Lipid-dependent Activation of Protein Kinase C-α by Normal Alcohols*

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Significant stimulation of protein kinase C-α (PKCα) by n-alcohols was observed in characterized lipid systems composed of phosphatidylcholine/phosphatidylserine/dioleoylglycerol (PC/PS/DO). The logarithm of the alcohol concentrations to achieve half-maximal PKC stimulation (ED$_{50}$) and of the maximal PKC stimulation by alcohols were both linear functions of alcohol chain length, consistent with the Meyer-Overton effect. Binding of phorbol esters to PKC was not significantly affected by octanol. Octanol increased, up to 4-fold, the affinity of PKC binding to the lipid bilayers in both the absence and presence of DO. However, octanol increased PKC activity much more significantly than it enhanced binding of the enzyme to the lipid bilayers, suggesting that the stimulation of PKC is not merely a reflection of the increase in PKC bilayer binding affinity. $^{31}$P NMR experiments did not reveal formation of non-lamellar phases with octanol. Differential scanning calorimetry suggested that alcohols, like diacylglycerol, induce formation of compositionally distinct domains and the maximal enzyme activity with alcohol resided roughly in the putative domain-coexistence region. These results suggest that alcohols are mimicking diacylglycerol in activating PKC, not by binding to the high-affinity phorbol ester binding site, but by altering lipid structure and by enhancing PKC-bilayer binding.

Despite extensive research in the past century, the mechanism of anesthesia is not well understood and the site(s) of action of anesthetics is still open to question (1, 2). A common feature of anesthetic action is the modulation of electrical signaling which is accomplished by altering membrane conductance through ion channels or ion channel-linked receptors (3). Two general hypotheses are proposed to account for the anesthetic actions on these membrane-associated protein systems (1, 3, 4). The protein binding hypothesis argues that anesthetics bind directly to hydrophobic regions of specific protein receptors. The membrane perturbation hypothesis argues that anesthetics alter physical properties of the membrane that are necessary for the normal operation of various membrane-associated proteins.

Protein kinase C (PKC) phosphorylates and regulates many of the membrane proteins that have been implicated in pathways affected by anesthetics (5–8), and both general and local anesthetics have been shown to modulate PKC activity (9–12). Moreover, PKC requires amphipathic molecules like diacylglycerol (DAG) and phosphatidylserine (PS) as cofactors that may activate PKC by both direct binding and by altering the physical properties of the membrane. Many anesthetics are amphipathic molecules which have been proposed to bind to PKC at certain sites (10) as well as to perturb the lipids in the bilayer (3). Thus PKC is a potential target of anesthetic action and a model protein for testing both the protein binding hypothesis and the membrane perturbation hypothesis.

PKC is a family of membrane-associated, serine/threonine kinases present in all tissues and especially abundant in the central nervous system (13). The PKC family consists of at least 11 isoforms which require negatively charged phospholipids (among which PS is much more effective than others), and for some of them, DAG and Ca$^{2+}$ to achieve full activation (14). Phorbol esters can replace the DAG requirement (15). In addition, the enzymes can be activated in a lipid-independent manner by protamine sulfate. While the role of cofactors in the PKC activation process is not clearly understood, a variety of studies has argued that physical properties of the lipid bilayers are important for the activation mechanism (16). The physical properties of lipid bilayers that are most important in PKC activation have not been elucidated. Those that have been suggested include lateral heterogeneity or domain formation (17–19), head group spacing (20, 21), lipid bilayer curvature (22), and tendency of the bilayer lipid to form non-bilayer phases (22, 23). The surface potential of lipid bilayers also can influence activation by, for example, sequestering Ca$^{2+}$ on the membrane surface (24, 25). Since anesthetics can alter many of these membrane properties (12, 26–28), it is our working hypothesis that anesthetics affect PKC activity, at least in part, by their effects on membrane physical properties, and that the anesthetic-modulated enzyme directly or indirectly regulates the ion channels involved in anesthesia.

Effects of anesthetics on PKC activation have been noted since the early 1980s (29), but the mechanism of and even the direction of the effects have remained elusive. Lester and Baumann (30) demonstrated stimulation of PKC by alcohols in the presence of phosphatidylcholine/phosphatidylserine (PC/PS) vesicles (30). Hemmings and Adamo (31) noted that varying...
anesthetic effects could be obtained in different lipid systems. Slater et al. (32) showed that interaction of n-alcohols and general anesthetics with PKCs results in dramatically different effects on protamine sulfate-activated enzyme activity versus lipid activated activity. Furthermore, the effects of the n-alcohols on lipid-associated PKC activity differ markedly depending on whether the activity is induced by diacylglycerol or phorbol ester and are dependent upon n-alcohol chain length (10, 32).

