Effects of Halothane and Propofol on Purified Brain Protein Kinase C Activation

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Background: Protein kinase C (PKC) has been implicated as a target for general anesthetic action in the central nervous system. Previous reports have described either stimulation or inhibition of PKC activity by general anesthetics. This study examines the effects of halothane and propofol on the activity of purified rat brain PKC under various assay conditions.

Methods: PKC was assayed in vitro using three previously characterized artificial substrates and three different lipid preparations in the absence or presence of halothane or propofol.

Results: Both halothane (50% effective concentration = 2.2 vol%) and propofol (50% effective concentration = 240 μM) markedly stimulated histone H1 phosphorylation by PKC in the presence of a lipid vesicle preparation consisting of phosphatidylcholine, phosphatidylserine, and diacylglycerol. Less marked or no stimulation of PKC by both anesthetics was observed in the presence of a phosphatidylserine/diacylglycerol dispersion or using protamine or polylysine, serine) as substrate. Neither anesthetic significantly stimulated PKC activity in the presence of phosphatidylserine/diacylglycerol/Triton X-100 mixed micelles. A mixture of histone H1, protamine or polylysine, serine) as substrate. Slight inhibition of PKC activity by halothane was observed under specific assay conditions with protamine as substrate. The activity of the catalytic fragment of PKC or of two lipid-independent second messenger-regulated protein kinases with conserved catalytic domains was not significantly affected by halothane.

Conclusions: Both halothane and propofol stimulated purified brain PKC activity in vitro assayed with physiologically relevant lipid bilayers in the absence or presence of Ca2+. This effect appears to be mediated through the lipid-binding regulatory domain of PKC. The potencies of halothane and propofol in stimulating PKC in vitro are consistent with submaximal activation of PKC at clinically effective anesthetic concentrations; the pharmacologic significance of this effect requires confirmation in an intact cellular system. (Key words: Anesthesia: theories. Anesthetics, intravenous: propofol. Anesthetics, volatile: halothane. Signal transduction: protein kinase C; protein phosphorylation.)

GENERAL anesthetics act primarily on synaptic transmission without affecting axonal conduction or neuronal excitability. The correlation between anesthetic potency and lipophilicity has implicated a hydrophobic site as the molecular locus for anesthetic action. Protein kinase C (PKC), an important molecule in the modulation of synaptic transmission, is activated by the lipid second messenger sn-1,2-diacylglycerol (DG) and by phospholipids through specific interactions with its regulatory domain and is therefore an attractive candidate as a target for the synaptic effects of general anesthetics.

Group A PKC subtypes α, β-1, β-2, and γ, which are abundant in mammalian brain, are activated physiologically by DG, a second messenger generated by phospholipase C activation in response to specific extracellular signals, and require phosphatidylserine (PS) and Ca2+ as cofactors. Activated PKC translocates to and possibly inserts into the cell membrane. Signal transduction occurs as active PKC phosphorylates specific substrate proteins, including many ion channels and receptors, and thereby alters substrate function and the physiologic function of the target cell. For example, phosphorylation by PKC has been implicated in the facilitation of neurotransmitter release, induction of long-term potentiation, regulation of intracellular Ca2+ levels, regulation of Ca2+ channels and K+ channels through changes in open probability, and regulation of neurotransmitter receptor desensitization.

A number of different lipid preparations and substrates have been used to analyze the activation of purified PKC in vitro. Initial studies of PKC used physically undefined dispersions of PS and DG as a source of lipids, whereas more recent studies have used stable

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PS/phosphatidylcholine (PC) bilayer vesicles or physically defined mixed micelles. Three distinct artificial substrate categories that differ in their cofactor requirements have been identified for PKC: prostamine represents a substrate that requires no cofactors, poly(lysine,serine 3:1) (PLS) represents a substrate that requires only phospholipid, and histone H1 represents a substrate that requires both Ca\(^{2+}\) and phospholipid, as do most natural substrates.

The results of previous studies on the effects of general anesthetics on PKC activity have been contradictory and inconclusive. We now report the results of a study of the effects of halothane and propofol on the activity of purified brain PKC performed by using three well-characterized assay conditions and substrates. Our results demonstrate that halothane and propofol affect PKC activity in vitro in a substrate- and cofactor-dependent manner, resulting primarily in augmentation of PS/DG-dependent enzyme activation.

