion channel activity, synaptic transmission, and [Ca²⁺], homeostasis. However, the underlying mechanism(s) of halothane action in these systems has remained ill defined. Our results demonstrate that halothane stimulates G-protein-dependent PLC activity and activates InsP₃ formation, and by inference diacylglycerol production, in intact erythrocytes. Both InsP₃-induced [Ca²⁺] release and diacylglycerol activation of PKC have been shown to be key regulators of cell function, including ion channel activity and [Ca²⁺], homeostasis (45, 46). Alterations in the formation of these second messengers could provide at least one mechanism whereby halothane could perturb membrane conductance. Thus, the ability of anesthetics to stimulate second messenger formation may provide a mechanism whereby these drugs can lead to a direct or indirect alteration of ion channel activity and cell excitability.

REFERENCES
1. Miller, K. W. (1985) Int. Rev. Neurobiol. 27, 1-61
2. Franks, N. P., and Lieb, W. R. (1987) Trends Pharmacol. Sci. 8, 169-174
3. Franks, N. P., and Lieb, W. R. (1982) Nature 300, 487-492
4. Nicoll, R. A., and Madison, D. V. (1982) Science 217, 1055-1057
5. Franks, N. P., and Lieb, W. R. (1986) Nature 323, 662-664
6. Franks, N. P., and Lieb, W. R. (1981) Science 214, 427-430
7. Nakahiro, M., Yeh, J. Z., Brunner, E., and Narahashi, T. (1989) FASEB J. 3, 1850-1854
8. Bean, B. P., Shragge, P., and Goldstein D. A. (1981) J. Gen. Physiol. 77, 233-253
9. Hayden, D. A., and Urban, B. W. (1985) J. Physiol. (Lond.) 341, 429-439
10. Pull, E., El Beheiry, H., and Baimbridge, K. G. (1990) J. Pharmacol. Exp. Ther. 250, 365-361
11. Bonekamp, Z. J., Supan, F. D., and Rusch, N. J. (1991) Anesthesiology 74, 340-346
12. Herrington, J., Stern, R., Evers, A. S., and Lingle, C. J. (1991) J. Neurosci. 11, 2226-2240
13. Klip, A., Hill, M., and Ramal, T. (1990) J. Pharmacol. Exp. Ther. 254, 352-359
14. Wheeler, D. M., Rice, T., Hansford, R. G., and Lakatta, E. G. (1988) Anesthesiology 69, 578-583
15. Iaizzo, P. A., Olsen, R. A., Seawald, M. J., Powis, G., Steir, A., and Van Dyke, R. A. (1990) Cell Calcium 11, 515-524
16. Danielli, J. D., and Harris, R. A. (1988) J. Pharmacol. Exp. Ther. 245, 1-7
17. Narayanan, T. K., Confer, R. D., Dennisson, R. L., Jr., Anthony, B. L., and Aronstam, R. S. (1988) Biochem. Pharmacol. 37, 1219-1223
18. Still, J. C., Scott, C. U., Eiskuri, S., Van Dyke, R., and Tarra, J. (1991) Mol. Pharmacol. 40, 1006-1013
19. Stern, R. C., Herrington, J., Lingle, C., and Evers, A. (1991) J. Neurosci. 11, 2217-2225
20. Harden, T. K., Stephena, L., Hawkins, P. T., and Downea, C. P. (1987) J. Biol. Chem. 262, 9057-9061
21. Harden, T. K., Hawkins, P. T., Stephens, L., Boyer, J. L., and Downea, C. P. (1988) Biochem. J. 253, 583-589
22. Boyer, J. L., Downea, C. P., and Harden, T. K. (1989) J. Biol. Chem. 264, 884-889
23. Boyer, J. L., Waldo, G. L., Evansa, T., Northupa, J. K., Downesa, C. P., and Harden, T. K. (1989) J. Biol. Chem. 264, 13917-13922
24. Roosey, T. A., Hager, R., and Thomas, A. P. (1991) J. Biol. Chem. 266, 15066-15074
25. Vezzi, C., and Dowessa, C. P. (1992) Biochem. J. 284, 917-922
26. Morris, A. J., Waldo, G. L., Downea, C. P., and Harden, T. K. (1990) J. Biol. Chem. 265, 13501-13507
27. Morris, A. J., Waldo, G. L., Downea, C. P., and Harden, T. K. (1990) J. Biol. Chem. 265, 13508-13514
28. Waldo, G. L., Boyer, J. L., Morris, A. J., and Harden, T. K. (1991) J. Biol. Chem. 266, 14217-14225
29. Roosey, T. A., Hager, R., Rubin, E., and Thomas, A. P. (1989) J. Biol. Chem. 264, 6817-6822
30. Stubbs, C. D., Tseng, W. M., Belin, J., Smith, A. D., and Johnson, S. M. (1980) Biochemistry 19, 2756-2762
31. Stubbs, C. D., Kinnella, K., Munkong, F. M., Quinn, P. J., and Ikegami, A. (1984) Biochem. Biophys. Acta 775, 374-386
32. Gratton, E., and Linkermann, M. (1988) Biophys. J. 44, 315-324
33. Lakowicz, J. R., and Haugland, R. P. (1985) Biophys. Chem. 21, 61-78
34. De Lenter, A., Munson, P. J., and Rodbard, D. (1978) Am. J. Physiol. 235, E97-E102
35. Beecher, J. M. (1990) Chem. Phys. Lipids 60, 237-252
36. Tolkovsky, A. M., and Levitzki, A. (1978) Biochemistry 17, 3796-3810
37. Schramm, M., and Rodbell, M. (1975) J. Biol. Chem. 250, 2232-2237
38. Seeman, P. (1970) Pharmacol. Rev. 24, 593-655
39. Stubbs, C. D. (1983) Essays Biochem. 19, 1-39
40. Taylor, S. J., Cane, H. Z., Rhee, S. G., and Goldstein D. A. (1991) Nature 350, 316-318
41. Boyer, J. L., Waldo, G. L., and Harden, T. K. (1992) J. Biol. Chem. 267, 52441-52446
42. Blank, J. L., Brattain, K. A., and Exton, J. H. (1992) J. Biol. Chem. 267, 23099-23075
43. Campa, M., Carboni, S., Schnabel, P., Scheer, A., Parker, P. J., and Jerschik, P. (1992) Nature 360, 684-686
44. Katz, A., Wu, D., and Simon, M. I. (1992) Nature 360, 686-689
45. Berdidge, M. J., and Irvine, R. F. (1988) Nature 334, 197-205
46. Shearman, M. S., Sekiguchi, K., and Nishizuka, Y. (1989) Pharmacol. Rev. 41, 211-237