We have examined the effects of several alcohol anesthetics on PKC activity and membrane binding in characterized lipid systems that support PKC activity and mimic many features of the cellular membrane. The saturated lipids DMPC and DMPS were used in this study so that the phase behavior of these defined lipid binary and ternary systems at various alcohol concentrations could be studied by differential scanning calorimetry (DSC). More physiological unsaturated lipid systems also were examined and similar effects were observed. The modulation of PKC activity by alcohols appears to be associated with alcohol effects on lipid structure, possibly via the induction of lateral heterogeneity or domain formation.

**EXPERIMENTAL PROCEDURES**

**Materials**—1,2-Dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphatidylserine (DMPS), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylserine (DPPS), 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine (POPS), 1,2-dioleoyl-sn-glycerol (DO), and dansyl-phosphatidyl ethanolamine (dansyl-PE) were from Avanti Polar Lipids (Birmingham, AL). All of the lipids were greater than 99% pure as determined by thin-layer chromatography on Adsorbosil-Plus plates from Alltech Associates, Inc. (Deerfield, IL) using the solvents described previously (19). MOPS, calcium chloride, potassium chloride, and EGTA were Chemika grade and the magnesium chloride was puriss grade from Fluka Chemical Corp. (Ronkonkoma, NY). [γ-32P]ATP (7000 Ci/mmol) was from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). [20-3H]phorbol 12,13-dibutyrate (10–20 Ci/mmol) was from NEN Life Science Products Inc. (Boston, MA). Lysine-rich histone (type III-s), ATP, and phorbol esters were from Sigma. Octanol, heptanol, hexanol, pentanol, and butanol were also from Sigma. Chloroform, methanol, and benzene were high performance liquid chromatography grade from Fisher Scientific Co. (Pittsburgh, PA). Grace’s culture medium, yeastolate, lactalbumin, and fetal calf serum for culture of S9 insect cells (Life Technologies, Inc., Grand Island, NY).

**Purification of Protein Kinase C—**S9 insect cells (~2 × 109 cells/ml in spinner culture) were infected with a PKCo baculovirus expression construct kindly provided by Drs. P. Parker, S. Stabel, and D. Fabbro. Infected cells were harvested when the viability dropped to approximately 85%. PKCo was purified from the cytosol by sequential chromatography on Q-Sepharose and phenyl-Sepharose columns, both from Amersham Pharmacia Biotech. Enzyme concentration was determined by assay of phorbol ester binding as described previously (33) and enzyme purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining and Western blotting using PKCo-specific rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The purified PKCo was then stored in 30% glycerol and 20 mM MOPS at −75 °C. Enzyme was thawed and diluted with MOPS/KCl buffer just prior to the kinase assays. The enzyme remained 90% intact after incubation for 1 h at 35 °C as described previously (19).

**Preparation of Lipid Vesicles**—The concentration of lipid stock solutions in chloroform was periodically determined by phosphate assay as described previously (34). Phospholipids and DO stored in chloroform were mixed together at the desired molar ratios and dried thoroughly under nitrogen then under vacuum for at least 1 h. The lipids were then lyophilized from benzene/methanol (19/1, v/v) for a minimum of 12 h in the dark. Each dried sample was hydrated in 20 mM MOPS, 100 mM KCl, 100 μM EGTA, pH 7.2 (MOPS/KCl buffer). Each sample was vortexed extensively above the main transition temperature for 20 min to make multimamellar vesicles (MLVs) dispersions. MLVs were subsequently extruded using a hand microextruder (Avanti Polar Lipids) to make large unilamellar vesicles (LUVs), as described (19). All lipid vesicles were stored in the dark under an argon atmosphere at room temperature.

**Assay of Protein Kinase C Activity—**Kinase activity was assessed by the ability of the enzyme to incorporate [γ-32P]ATP into histone. The reaction mixture (75 μl total) contained 5 mM MgCl2, 300 μM CaCl2, 1 mg/ml bovine serum albumin, 2 mM dithiothreitol, 40 μM [20-3H]PDBu, MOPS/KCl buffer, 5 mM PKCo, 1 mM lipids, with 1.5 μM non-labeled PDBu or 0.15% ethanol vehicle, and alcohols at concentrations as indicated in the figure legends. The reaction was conducted for 5 min at 30 °C or 1 h at 4 °C, and bound [20-3H]PDBu was separated from free PDBu by rapid passage through Whatman 934-AH glass fiber filters. The filters were washed three times with ice-cold phosphate-buffered saline (PBS), 1.4 mM NaHCO3, 3 mM NaCl, 0.1 mM EGTA, and eluted in a Beckman scintillation counter. The specific binding of PDB to PKCo was calculated as the difference between binding in the presence and absence of excess unlabeled PDB.