Materials and Methods

Determination of Protein Kinase C Activity

PKC (consisting of the major brain isoforms \(\alpha, \beta I, \beta II\) and \(\gamma\)) was purified from rat forebrain and stored in liquid \(N_2\). Initial rate assays of PKC activity (rate limited by enzyme concentration) were performed at 30°C or 37°C (as indicated) in a reaction volume of 100 \(\mu l\) containing (final concentrations) 50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) (pH 7.4), 1 mM ethylene glycol bis (\(\beta\)-aminoethyl ether) N,N,N’,N’-tetraacetic acid (EGTA), 10 mM MgCl\(_2\), 1.5 mM CaCl\(_2\) (when indicated), 100 \(\mu\)M \([\gamma-\text{P}]\)adenosine triphosphate (\([\gamma-\text{P}]\)ATP) (100–250 cpmp/mol; DuPont–New England Nuclear, Boston, MA), 0.1–1 \(\mu\)g/ml PKC, 20 \(\mu\)g/ml bovine serum albumin (Baker, Phillipsburg, NJ, fraction V), and 0.1 mM dithiothreitol. These studies were carried out with three separate preparations of PKC, each prepared from 100 rat brains, with similar results. Either 0.2 mg/ml lysine-rich histone H1 (histone HL, Worthington Biochemical, Freehold, NJ), 0.2 mg/ml protamine sulfate (Sigma, St. Louis, MO) or 80 \(\mu\)g/ml poly(lysine, serine 3:1) (Sigma) was added as substrate without or with one of the following freshly prepared lipid preparations, chosen to conform with previous studies of PKC activation: (1) PS/DG, a dispersion of 0.5 mg/ml bovine brain \(\iota\)-a-PS (Avanti Polar Lipids, Alabaster, AL) and 40 \(\mu\)g/ml

\(sn-1,2\)-dioleoylglycerol (Avanti); (2) PC/PS/DG, bilayer vesicles consisting of 20 \(\mu\)M (20 mol%) bovine brain PS, 80 \(\mu\)M (80 mol%) egg \(\iota\)-o-PC and 2 \(\mu\)M \(sn-1,2\)-dioleoylglycerol; or (3) Triton X-100 (TX-100)/PS/DG, mixed micelles consisting of 0.3% (w/v) TX-100 (Baker), 344 \(\mu\)M (8 mol%) synthetic \(1,2\)-dioleoyl-sn-glycero-3-phosphoserine (Avanti) and 86 \(\mu\)M (2 mol%) \(sn-1,2\)-dioleoylglycerol.

Reactions in the presence of halothane (thymol-free, Halocarbon Products, North Augusta, SC) were carried out in 8.8-mI glass vials with Teflon–silicone elastomer (Silastic) septa through which liquid halothane and other reactants were injected directly; vapor phase halothane concentrations were determined by gas chromatography after equilibration, which occurs within 5 min. This technique allowed reproducible and stable halothane concentrations using a small reaction volume. Reactions in the presence of propofol were carried out in open vials containing 5% (vol/vol) ethanol (vehicle control necessary for propofol solubilization) with or without propofol (2,6-diisopropylphenol, Aldrich, Milwaukee, WI, or Zeneca Pharmaceuticals, Maclesfield, UK). Mixtures were equilibrated with halothane or propofol for 5 min, and reactions were initiated by the addition of ATP and terminated by the addition of 10 \(\mu\)l glacial acetic acid. An aliquot of the reaction mixture was then applied to phosphocellulose paper (P81, Whatman, Maidstone, UK), and the papers were washed with three changes of 0.5% (vol/vol) \(H_2PO_4\). Phosphorylation was quantified by measuring retained Cerenkov radiation; values were corrected for a blank assay performed without substrate.

Each independent experiment consisted of a complete set of duplicate assays of each reaction condition for each substrate and was performed as a group with and without anesthetic using freshly thawed PKC. A single preparation of PKC was used for all experiments involving each substrate to reduce variation in specific activity due to PKC instability. Differences between mean values with or without anesthetic were analyzed by the two-tailed \(t\) test (SAS System, release 6.03, SAS Institute). For concentration–effect determinations, PKC was assayed with varying concentrations of halothane or propofol using histone H1 as substrate in the presence of PC/PS/DG vesicles plus Ca\(^{2+}\). Data were analyzed using a graded dose–response program (PHARM/PCS Pharmacologic Calculation System, Version 4.2).
Miscellaneous Methods

Purified brain PKC was assayed with arachidonic acid (BIOXOM, Research Laboratories, Plymouth Meeting, PA) or oleic acid (Sigma) in aqueous solution as the lipid addition using histone H1 as substrate in the presence of Ca\(^{2+}\). The activity of purified PKC using phorbol ester instead of DG was determined using a 100 \(\mu\)M lipid vesicle preparation consisting of 80 mol% PC, 20 mol% PS with or without 10 nm phorbol 12,13-dibutyrate (Sigma) using histone H1 as substrate in the presence of Ca\(^{2+}\). The reactions were carried out and the data were analyzed as described above.

