Interaction of Alcohols and Anesthetics with Protein Kinase Cα*

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The key signal transduction enzyme protein kinase C (PKC) contains a hydrophobic binding site for alcohols and anesthetics (Slater, S. J., Cox, K. J. A., Lombardi, J. V., Ho, C., Kelly, M. B., Rubin, E., and Stubbs, C. D. (1993) Nature 364, 82–84). In this study, we show that interaction of n-alkanols and general anesthetics with PKCα results in dramatically different effects on membrane-associated compared with lipid-independent enzyme activity. Furthermore, the effects on membrane-associated PKCα differ markedly depending on whether activity is induced by diacylglycerol or phorbol ester and also on n-alkanol chain length. PKCαs contain two distinct phorbol ester binding regions of low and high affinity for the activator, respectively (Slater, S. J., Ho, C., Kelly, M. B., Larkin, J. D., Taddeo, F. J., Yeager, M. D., and Stubbs, C. D. (1996) J. Biol. Chem. 271, 4627–4631). Short chain n-alkanols competed for low affinity phorbol ester binding to the enzyme, resulting in reduced enzyme activity, whereas high affinity phorbol ester binding was unaffected. Long chain n-alkanols not only competed for low affinity phorbol ester binding but also enhanced high affinity phorbol ester binding. Furthermore, long chain n-alkanols enhanced phorbol ester-induced PKCα activity. This effect of long chain n-alkanols was similar to that of diacylglycerol, although the n-alkanols alone were weak activators of the enzyme. The cellular effects of n-alkanols and general anesthetics on PKC-mediated processes will therefore depend in a complex manner on the locality of the enzyme (e.g. cytoskeletal or membrane-associated) and activator type, apart from any isoform-specific differences. Furthermore, effects mediated by interaction with the region on the enzyme possessing low affinity for phorbol esters represent a novel mechanism for the regulation of PKC activity.

Driven by the need to improve anesthetic efficacy and to remove deleterious side effects, much effort has been expended in determining the site and mechanism of anesthetic action, yet both have remained elusive (1, 2). Current theories of anesthesia are based on the observations made by Meyer (3) and Overton (4) at the beginning of this century that anesthetic action was thought to reside within the hydrophobic cell membrane interior (1). However, these “lipid theories” have been criticized on the grounds that the magnitude of effects on the physical properties of membrane lipids at clinically relevant concentrations are too small to be responsible for inducing anesthesia (2). The alternative “protein theory” proposes that a hydrophobic binding site for anesthetics resides within a protein (5). Strong support for this came from a series of important studies that showed that the soluble enzyme firefly luciferase contains a hydrophobic binding site for alcohols and anesthetics, which mediated effects on enzyme activity that obeyed the Meyer-Overton rule (5, 6).

The challenge now is to find a site for anesthetics on a central nervous system protein that could be relevant to the mechanism of anesthesia. Efforts in this direction have shown that a number of membrane proteins localized in the central nervous system are sensitive to clinically relevant concentrations of alcohols and anesthetics. Examples include voltage- and ligand-gated ion channels, synaptic receptors, and G-protein-mediated second messenger systems (e.g. Refs. 2 and 7–14). Most of the effects have been observed on intact cell systems; therefore, ascertaining which protein in a cascade of signaling events may actually be the target is difficult. In addition, membrane lipids are required for protein function in all of these systems, so it is difficult to discount a perturbation of the membrane being sensed by the protein leading to the observed effect. Furthermore, there is also the possibility that the membrane protein-lipid interface maybe involved in anesthetic action (1, 15, 16).