**Binding of PKCo to the Lipid Bilayers—**Affinity of PKCo binding to the lipid bilayers was estimated using fluorescence energy transfer from tryptophans in PKCo to a dansyl-PE probe with a SLM 8100 fluorometer (SLM-Aminco, Urbana, IL), as modified from Bazzi and Nelsestuen (35). The excitation and the emission wavelengths were 283 and 510 nm, respectively. Large unilamellar vesicles (LUVs) containing PC/PS/DO and 2 mol% dansyl-PE at various lipid concentrations were incubated with 33 or 67 nM PKCo. The experiments were carried out in the same buffer as in the PKCo activity assay but histone and ATP were omitted.

**31P Nuclear Magnetic Resonance Spectroscopy—**MLVs (20 μl total lipid) were prepared with hydrating buffer containing 50% (v/v) D2O. After a minimum of 2 days of hydration, each sample was transferred to a 10-mm diameter NMR tube (Wilmad Glass Co., Buena, NJ). Proton-decoupled free induction decays were collected at 30 °C using a Varian 500 Unity Plus spectrometer operating at 202.4 MHz with the following instrumental setting. S/1 sweep width, 30 kHz; pulse width, 2 μs; delay time, 0.4 s; receiver delay, 0.65 s; number of transients, 10,800. An exponential multiplication corresponding to line broadening of 10 Hz was applied to the accumulated free induction decays before Fourier transformation.

**Differential Scanning Calorimetry—**Excess heat capacity measurements of lipid and lipid-alcohol dispersions (1.4 ml of 5 mM total lipid) were performed on a MicroCal MC-2 (Microcal Inc., Northampton, MA) differential scanning calorimeter at a nominal scan rate of 10 °C/min. The experimental data were analyzed with the MicroCal’s Origin graphic software program.

**Determination of Partition Coefficient—**Partition coefficients of alcohols in DMPC/DMPS/DO systems were determined by injection titration calorimetry (36) and by measuring the depression of the gel-fluid transition temperature (37), as described elsewhere. Injection titration calorimetry was performed on a MicroCal Omega calorimeter. Solvent blanks or lipids (5 mM) combined with alcohol were injected into alcohol solutions at 35 or 4 °C with an injection volume of 30 μl for a duration of 20 s. The syringe spin rate was 300 rpm. The free alcohol concentration in an alcohol-lipid-water suspension was determined by a solvent null method (37), heat is absorbed (released) if the free alcohol concentration in the cell is higher (lower) than that in the suspension; when the concentration in the cell matches the free concentration in the suspension, no heat is generated upon mixing. The freezing point depression method was used to obtain the difference between the partition coefficients of the alcohol into lipids in the fluid and gel state, using the transition temperature shift induced by alcohol. The relationship between the difference of partition coefficients and the shift of the phase transition temperature is:

\[
\frac{1}{T_m} - \frac{1}{T_m^0} = R \times (K_p - K_{p,x}) \times \frac{X}{3H}
\]

(Eq. 1)
where $T_m$ and $T_m^*$ are the transition temperatures in the absence and presence of alcohol, respectively, $K_{p,f}$ and $K_{p,g}$ are the mole fraction partition coefficients of the alcohol in fluid and gel state lipids, respectively; $R$ is the gas constant; $\Delta H^\circ$ is the enthalpy per mole of lipid that undergoes a gel to fluid phase transition in the absence of alcohols; $X_{a,w}$ is the mole fraction of alcohol in the aqueous solution at $T_m^*$.

RESULTS

Alcohols Activate PKC in DMPC/DMPS/DO—The effect of alcohols on PKC activation was examined first in DMPC/DMPS/DO ((80-X)/20/X). PKC activity increased from 0 to 25–30 mol % DO and decreased above 30 mol % DO (data not shown). Addition of alcohols to this lipid system increased PKC activity (Fig. 1). In the presence of 15 and 25 mol % DO, alcohols activated PKC synergistically with DO until maximal enzyme activity was achieved. With shorter chain alcohols, PKC activation decreased significantly at higher alcohol concentrations (Fig. 1, D and E). Even in the absence of DO, high concentrations of alcohols activated PKC. With longer chain alcohols (Fig. 1, A–C), this activation was greater than that achieved with 25 mol % DO, which maximally activated PKC in the absence of alcohol (see above).

The alcohol concentration to achieve half-maximal stimulation of PKC activity (ED$_{50}$) correlated quantitatively with the chain length of the alcohol (Fig. 2A), with the longer chain alcohols requiring lower concentrations for half-maximal activation of PKC. For each alcohol, the ED$_{50}$ was smaller in the presence of DO than in the absence of DO, but the slope of the log ED$_{50}$ versus chain length appeared to be invariant. Since the alcohol chain length correlates linearly with the logarithm of alcohol partition coefficient between lipids and buffer, a quantitative correlation between the ED$_{50}$ and the partition coefficient of alcohols was obtained as expected (inset of Fig. 2A).