The catalytic fragment of PKC was prepared by a modification of published methods.\(^{25,26}\) PKC (30 \(\mu\)g) was incubated at 30\(^\circ\)C for 15 min in 0.4 ml of a solution containing 25 mM HEPES (pH 7.4), 0.5 mM EGTA, 5 mM MgCl\(_2\) and 100 \(\mu\)g/ml ethyl chloromethyl ketone-trypsin (Worthington). The reaction mixture was then chilled and terminated by the addition of (final concentrations) 0.1 mg/ml soybean trypsin inhibitor (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Calbiochem, La Jolla, CA). PKC activity was determined using 5 \(\mu\)g/ml trypsinized PKC and histone H1 as substrate as described above except that lipids and Ca\(^{2+}\) were omitted.

Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) was purified from rat brain\(^{27}\) and its activity was determined using 10 \(\mu\)M bovine synapsin 1-Tyr\(^{586}\) (586–609) as substrate in a final reaction volume of 100 \(\mu\)l containing 5 \(\mu\)g/ml CaMKII, 50 mM HEPES (pH 7.4), 10 mM MgCl\(_2\), 1 mM EGTA, 1.5 mM CaCl\(_2\), 30 \(\mu\)g/ml bovine brain calmodulin, 30 \(\mu\)g/ml bovine serum albumin and 100 \(\mu\)M \(\gamma\)-32P]ATP as described.\(^{11}\) The catalytic subunit of cyclic adenosine monophosphate–dependent protein kinase (PKA) was purified from bovine heart\(^{28}\) and its activity determined with 0.2 mg/ml histone H2B (Worthington) as a substrate and 0.5 \(\mu\)g/ml PKA.\(^{29}\) The reactions in the presence of anesthetics were carried out as described above for PKC.

Protein concentrations were determined by the bicinchoninic acid method\(^{30}\) using bovine serum albumin as a standard.

These studies were approved by the Cornell University Medical College Institutional Animal Care and Use Committee.

Results

Characterization of the Protein Kinase C Assay

Phosphorylation of histone H1 by PKC using a lipid vesicle preparation of PC/PS/DG\(^{14}\) showed a time- and temperature-dependent decay in reaction rate due to the extreme thermolability of this enzyme\(^31\) (fig. 1). At 37\(^\circ\)C (fig. 1A), loss of enzyme activity was evident after a reaction time of 2 min as the reaction rate became nonlinear. A reaction time of 5 min at 30\(^\circ\)C was chosen for subsequent experiments to minimize enzyme inactivation during the course of the assay and to allow an adequate number of assays to be performed in each independent experiment. Under these conditions, histone phosphorylation was linear versus time with a standard deviation of ±8% (fig. 1B).
Effects of Halothane on Protein Kinase C Activity

The effects of halothane on the activity of purified brain PKC in the absence of cofactors or using three standard assay conditions, each characterized by a different lipid preparation, are shown in Table 1. In the absence of lipids or Ca\(^{2+}\), halothane (2.4 vol% at 30°C; equivalent to approximately 3.1 vol% at 37°C) had no significant effect on the low basal rate of phosphorylation of histone H1 or PIS by PKC, but produced a small but significant inhibition of the high basal rate of phosphorylation of proteamine by PKC. This inhibitory effect was also evident at a higher proteamine concentration (0.4 mg/ml; data not shown), and thus did not appear to result from decreased substrate affinity. The addition of Ca\(^{2+}\) alone had no significant effects on PKC activation in the absence of lipid cofactors (data not shown).

When assayed with a dispersion of PS/DG, the rate of phosphorylation of histone H1 by PKC was much greater than in the absence of lipid cofactors. Halothane further stimulated histone H1 phosphorylation in the absence or presence of Ca\(^{2+}\). The phosphorylation of proteamine was inhibited slightly, whereas PIS phosphorylation was not affected by halothane.

The rate of phosphorylation of histone H1 by PKC assayed with PC/PS/DG vesicles was much greater in the presence of Ca\(^{2+}\) than in the absence of Ca\(^{2+}\) or of lipid cofactors. Histone H1 phosphorylation by PKC was markedly stimulated by halothane in the presence of Ca\(^{2+}\) using PC/PS/DG vesicles. Phosphorylation of proteamine or PIS by PKC was stimulated by halothane in the absence but not the presence of Ca\(^{2+}\).