Strong support for the protein theory of anesthesia was provided in a recent report from this laboratory (17), which showed that the regulatory region of protein kinase C (PKC), a key enzyme in intracellular signal transduction (18, 19), contains a hydrophobic binding site for alcohols and anesthetics. Furthermore, it has been shown that inhibition of PKC by staurosporine decreases the EC₅₀ for anesthesia in tadpoles, supporting a role for the enzyme in the anesthetic process (20, 21). The discovery of the n-alkanol and anesthetic binding site was based on the finding that the in vitro activity of a rat brain preparation consisting of the “conventional,” Ca²⁺-dependent PKCα, β₁/III, and γ isoforms was inhibited, in a lipid-free assay, by a homologous series of n-alkanols along with halothane and enfurane, with a potency that followed the Meyer-Overton rule (17). An inhibitory effect of anesthetics on rat brain Ca²⁺-dependent PKC has also been demonstrated in other in vitro

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1 The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; dapsone-PF, N-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphocholine; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-PC; SADP, sapindox; TPA, 4β,12-O-tetradecanoylphorbol-13-acetate; C₆-NBD-PC, 1-palmitoyl-2-(7-nitro-2-oxa-1,3-diazole-4-ylaminohexanoyl)-PC; F₅, fluorescence intensity; F₀, in the absence of SAPD.
The n-alkanols and anesthetics were also observed to inhibit membrane-associated PKC activity. However, the question remains whether this effect on membrane-associated PKC is mediated by a hydrophobic n-alkanol and anesthetic binding site similar to that present on the lipid-free enzyme form and whether, in each case, a common mechanism of action is involved.

The activity of PKC is regulated by interaction with cofactors, including Ca\(^{2+}\) and anionic phospholipids, along with its main natural activator, diacylglycerol (19). Binding of this activator and the tumor-promoting phorbol esters has been shown to be confined to two zinc finger motifs (cys1 and cys2), which constitute the conserved C1 domain contained within the regulatory region of the enzyme (23–28). Using peptide fragments, it has been shown that both of these subdomains bind phorbol esters, the former with a relatively low affinity (26).

Furthermore, recent studies have provided evidence that this asymmetry in phorbol ester binding to the cys1 and cys2 subdomains is carried over into the intact enzyme (29, 30). Based on a highly sensitive phorbol ester binding assay recently developed in this laboratory (31), the low and high phorbol ester binding affinities for these sites on intact PKCa were directly determined. Using this technique it was found that DAG interacts preferentially with the low affinity phorbol ester binding site (31). Furthermore, this interaction of diacylglycerol with the low affinity phorbol ester binding region resulted in a cooperative enhancement of high affinity phorbol ester binding. In keeping with this, the level of PKC activity induced by diacylglycerol and phorbol ester together was found to be greater than that achievable for either activator singly, each activator being present at a concentration that when alone was sufficient to induce maximal activity (31, 32).

In the present study, it was hypothesized that alcohol and anesthetics might interact with either the low or high affinity activator binding sites, or both, based on the finding that the alcohol and anesthetic binding site on PKC is also located within the regulatory domain (17, 22). First, it was confirmed that alcohols and anesthetics inhibited membrane lipid-independent PKCa with a potency that was a linear function of hydrophobicity. By contrast, effects of these agents on membrane-associated PKCa differed markedly dependent on whether activity was induced by phorbol ester or diacylglycerol and also on n-alkanol chain length (i.e. hydrophobicity). Thus, short chain alcohols inhibited membrane-associated activity induced by either phorbol ester or diacylglycerol. However, long chain alcohols and anesthetics potentiated phorbol ester-induced activity but had biphasic effects on diacylglycerol-induced activity. Evidence for direct competition between n-alkanols and phorbol esters for low affinity phorbol ester binding is presented.

**Experimental Procedures**

**Materials**—A peptide substrate corresponding to the PKC phosphorylation site of myelin basic protein (KPRPSRWSKLY) was custom synthesized by the Jefferson Cancer Institute Protein Chemistry Facility. Lipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), except N-(5-dimethylaminonapthalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (dansyl-PE), which was from Molecular Probes (Eugene, OR). SAPD was from Calbiochem. Tryptamine sulfate and 4β-12-O-tetradecanoylphorbol-13-acetate (TPA) were from Sigma. ATP was from Boehringer Mannheim. And [γ-32P]ATP was from DuPont-New England Nuclear. The n-alkanols and propofol were obtained from Aldrich. Enufarine was from Anquest (Madison, WI). All other chemicals were of analytical grade and obtained from Fisher.