A similar correlation was obtained between the alcohol chain length and the maximal alcohol-enhanced PKC activity, defined as the fold stimulation of maximal PKC activity with alcohol over that with 25 mol % DO without alcohol (Fig. 2B). The maximal alcohol-enhanced PKC activity was greater with higher DO concentrations for individual alcohols, but maximal PKC activity appeared to increase with alcohol chain length more rapidly at lower DO concentrations, as indicated by the increase of the slope when DO mol % was decreased (Fig. 2B).

Octanol Activates PKC in DPPC/DPPS/DO and POPC/POPS/DO Systems—To determine whether the phospholipid composition, like the DO mol %, affected alcohol activation of PKC, we examined two additional lipid systems. DPPC/DPPS/DO was selected because this system is similar to DMPC/DMPS/DO with saturated acyl chains on the phospholipids, but it provides a gel state environment because its gel-liquid crystalline phase transition occurs at temperatures higher than the PKC activity assay temperature (30 °C). POPC/POPS/DO was selected because this system contains lipids with saturated and unsaturated acyl chains that are similar to cell membrane lipids and exist in the fluid state under our assay conditions. It also has been shown to activate more effectively than DMPC/DMPS/DO at low DO concentrations (20). This system also supports PKC-phorbol ester binding which will be utilized in later experiments whereas neither
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Addition of octanol to the DPPC/DPPS system enhanced protein kinase C (PKC) activity similarly to that in DMPC/DMPS system (Fig. 1B). The presence of DO in the DPPC/DPPS system in the absence of DO raised the possibility that alcohols might activate PKC by interacting with the binding sites for DO on the enzyme, presumably the cysteine-rich domains which also bind phorbol esters with high affinity. To test this hypothesis, we examined the effects of octanol on the binding of phorbol esters to PKC.

Octanol Increases Membrane Binding of PKC in the DMPC/DMPS System—PKC has to be associated with the bilayer to activate PKC. Second, the enzyme activity exhibits a maximum as a function of alcohol or DAG concentration. Third, in the unsaturated lipid system much lower concentrations of either alcohol or DAG are required to activate PKC.

The observation that alcohols activate PKC in the absence of DO raised the possibility that alcohols might activate PKC by interacting with the binding sites for DO on the enzyme, presumably the cysteine-rich domains which also bind phorbol esters with high affinity. To test this hypothesis, we examined the effects of octanol on the binding of phorbol esters to PKC. In POPC/POPS (50/50) MLVs, PDBu bound to 5 nM PKC with an apparent dissociation binding constant (Kd) of about 20 nM (Fig. 4A). We had to reduce the concentration of POPC from 80 to 50 mol % because we found that the nonspecific binding of PDBu to the membrane was greatly enhanced when POPC is greater than 70% (data not shown). High concentrations of octanol (1 and 2 mM) did not have inhibitory effects on the binding over a range of PDBu concentrations up to 10-fold the apparent Kd (Fig. 4A). Instead, octanol appeared to slightly enhance maximal PDB binding to PKC. To further investigate this enhancement, a series of octanol concentrations were applied to POPC/POPS (50/50) with 75 nM PDBu, under which conditions the high affinity phorbol ester-binding site of PKC was essentially saturated. No significant effect on the degree of PDB-PKC binding was observed (Fig. 4B). The inset of Fig. 4 shows that octanol activated PKC over this alcohol concentration range in this POPC/POPS (50/50) system as it did in POPC/POPS (80/20) (Fig. 3). Similar results were found in the presence of 5 mol % DO (POPC/POPS/DO (45/50/5)) with dramatic activation of PKC by octanol but with no effect on PDB binding to PKC (data not shown). PKC-a has a putative second phorbol ester-binding site with a Kd of about 200 nM, which is much lower than that for the high affinity binding site (38). Due to high nonspecific membrane binding of PDBu at high concentrations, our binding assay was not able to assess low affinity binding of phorbol ester to PKC.
DMPS/DO system and bilayer binding of PKC was detected by measuring fluorescence energy transfer from the Trp in PKC-α to dansyl-phosphatidylethanolamine (dansyl-PE) probes incorporated into the vesicles. Dansyl-PE at high concentration (≥10 mol%) has been shown to alter membrane structure and in turn to activate PKC (39). In this experiment we used 2 mol % which did not affect PKC activity (data not shown).