A mixed micelle preparation of TX-100 containing PS and DG (TX-100/PS/DG) provides another physically defined lipid preparation that has been used to study PKC–lipid interactions.\(^{15}\) Phosphorylation of histone H1 by PKC showed the greatest activity and Ca\(^{2+}\) dependence using this assay method. Halothane stimulated the low rate of histone H1 phosphorylation in the absence of Ca\(^{2+}\) and the high rate of histone H1 phosphorylation in the presence of Ca\(^{2+}\), although these effects did not achieve statistical significance. Halothane had no effect on proteamine or PIS phosphorylation by PKC. The less marked stimulation of PKC activity by halothane using mixed micelles compared with PS/DG dispersions or PC/PS/DG vesicles appears to be due to differences in the relative lipid cofactor concentrations in the different assay conditions (vide infra).

A concentration–effect analysis of the potentiation of PKC activity by halothane was determined using PC/PS/DG vesicles plus Ca\(^{2+}\) with histone H1 as substrate, conditions that produced maximal halothane-stimulated PKC activation, by a modification of the standard assay that allowed the use of lower halothane concentrations. The lowest concentration of halothane that

Table 1. Effects of Halothane on Protein Kinase C Activity

<table>
<thead>
<tr>
<th>Lipid Cofactors</th>
<th>Ca(^{2+})</th>
<th>Halothane</th>
<th>Ratio†</th>
<th>Protamine</th>
<th>Poly (Lys, Ser)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PKC Activity* (pmol/min)</td>
<td>PKC Activity (pmol/min)</td>
<td>PKC Activity* (pmol/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.69 ± 0.12</td>
<td>1.92 ± 0.62</td>
<td>114</td>
<td>63.2 ± 7.8</td>
<td>52.5 ± 10†</td>
</tr>
<tr>
<td>PS/DG dispersion</td>
<td>20.4 ± 4.4</td>
<td>26.7 ± 4.8**</td>
<td>131</td>
<td>130 ± 9.8</td>
<td>140 ± 21</td>
</tr>
<tr>
<td></td>
<td>32.2 ± 3.0</td>
<td>46.0 ± 5.6**</td>
<td>143</td>
<td>92.2 ± 6.5</td>
<td>82.0 ± 12†</td>
</tr>
<tr>
<td>PC/PS/DG vesicles§</td>
<td>3.90 ± 0.48</td>
<td>4.88 ± 0.54**</td>
<td>125</td>
<td>74.0 ± 6.8</td>
<td>101 ± 15†</td>
</tr>
<tr>
<td></td>
<td>16.7 ± 2.6</td>
<td>35.2 ± 10.4**</td>
<td>211</td>
<td>91.5 ± 10</td>
<td>92.8 ± 23</td>
</tr>
<tr>
<td>PS/DG Triton X-100</td>
<td>5.55 ± 0.89</td>
<td>7.39 ± 1.76</td>
<td>133</td>
<td>173 ± 22</td>
<td>182 ± 25</td>
</tr>
<tr>
<td>mixed micelles†</td>
<td>51.6 ± 7.3</td>
<td>57.0 ± 4.8</td>
<td>110</td>
<td>198 ± 22</td>
<td>160 ± 14</td>
</tr>
</tbody>
</table>

* PKC activity was assayed at 30°C using 0.25 µg/ml [for histone H1 and poly(Lys, Ser)] or 0.05 µg/ml (for proteamine) purified PKC. Values for proteamine assays have been normalized to an enzyme concentration of 0.25 µg/ml to allow direct comparison to other substrates. Data are mean ± SD; n = 5. Each experiment included duplicate assays of each experimental condition in the absence or presence of 2.4 vol% halothane.
† Values are expressed as percent PKC activity in the presence of halothane normalized to activity in the absence of anesthetic.
‡ Assayed by the method of Takai et al.\(^{16}\)
§ Assayed by the method of Boni and Rando.\(^{13}\)
† Assayed by the method of Hannum et al.\(^{14}\)

Differences between means were analyzed by the two-tailed t test: **P < 0.02; ††P < 0.05.