**Preparation, Purification, and Assay of PKCa—Recombinant PKCa** (bovine brain) was prepared using the baculovirus Sf9 insect cell expression system (33) and purified as described previously (34). Assays of membrane-associated, lipid-dependent activity followed a previously described method (32, 35). Large unilamellar vesicles of defined size (100 nm) and composition (36), prepared as described elsewhere (37), consisted of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC); bovine brain phosphatidylethanolamine (4:1 molar, 150 µmol), incorporating DAG at a level of 4 mol % of total lipid concentration (POPC composition was modified accordingly) or 0.5 µM TPA as indicated. For determinations of lipid-independent activity, PKCa (10 ng) was assayed in the presence of 0.4 mg ml\(^{-1}\) proteamine sulfate. Additions of n-alkanols and anesthetics were made from concentrated stock buffer solutions. Loss of volatile anesthetics due to evaporation from reaction tubes has been previously determined under assay conditions similar to those used here and found to be negligible (17).

**Determination of SAPD Binding to PKCa**—Binding was quantified using fluorescence resonance energy transfer from PKCa transphosphatidylcholine to the 2-(N-methylamino)benzoyl fluorophore of SAPD, as described previously (31). Briefly, the fluorescence intensities at the emission maxima of PKC transphosphatidylcholines and SAPD (333 and 425 nm, respectively), obtained on excitation of the transphosphatidylcholines at 290 nm, were determined using a PTI Alphalas fluorimeter (Photon Technology International, Princeton, NJ). The assay conditions were identical to those used for PKC activity determinations, except that the assay volume was 2 ml, and the PKC concentrations was 5 ng/µl. Saptoxin-D was titrated into the assay system, and the fluorescence intensities were recorded as a function of the phorbol ester concentration. The direct excitation of the SAPD fluorophore was corrected for by measuring the fluorescence intensity corresponding to each SAPD concentration in the presence of all assay components (except alcohols and anesthetics where added) except PKCa. This signal was then subtracted from the corresponding fluorescence intensities observed in the presence of the enzyme, therefore isolating the contribution of resonance energy transfer to the observed signal. The resultant fluorescence intensity (F) values were normalized to that observed in the presence of all assay components except for the phorbol ester (F\(_{0}\)) according to: (F\(_{E}\) – F\(_{0}\))/F\(_{0}\). The Hill coefficients corresponding to low and high affinity SAPD binding were obtained by fitting binding data using nonlinear least squares analysis to a modified Hill equation (31).

**Determination of Membrane Partitioning of SAPD—Partitioning of SAPD** was determined from Stern-Volmer plots, as described previously (38). Briefly, the emission fluorescence intensity at 530 nm of the fluorophore-labeled phospholipid 1-palmitoyl-2-(7-nitrobenz-2-oxa-1,3-diazeole-4-yl)methanimidatyl-PC (C\(_{2}N\)-NBD-PC; 1 mol % of the total lipid concentration) on excitation at 470 nm was measured as a function of SAPD concentration. Conditions were identical to those used in the activity and binding assays and included all assay components, except PKCa. The probe was also used to assess the effect of SAPD on head group organization by measuring the fluorescence anisotropy (38).

**Results**

In previous a study from this laboratory, evidence for a hydrophobic binding site within the regulatory domain of PKC for alcohols and anesthetics was provided, based on an inhibitory effect of these agents on a rat brain PKCa, β, and γ isoform mixture in a lipid-free assay system (17). In the present study, the effects of a homologous series of n-alkanols on membrane-associated phorbol ester compared with DAG-induced PKC activity were determined. The aim here was to determine the site of interaction of n-alkanols and to gain some insight into the mechanism of the effects of these agents on PKC activity, which has not been resolved in previous studies. Based on the asymmetric binding of phorbol esters and diacylglycerols to the two activator binding sites on PKCa, it was predicted that interaction of alcohols and anesthetics with these sites would result in disparate effects of these agents on PKC activity, dependent on activator type. In this study, a single Ca\(^{2+}\)-dependent PKCa isoform, prepared using the baculovirus Sf9 insect cell expression system (33), was used to circumvent the effects of variation in mixed isoform preparations that tend to complicate the interpretation of results.