In the absence of DO, the binding was poor and little fluorescence energy transfer was measurable (Fig. 5A). An assay of competitive binding between DMPC/DMPS (72.5/20/7.5) vesicles provided an estimate of the apparent dissociation constant of reference vesicles ($K_d$) in the absence and the presence of the vesicles of interest ($V_p$). The apparent $K_d$ of $V_p$ is estimated as $[L]/([K_d]/K_d)\times K_d$, where $[L]$ is the concentration of $V_p$ and $K_d$, $K_d$, and $K_d$ are the apparent dissociation constants of $V_p$ in the presence and absence of $V_p$, respectively. When 3 mM octanol was added to DMPC/DMPS (80/20), $K_d$ was estimated to be ~170 μM (Fig. 5A). LUVs were used in the fluorescence energy transfer experiments whereas MLVs were used in PKC activity assays shown in Fig. 1. About 10% of the total lipids are in the outermost layer of MLVs with which PKC is associated. With the 1 mM MLVs used in Fig. 1, PKC is mostly bound to the bilayers in the absence of DO and octanol because the $K_d$ (~450 μM) is much greater than the accessible MLV concentration (100 μM), whereas in the presence of 5 mM octanol some fraction of the enzyme is bound to the bilayer because the $K_d$ (~170 μM) is close to the accessible MLV concentration. These results suggest that in the absence of DO, octanol may, at least in part, promote PKC activity by enhancing the membrane binding of the enzyme. They cannot account for the fact that the maximal activity in the presence of octanol is 2–4-fold greater than the maximal activity obtained with DO alone.

In the presence of 7.5 mol % DO, the affinity of PKC binding to the bilayer was increased with the addition of octanol (Fig. 5B). The apparent $K_d$ was reduced by about 2-fold with 0.6 mM octanol (from 5.7 to 3.3 μM) and by about 4-fold with 3 mM octanol (from 5.7 to 1.6 μM). Octanol activated PKC in this lipid system (data not shown) as it did in DMPC/DMPS/DO (65/20/15) and (55/20/25) (Fig. 1A). The $K_d$ was less than 0.5 mM octanol and 1 mM octanol was able to maximally stimulate PKC activity by more than 12-fold, about 4-fold higher than that induced by 25 mol % DO alone.

In DMPC/DMPS/DO (65/20/15), 200 μM octanol was able to increase the enzyme activity by 3-fold (Fig. 1A) but only slightly enhanced the affinity of PKC binding to the bilayers (Fig. 5C). A similar result was observed in the DMPC/DMPS/DO (55/20/25) system (data not shown), although the apparent dissociation constant $K_d$ was so small (≤2.5 μM) that changes in $K_d$ would be in the range of errors of measurement.

Octanol Does Not Induce Micellar or Hexagonal II Phases—PCs or PSs with acyl chains shorter than eight carbons can form micelles and eliminate the requirement for PS or DAG in activating PKC (40). Bilayers in the cubic phase or with a tendency to form the HII phase, but not the HII phase itself, have been proposed to activate PKC (21, 41). To test the hypothesis that alcohols activate PKC by inducing non-lamellar phases of the lipids, 31P NMR spectroscopy was employed to detect the isotropy of the DMPC/DMPS/DO systems with octanol. At 30 °C, a typical, broad anisotropic spectrum indicative of a lamellar structure of MLVs of DMPC/DMPS/DO (80/20/0, 65/20/15), and (55/20/25) was observed. A small, isotropic peak indicative of a slight contamination (3–5%) by SUV also was observed on occasion. No nonlamellar isotropic resonances were observed in any lipid system with the addition of different octanol concentrations up to the octanol aqueous solubility limit of 4.5 mM (data not shown). Interestingly, octanol acted similarly to DO in shifting the 31P resonance downfield and broadening the MLV spectrum. These results suggest that...
alcohol does not induce the phospholipids to form nonlamellar phases or regions of high bilayer curvature.

**Oc**

**tanol and Pentanol Change the Phase Behavior and Induce Lateral Heterogeneity in DMPC/DMPS System**—Another possible mechanism by which alcohols may mimic DAG in activating PKC is that they both may alter the phase behavior of the lipid bilayers. Lipid lateral domain heterogeneity has been related to PKC activation by DAG (19, 42). To investigate the effects of alcohols on the phase behavior of the lipid bilayers, we used DSC and examined the DMPC/DMPS system with octanol and pentanol (Fig. 6). DSC measures the excess heat capacity of the lipid system as a function of temperature and provides direct information about the lipid gel to liquid-crystalline phase transition temperature ($T_m$) and the enthalpy of the phase transition.