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could be reproducibly achieved with the standard assay system (used to obtain the data in table 1) was 2.4 vol%. To analyze lower concentrations of halothane, experiments were performed with halothane diluted in ethanol, which alone had no significant effect on PKC activity at the concentration used (3% (vol/vol) ethanol; data not shown and vide infra). The 50% effective concentration (EC50) for activation of PKC by halothane (range of halothane concentrations tested: 0.6–4.8 vol%) was 2.2 ± 0.2 vol% at 30°C (n = 4; correlation coefficients of log concentration–effect curves > 0.98).

**Effects of Propofol on Protein Kinase C Activity**

Propofol, an intravenous anesthetic chemically unrelated to the volatile agents, also produced significant stimulation of PKC activity under several conditions (table 2). Ethanol was chosen as a vehicle for solubilization of this extremely hydrophobic compound based on a previous study that found no significant effects of high concentrations of ethanol on purified PKC activity. In control assays, 5% (vol/vol) ethanol alone had relatively minor substrate-dependent effects on PKC activity assayed with PC/PS/DG vesicles and Ca2+: no significant effect on histone H1 phosphorylation (0.99 ± 0.17 μmol/min/mg without versus 0.90 ± 0.19 μmol/min/mg with ethanol, P > 0.05, n = 5); slight stimulation of protamine phosphorylation (3.15 ± 0.33 μmol/min/mg without versus 3.82 ± 0.38 μmol/min/mg with ethanol, P < 0.05, n = 3); and slight inhibition of PLS phosphorylation (0.52 ± 0.04 μmol/min/mg without versus 0.43 ± 0.18 μmol/min/mg with ethanol, P < 0.01, n = 3).

In the absence of lipid activators, propofol inhibited the low rate of basal histone H1 phosphorylation, but this effect did not achieve statistical significance. In contrast, stimulation of protamine (not statistically significant) and of PLS phosphorylation was observed in the absence of cofactors. The addition of Ca2+ alone had no significant effects on PKC activation in the absence of lipid cofactors (data not shown).

Modest stimulation of PKC activity was observed with all three substrates using the PS/DG dispersion; however, only the effect on histone H1 phosphorylation in the absence of Ca2+ was statistically significant.

Marked stimulation by propofol of both histone H1, protamine (in the absence of Ca2+) and PLS (in the absence of Ca2+) phosphorylation by PKC was observed using PC/PS/DG vesicles.

Propofol had small but statistically significant inhibitory effects on histone H1 and PLS phosphorylation by PKC assayed using TX-100/PS/DG mixed micelles.

A concentration–effect analysis of the potentiation of PKC activity by propofol was determined using PC/PS/

<table>
<thead>
<tr>
<th>Lipid Cofactors</th>
<th>Ca2+</th>
<th>Propofol</th>
<th>Ratio† (%)</th>
<th>Propofol</th>
<th>Ratio† (%)</th>
<th>Propofol</th>
<th>Ratio† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>3.40 ± 0.74</td>
<td>2.80 ± 0.80</td>
<td>82.4</td>
<td>66.1 ± 6.7</td>
<td>92.6 ± 29.5</td>
<td>140</td>
</tr>
<tr>
<td>PS/DG dispersion</td>
<td>–</td>
<td>31.1 ± 2.5</td>
<td>37.4 ± 5.1**</td>
<td>120</td>
<td>190 ± 26</td>
<td>216 ± 42</td>
<td>114</td>
</tr>
<tr>
<td>PC/PS/DG vesicles</td>
<td>–</td>
<td>32.2 ± 5.6</td>
<td>43.7 ± 4.1**</td>
<td>138</td>
<td>128 ± 23</td>
<td>174 ± 44</td>
<td>128</td>
</tr>
<tr>
<td>PS/DG/Triton X-100 &amp; mixed micelles§</td>
<td>–</td>
<td>42.8 ± 7.6</td>
<td>49.3 ± 6.8**</td>
<td>160</td>
<td>120 ± 29</td>
<td>190 ± 14**</td>
<td>158</td>
</tr>
</tbody>
</table>

*PKC activity was assayed at 30°C using 0.25 μg/ml [for histone H1 and poly(Lys, Ser)] or 0.05 μg/ml (for protamine) purified PKC. Values for protamine assays have been normalized to an enzyme concentration of 0.25 μg/ml to allow direct comparison to other substrates. Data shown are mean ± SD; n = 5. Each experiment included duplicate assays of each experimental condition in the absence or presence of 200 μM propofol.

† Values are expressed as percent PKC activity in the presence of propofol normalized to activity in the absence of anesthetic.

‡ Assayed by the method of Takai et al.12
§ Assayed by the method of Boni and Randone.13
¶ Assayed by the method of Hamun et al.14

Differences between means were analyzed by the two-tailed t test: **P < 0.02; ††P < 0.05.
Table 3. Effects of Halothane and Propofol on Protein Kinase C Activation by Phorbol Ester and cis Unsaturated Fatty Acids

<table>
<thead>
<tr>
<th>Lipid Cofactor</th>
<th>PKC Activity* (pmol/min)</th>
<th>PKC Activity* (pmol/min)</th>
<th>Ratio† (%)</th>
<th>PKC Activity* (pmol/min)</th>
<th>PKC Activity* (pmol/min)</th>
<th>Ratio† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Halothane</td>
<td>Protopof</td>
<td></td>
<td>Halothane</td>
<td>Protopof</td>
<td></td>
</tr>
<tr>
<td>PC/PS/DG vesicles†</td>
<td>24.0 ± 4.5</td>
<td>52.8 ± 5.4†</td>
<td>220</td>
<td>30.4 ± 3.8†</td>
<td>60.4 ± 7.8†</td>
<td>198</td>
</tr>
<tr>
<td>Phorbol 12,13-dibutyrate§ (10 nm)</td>
<td>25.2 ± 2.0</td>
<td>43.3 ± 2.4†</td>
<td>172</td>
<td>28.9 ± 2.9</td>
<td>44.9 ± 6.2†</td>
<td>155</td>
</tr>
<tr>
<td>Arachidonic acid§ (100 μM)</td>
<td>21.8 ± 2.2</td>
<td>23.1 ± 1.2†</td>
<td>106</td>
<td>21.8 ± 1.9</td>
<td>18.0 ± 1.0</td>
<td>82.6</td>
</tr>
<tr>
<td>Oleic acid§ (100 μM)</td>
<td>36.9 ± 1.2</td>
<td>37.8 ± 2.0</td>
<td>102</td>
<td>36.1 ± 2.4</td>
<td>33.3 ± 1.5</td>
<td>92.2</td>
</tr>
</tbody>
</table>

* PKC activity was assayed at 30°C using 0.25 μg/ml purified PKC and 0.2 mg/ml histone H1 in the presence of Ca²⁺. Data are mean ± SD; n = 3. Each experiment included duplicate assays of each experimental condition in the absence or presence of 2.4 μM halothane or 200 μM propofol as indicated.
† Values are expressed as percent PKC activity in the presence of anesthetic normalized to activity in the absence of anesthetic.
‡ Assayed by the method of Boni and Rando.¹⁰
§ Assayed as described in materials and methods.
Differences between means were analyzed by the two-tailed t test: *P < 0.02.

DG vesicles plus Ca²⁺ with histone H1 as substrate. The EC₅₀ for activation of PKC by propofol (range of concentrations tested: 25–800 μM) was 240 ± 40 μM at 30°C (n = 3; correlation coefficients for log concentration–effect curves > 0.98).

**Activation of Protein Kinase C by Phorbol Ester or cis-unsaturated Fatty Acids**

Phorbol esters are potent tumor promoters that interact with PKC at the same site as DG,¹⁶ and that appear to activate the enzyme by a similar mechanism.³ The activation of PKC by halothane or propofol in the presence of PC/PS/DG occurred to a similar extent when phorbol 12,13-dibutyrate, a potent pharmacologic activator of PKC, replaced DG, the endogenous activator of PKC, in the enzyme assay (table 3).

The cis-unsaturated fatty acids arachidonic acid and oleic acid activate PKC by a mechanism that is kinetically distinct from that of PS and DG.³⁵ Halothane or propofol did not stimulate the activation of PKC by either 100 μM arachidonic acid or 100 μM oleic acid.

**Effect of Halothane on the Activity of Trypsinized Protein Kinase C and of Other Protein Kinases**

PKC consists of an amino-terminal regulatory domain, that contains the binding sites for phospholipid, DG, phorbol ester and Ca²⁺, and a carboxy-terminal catalytic domain, that contains the ATP and substrate binding sites.⁵ Limited proteolysis with trypsin of intact PKC produces a catalytically active fragment (50 kDa) and a lipid binding regulatory fragment (32 kDa).⁵ Halothane had no significant effect on histone H1 phosphorylation by the cofactor-independent catalytic fragment of PKC. Likewise, halothane had no significant effect on the activity of CaMKII or of the catalytic subunit of PKA (table 4).