As octanol or pentanol concentration was increased up to $\chi_{\text{octanol}} = 0.29$ or $\chi_{\text{pentanol}} = 0.27$ in gel state lipids, the $T_m$ decreased and the transition broadened. However, at higher octanol or pentanol concentrations the $T_m$ continued to decrease but the transition peak retained its shape with the increase of octanol and became sharper with the increase of pentanol (Fig. 6, A and B). In the presence of pentanol the transition exhibited two maxima suggestive of possible phase separation. The onset and offset temperatures of the main transition rapidly decreased at lower pentanol concentration and at a much reduced rate above $\chi_{\text{pentanol}} = 0.2$ and 0.38 for onset and offset temperatures, respectively. The partial phase diagram, shown in Fig. 6D, is suggestive of possible lipid demixing in the presence of pentanol. A similar partial phase diagram for octanol is less clear (Fig. 6C), although demixing above $\chi_{\text{octanol}} = 0.19 - 0.29$ (onset) and 0.36 (offset) is suggested. The enthalpy change associated with the transition, obtained from the total area under the transition curve, did not vary significantly with the concentration of either alcohol (data not shown). DAG was omitted here to avoid the interference of DAG-induced alteration of lipid structure, which shares many features with the alcohol results shown here (19). Nonetheless, the effect of octanol also was tested in the DMPC/DMPS/DO and DPPC/DPPS/DO systems with 0, 15, and 25 mol % DO and results similar to those in Fig. 6 were observed (data not shown).

When PKC activity was measured over the same alcohol concentration range as used in the experiments described in the legends to Fig. 6, A and B, two effects resembled those of DAG on PKC activation previously reported (19, 42). First, with both gel (4 °C) and fluid (35 °C) state lipid bilayers, PKC activity exhibited a maximum at a pentanol concentration that resides roughly in the putative coexistence region (cf. Fig. 6, D with F). Second, a lower mole fraction of alcohol in the lipid was required to activate PKC and achieve maximal enzyme activity in gel state than in fluid state (Fig. 6, E and F).

The peak in PKC activity is more pronounced with pentanol than with octanol (cf. Fig. 6, E and F). It appears that in gel state lipids with octanol, the enzyme activity exhibits a plateau, and a decrease of PKC activity is not observed in either gel or fluid state lipids with octanol. However, Fig. 6, E and F, show that the dependence of PKC activity on $\chi_{\text{octanol}}$ in the lipid is similar to that on $\chi_{\text{pentanol}}$ in the lipid before the maximal enzyme activity is achieved. With 5 mM lipids, octanol concentrations above $\chi_{\text{octanol}} = 0.42$ in gel state (or 9 mM total octanol concentration, the highest in the experiment) were not accessible due to the aqueous solubility limit (4.5 mM) of octanol.

**DISCUSSION**

Here we report the activation of PKCα by several n-alcohols in a saturated lipid system (Fig. 1) and by octanol in both saturated and unsaturated lipid systems (Figs. 1A and 3). In both systems alcohols activate PKC synergistically with DO and more effectively than DO. PKC activity as a function of alcohol concentration in either saturated or unsaturated lipids resembles that as a function of DO.

As noted previously (19–20, 42–43), the mole % DAG required for maximal PKC activity is much higher in saturated versus unsaturated lipid systems. Although these concentrations would appear to be supraphysiological when expressed as a function of total cellular lipid, it must be remembered that...
the local concentration of DAG would be much higher at sites of phospholipase action where PKC might be activated. Similarly, higher alcohol concentrations are required to activate PKC in the saturated systems (Figs. 1 and 3). Thus, like other lipid-soluble modulators of PKC activity (31, 44), the concentrations of alcohols required depend on the lipid context.

Our results for octanol and heptanol in DMPC/DMPS/DO (80-X/20/X) are similar to those of Slater et al. (32) in POPC/brain PS/DO (76/20/4). However, our results for shorter chain alcohols (butanol to hexanol) demonstrate activation without DO and activation followed by inhibition with DO, whereas they observed only inhibition of PKC (10, 32). Some differences in lipid compositions (unsaturated mixed lipids versus saturated or unsaturated mixed lipids here), lipid vesicle types and concentrations (150 μM LUVs versus 1 mM MLVs here), and substrates (peptide corresponding to the consensus sequence of myelin basic protein versus histone here) exist between their studies and this one and could be the basis of the difference in PKC activity.