**Discussion**

Previous studies of the effects of general anesthetics on PKC activity have reported either stimulation or inhibition.¹⁷⁻²¹ The results of this study suggest that these variable effects may have resulted from the use of different assay conditions. For example, a preliminary study of the effect of extremely high concentrations of halothane (20 mm) on crude brain PKC activity¹⁷ found a stimulatory effect using the PC/PS/DG lipid bilayer assay method.¹⁰ In contrast, a recent study¹⁸ described

Table 4. Effects of Halothane on the Activity of Trypsinized Protein Kinase C and of Other Protein Kinases

<table>
<thead>
<tr>
<th>Protein Kinase</th>
<th>Halothane</th>
<th>Ratio† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Kinase</td>
<td>Halothane</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Trypsinized PKC</td>
<td>7.8 ± 4.9</td>
<td>7.5 ± 2.4</td>
</tr>
<tr>
<td>Ca²⁺/calmodulin-dependent protein</td>
<td>78.4 ± 4.9</td>
<td>71.4 ± 3.1</td>
</tr>
<tr>
<td>kinase II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclic AMP-dependent protein kinase</td>
<td>81.3 ± 8.0</td>
<td>80.8 ± 1.8</td>
</tr>
</tbody>
</table>

* Assayed at 30°C as described in materials and methods. Data are mean ± SD; n = 3. Each experiment included duplicate assays in the absence or presence of 2.4 μM halothane as indicated. No significant differences between means were detected by the two-tailed t test (P > 0.05).
† Values are expressed as percent protein kinase activity in the presence of halothane normalized to activity in the absence of anesthetic.

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inhibition of brain PKC by halothane and various alkanols, including ethanol, when assayed in the absence of lipids with a mixture of histone and protamine. This inhibitory effect of halothane (ref. 18 and table 1) may be related to the anomalous mechanism of activation of PKC by protamine in vitro that involves enzyme-substrate aggregation.10 Another study found no significant effect of ethanol on partially purified brain PKC activity using an artificial mixed micelle assay with histone as substrate.19 We also failed to observe marked effects of halothane (vide infra) or ethanol (data not shown) on PKC activity with the mixed micelle assay. These divergent results obtained with various artificial assay conditions emphasize the importance of assaying PKC in the presence of its natural lipid cofactors and with various substrates when analyzing anesthetic effects in vitro.

Our data demonstrate that halothane and propofol enhance the activation of purified brain PKC in vitro using a PS/DG dispersion or PC/PS/DG vesicles as lipid cofactors. Although the PS/DG dispersion has been widely used in the study of PKC, PS does not exist as a stable lipid bilayer in the presence of high cation concentrations.14 Vesicles consisting of a 1:4 molar ratio of PS:PC form physiologically relevant bilayers and support Cα2+- and DG-dependent PKC activity without aggregation in the presence of high cation concentrations.14 Thus, vesicles formed from PC/PS/DG more closely approximate in vitro the biologic conditions for PKC activation in providing a physiologically relevant bilayer16 composed of PS and PC as well as containing the endogenous second messenger DG. Using PC/PS/DG bilayers as lipid activators of PKC, we observed consistent stimulation of PKC activity by both halothane and propofol with histone H1, protamine, or PLS as substrate. The greatest stimulation of PKC activity by either anesthetic was observed with this lipid preparation and histone H1.

Under certain conditions, modest effects of halothane or propofol on PKC activity were observed that were not statistically significant. The lack of statistical significance in these cases is likely due primarily to the inherent variability in absolute PKC activities between independent experiments; this, however, does not alter the principal conclusions of this study.

The ability of halothane or propofol to stimulate PKC activity depended on both the substrate and the lipid preparation used in the assay. The lipid dependency of the anesthetic effect may be related in part to the relative lipid concentrations in the different assays. For example, the TX-100/PS/DG mixed micelle assay uses 8 mol% PS and 2 mol% DG, which maximally activate PKC (ref. 14 and data not shown). In contrast, the PS/DG dispersion and PC/PS/DG vesicle assay methods contain submaximal concentrations of PS and DG with regard to PKC activation, though they are closer to physiologic levels. Thus, both halothane and propofol activated PKC only under conditions of subsaturating amounts of PS and DG, possibly because of an effect on the sensitivity of PKC to these cofactors, whereas they had no effect or inhibited PKC under conditions of saturating and supraphysiologic amounts of PS and DG.