**Alcohol Stimulation of Protein Kinase C**

**Fig. 6. Effects of octanol and pentanol on excess heat capacity functions for DMPC/DMPS LUVs and PKCα activity in the lipids.** The excess heat capacity with various concentrations (mole fractions in the lipids) of octanol (A) and pentanol (B) as a function of temperature was determined in DMPC/DMPS = 50/50 LUVs. The thermograms were up-scans from 2 to 40 °C at 10 °C/h. Lipids (5 mM) were hydrated in 20 mM MOPS, 100 mM KC1, 100 μM EGTA, pH 7.2. Onset (△) and offset (▲) temperatures of the gel-fluid phase transition are plotted as a function of octanol (C) and pentanol (D) mole fractions in the lipids. Lipid samples with octanol and pentanol were subsequently used for PKC activity measurements at 4 °C (○) and 35 °C (●) and the results are plotted in E and F. The reaction times for 4 and 35 °C were 1 h and 2.5 min, respectively. PKC activity is normalized to the maximal activity at each temperature in each alcohol. Partition coefficients for octanol are 7.4 × 10^3 and 1.4 × 10^4 at 4 and 35 °C, respectively, and partition coefficients for pentanol are 350 and 600 at the two temperatures. Figures are representative of two to three independent experiments.
the observations. Lester and Baumann (30) did observe a slight activation of rat brain PKC with ethanol in 100 μM egg PC/bovine spinal cord PS/liver DAG (80/20/10) vesicles using histone as substrate.

We have observed that octanol enhances the binding of PKC to lipid bilayers in both the absence and presence of DO (Fig. 5). This enhancement appears to account, at least partially, for PKC activation by octanol in the absence of DO. However, the increase in PKC activity with 3 mM octanol is at least 2 orders of magnitude greater than the basal activity whereas the increase of PKC-bilayer binding affinity is only 3-fold, suggesting that the increased binding is insufficient to explain the promotion of PKC activity by octanol. Similarly, in the presence of DO, binding of PKC to the bilayers is only slightly increased with low concentrations of octanol which dramatically activate PKC (cf. Figs. 1A and 5). It should be noted, however, that different lipid vesicles are used in the activity assays (MLVs) and bilayer binding measurements (LUVs) as well as different enzyme:lipid ratios (5 nM PKC with 1 mM total lipid in kinase assays versus 33 or 67 nM PKC with 3–400 μM lipid in bilayer binding assays). The enzyme:lipid ratio is higher in the bilayer binding assays under all conditions even with accounting for the MLV/LUV difference. Since a peak rather than a plateau occurs in PKC activity assays as a function of lipid concentration and composition (43), bilayer binding and activity cannot be compared quantitatively unless the assays are conducted under identical conditions and this was not possible here due to the differing sensitivities of the two assays.

One of the two major hypotheses for the mechanism of anesthetic actions argues for the existence of a specific binding site(s) in a protein for anesthetics (the specific protein binding model). Since (i) alcohols mimic DAG in activating PKC and enhancing the binding of PKC to lipid bilayers and (ii) DAG interacts directly with PKC by binding to one of the cysteine-rich domains on the enzyme, PKC would be an example of the specific protein binding model if alcohols bind to the DAG, or more specifically phorbol ester, the binding sites. Activation of PKCα by alcohols does not seem to be attributable to the interaction of alcohols with the high affinity phorbol ester-binding site on PKCα, as shown in Fig. 4. No significant effect on PDB-PKCa binding is observed with octanol over a wide range of PDB concentrations (Fig. 4A). There is no competition between octanol and PDB in binding to PKCα over a range of octanol concentrations which dramatically enhance PKCα activity (Fig. 4B).

Slater et al. (32) observed that octanol enhances the interaction of the phorbol ester sapintoxin D with PKCα at the high affinity binding site. However, they also observed no effect of butanol on high affinity binding of sapintoxin D to PKCα. They proposed that DAG and alcohols compete for binding to a putative low affinity phorbol ester-binding site, and that high affinity binding of phorbol ester is in turn enhanced by DAG or long chain alcohols but not by short chain alcohols. They suggested that the activation of PKC by DAG or long chain alcohols resulted from enhancement of phorbol ester binding to the high affinity site on the enzyme.

An alternative explanation for the enhancement of high affinity phorbol ester binding is an increase in PKC bilayer association when DAG or octanol are present. The apparent dissociation constant (K_d,app) of phorbol esters from the high affinity site on PKCα is determined by the association of PKCα with the bilayer and the interaction of phorbol esters with bilayer-associated PKCα. If some of the enzyme is not bound to the bilayer, K_d,app will be greater than the K_d of phorbol ester with bilayer-bound PKCα and any compound that promotes the association of soluble PKCα with the bilayer will decrease K_d,app. On the other hand, if all of the enzyme is bound to the bilayer, K_d,app is the same as the K_d of phorbol ester from bilayer-bound PKC and would not be affected by any compound that facilitates the bilayer association process. The K_d,app of sapintoxin D and PDB binding to PKCα in a detergent/lipid mixed micellar system are ~2.5 and ~20 nm, respectively (45). However, the K_d,app of sapintoxin D from PKC (~100 nm) in our lipid system used by Slater et al. (32) (POPC/BPS = 4/1 molar ratio, 150 μM) is 40-fold greater than that in a micellar system, whereas the K_d,app of PDB from PKC (~20 nm) in our lipid system (POPC/BOPS = 1/1 molar ratio, 1 mM) is close to that in the micellar system. This suggests that PKCα is not all bound to the bilayers under the conditions used in the experiments described by Slater et al. (32) but that it is mostly associated with the lipid system described here. In support of this conjecture, it has been shown that 20 mol % PS (used in the Slater et al. (32) report) was insufficient to support significant PKC binding to the POPC/POPS or egg PC/BPS systems, while 50 mol % PS (used in the phorbol ester binding experiments of Fig. 4) was able to facilitate binding of more than 75% of PKC to the bilayer (39, 46, 47). Octanol can increase the membrane binding of PKC in the absence and presence of DAG, as shown in Fig. 5, and DAG is known to promote significantly bilayer association of PKC (39).