Assays of PKC activity were carried out at 30°C because of the extreme thermolability of this enzyme in its purified form (ref. 31 and fig. 1). To compare the effective anesthetic concentrations at 30°C in vitro with the EC50 (MAC) of halothane at 37°C in vivo (0.88–1.24 vol% in rats),21–28 the MAC at 30°C can be approximated from the ratio of the partial pressures of halothane at 37°C and 30°C29 to be 23% less than the MAC at 37°C, or 0.68–0.95 vol%. Extrapolation of direct measurements of the effects of hypothermia on halothane MACin the rat30 predict a slightly lower MAC for halothane of 0.58–0.77 vol% at 30°C. The EC50 for activation of PKC by halothane using the PC/PS/DG assay was 2.2 vol% at 30°C (equivalent to 2.9 vol% at 37°C), or threefold higher than the calculated MAC. This potency is comparable to that observed for effects of halothane on other potential anesthetic targets in vitro, such as inhibition of the high voltage-activated Cα2+ current in GH3 pituitary cells (concentration for 50% inhibition [IC50] 0.85 mM or 3.4 vol% at 37°C).31 The EC50 for activation of PKC by propofol at 30°C using the PC/PS/DG assay was 240 µmol, or fivefold higher than the EC50 of propofol at 37°C in humans of 45 µmol; similar data on the EC50 of propofol in the rat or on the effects of hypothermia on its EC50 are not available.

Although it was not determined directly, it is possible to estimate from the concentration-effect curve the degree of PKC activation produced by a clinically effective concentration of halothane at 37°C. A concentration of 0.8 vol% halothane (~1 MAC in the rat at 30°C) would produce a 15% increase in PKC activation, whereas 45 µmol propofol (the EC50 in humans at 37°C) would produce a 12% increase in PKC activation. The pharmacologic significance of these modest degrees of PKC activation will require studies of anesthetic effects on PKC activation in intact cells. Sub-

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maximal stimulation of PKC activity *in vivo* could have functional significance as a result of signal amplification by PKC, an early component in the phospholipase C-linked signal transduction cascade. This possibility can be examined by analyzing the effects of anesthetics on the phosphorylation of specific endogenous substrates for PKC, the downstream effectors of PKC activation, in an intact system.

Results from two previous studies are consistent with activation of PKC by general anesthetics in intact cells. Volatile anesthetics potentiated phorbol ester-evoked neurotransmitter release from PC12 cells, although a direct effect on PKC activity was not ruled out. Halothane was also found to stimulate phorbol ester-induced superoxide generation and protein phosphorylation in neutrophils through activation of PKC. Additional experiments will be necessary to identify specifically a direct versus an indirect effect of anesthetics on PKC activation in neuronal tissue, because multiple mechanisms are involved in the cellular regulation of PKC activity.

The augmentation by halothane of PKC activity occurs with intact PKC but not with its catalytic fragment. This result suggests that the effect is mediated through the lipid-binding regulatory domain of PKC, and not through the catalytic domain or at the substrate level. Additional evidence that the stimulatory effect of halothane on PKC activity is mediated through an interaction with the regulatory domain is provided by the absence of a effect of halothane on PKA or CaMKII, both of which share similar amino acid sequences with PKC in their catalytic domains, but which have unique regulatory domains that do not interact with lipids. Furthermore, the stimulatory effect of general anesthetics on PKC activity does not occur with *cis*-unsaturated fatty acids, and is therefore specific for activation by PS and DG or phorbol ester. This result is consistent with previous evidence for distinct mechanisms of activation of PKC by PS/DG and phorbol ester versus *cis*-unsaturated fatty acids. Taken together, these data support a stimulatory effect of halothane on PKC activation mediated through a specific interaction with the PS and/or DG binding sites on its unique regulatory domain, rather than a direct effect on the active site or a substrate-mediated effect. Identification and mutational analysis of the PKC domain involved in binding PS and DG will allow a more detailed analysis of the interaction between general anesthetics and PKC, and could provide a useful model for analyzing the interactions of general anesthetics with a functional lipid binding site on a key signal transduction enzyme.

In conclusion, our results demonstrate the ability of two distinct classes of general anesthetics to stimulate the activity of PKC with three different artificial substrates *in vitro*. This finding implicates a direct effect of general anesthetics on PKC activation as a potential target for their effects on synaptic transmission. Differences in the results of previous studies may be explained in part by the use of different assay conditions. Further experiments carried out with more intact cellular preparations will be required to determine the pharmacologic significance of this effect and to determine the relative importance of direct effects of anesthetics on PKC activation as opposed to potential indirect effects proximal to (e.g., due to changes in intracellular Ca²⁺ concentration or in phospholipase C or phospholipase A₂ activity) or distal to (e.g., at the level of substrate phosphorylation or dephosphorylation) PKC activation in the PKC signal transduction cascade.

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