To test the specific protein binding hypothesis for alcohol effects on PKC, Slater et al. (32) also used protamine sulfate and observed inhibition of PKC by alcohols, instead of activation as observed in the presence of lipids. Proamine sulfate is commonly used in place of lipids to monitor the lipid independent activity of PKC (48) and Slater et al. (32) suggested that the inhibitory effect involves the direction interaction between PKC and alcohols which attenuates a conformational change of PKC induced by interaction with protamine sulfate (32). It should be noted that protamine sulfate also can be phosphorylated by PKC and is sometimes used as a PKC substrate. We have used protamine sulfate to replace lipids and observed octanol effects on PKC activation in the presence and absence of histone. Interestingly, phosphorylation of protamine sulfate in the absence of histone is increased with octanol, whereas the total phosphorylation of protamine sulfate plus histone is decreased; the phosphorylation of histone in the presence of protamine sulfate is therefore decreased with octanol (data not shown). The mechanism of protamine sulfate activation of PKC is poorly understood and Orr and Newton (49) have shown that this activator does not induce the same conformational change in PKC as do lipid activators. Possible alternative interpretations of the inhibition of PKC by alcohols with protamine sulfate are disruption of essential protamine sulfate aggregates (50) by the alcohols or irreversible damage to PKC, as may occur with some organic solvents in the absence of lipids (16).

The other major hypothesis for the mechanism of anesthetic actions argues for nonspecific effects on the lipid bilayers (the general membrane perturbation model). In addition to direct binding to PKC, DAG plays an important role in changing the physical properties of bilayers that are important for PKC activation. A relationship between PKC activity and changes in bilayer physical properties induced by the DAG-mimicking alcohols would support the general membrane perturbation model.

31P NMR experiments did not reveal formation of any nonlamellar structures over the octanol concentration range used in our PKC activity assays, arguing that octanol does not activate PKC by inducing nonlamellar phases like micelles, cubic phase, or HII phase. Epand et al. (21) suggested that the HII phase itself does not activate PKC, but the propensity of the
lipsids to form H$_2$O does. This propensity, however, cannot be revealed by $^{31}$P NMR.

Another structural change that alcohols with carbon chains shorter than 7 or 8 may induce in lipid bilayers is interdigitation of the lipids (51). Alcohol-induced lipid interdigitation can be ruled out here for two reasons: 1) interdigitation only occurs in gel state lipids but many of our experiments were conducted with fluid state lipids; 2) more importantly, the main transition temperature monotonically decreases with alcohol (e.g., Fig. 6) rather than increases as observed with alcohol-induced interdigitation (51).

DSC experiments suggest that alcohols, like DAGs (19, 42), may promote domain formation/demixing in DMPC/DMPS systems (Fig. 6). In the case of pentanol, a maximal PKC activation was observed with alcohol mole fractions where maximal coexistence of distinct domains would exist. If these putative alcohol-induced domains are similar to DAG-induced domains in facilitating the binding/insertion of PKC to the membrane and subsequent PKC substrate or PKC-PKC aggregation, then a further common basis for understanding alcohol and DAG activation of PKC exists.

Although DAG can bind directly to PKC (reviewed in Ref. 16) and is well known to enhance PKC-bilayer association (39, 46), activation of PKC exists. A further common basis for understanding alcohol and DAG activation of PKC might be facilitated by the binding/insertion of the enzyme at either the DO-rich or the DO-poor phase. Recent studies of PKC activity as a function of mole fraction of PS, lipid concentration revealed an activity maximum rather than increases as observed with alcohol-induced interdigitation (51).

In summary, alcohols can both lower and replace the DAG requirement for PKC activation in part by enhancing the affinity of enzyme binding to the bilayers and also in a manner dependent upon bilayer composition. The latter effect, we suggest, is due to the alcohol-induced formation of certain types of lipid domains required for PKC activation.

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