Lipid-independent PKCa activity, induced by protamine sulfate, was found to be inhibited in a concentration-dependent manner by a homologous series of n-alkanols. The relationship between the concentration of each n-alkanol required to inhibit activity by 50% (EC\(_{50}\) and chain length was linear (Fig. 1), confirming the existence of a hydrophobic inhibitory site for
Interaction of Alcohols and Anesthetics with Protein Kinase Cα

Fig. 1. Potency of the inhibition of protamine sulfate-induced PKCa activity by a homologous series of n-alkanols (EC$_{50}$) as a function of n-alkanol chain length (hydrophobicity). Values of EC$_{50}$ were derived from anesthetic or n-alkanol dose-response curves (not shown) and are representative of triplicate determinations. Inset, same data plotted against n-alkanol octanol:water partition coefficients (P$_{oct}$; see Ref. 46). Included are values of EC$_{50}$ for inhibition of protamine sulfate-induced activity by endurane (△) and propofol (□). Other details are described under “Experimental Procedures.”

Fig. 2. Distinct activator-dependent effects of a homologous series of n-alkanols on membrane-associated PKCa activity induced by DAG or TPA. DAG was incorporated into bovine brain phosphatidylserine:POPC large unilamellar vesicles (1:4, molar) at a level of 4 mol % of the total lipid concentration (150 μM; ○), and TPA was present at 0.5 μM (●). Activities obtained under each condition are expressed as percentages of those determined in the absence of n-alkanols and are averages of triplicate determinations ± S.D. (bars). The specific activity of PKCa was ~1 nmol of ATP min$^{-1}$ mg$^{-1}$. Other details were as described under “Experimental Procedures.”

n-alkanols on the single PKCa isoform, as previously shown for the rat brain mixed isoform preparation (17). In addition, as shown in Fig. 1 (inset), it was also found that the general anesthetics enflurane and propofol inhibited lipid-independent activity with potencies (EC$_{50}$) that fitted closely with those of n-alkanols of comparable hydrophobicity (i.e. octanol:water partition coefficient).

The chain length-dependent effects of n-alkanols on membrane-associated PKCa activity induced by DAG and phorbol ester (TPA) are shown in Fig. 2. It was found that n-alkanols with chain lengths less than that of n-pentanol inhibited DAG-induced activity, whereas TPA stimulation was unaffected. For n-alkanols with chain lengths greater than that of n-hexanol, TPA-induced activity was potentiated in a concentration-dependent manner, whereas effects on DAG stimulation were biphasic, consisting of a potentiation at low alcohol concentrations followed by a reduction in this potentiation effect at higher alcohol concentrations. A plot of the potency of the amplifying effect of long chain n-alkanols on TPA-induced activity (n-alkanol concentration required to increase TPA-induced activity by 50%; EC$_{50}$) against n-alkanol chain length was linear (Fig. 3). Furthermore, as shown in Fig. 3 (inset), the anesthetics enflurane and propofol behaved in a manner equivalent to that of the long chain alcohols in that TPA-stimulated activity was enhanced by these agents, with potencies proportional to the corresponding value of the membrane:buffer partition coefficient.

In a previous study, it was found that the dose-response curves for the interaction of the phorbol ester SAPD to PKCa and for the induced activity were “dual sigmoidal,” indicating the existence of low and high affinity binding domains for this phorbol ester on PKCa (31). This is again shown to be the case in the present study for TPA-induced activity (Fig. 4A). Addition of the short chain n-alkanol n-butyl alcohol resulted in an inhibition of TPA-induced activity within a concentration range corresponding to the low affinity phorbol ester interaction (~5 μM). This inhibition of membrane lipid-dependent, TPA-induced PKC activity by short chain n-alkanols was also observed in a previous study from this laboratory (17), although the inhibition was observed at a phorbol ester concentration of 500 nM, which under the present assay conditions was unaffected by n-butyl alcohol. However, the previous study was performed on a PKCa, β, and γ isoform preparation for which it has recently been shown that phorbol ester affinity varies considerably (39, 40), which may explain the differing sensitivity of TPA-induced activity to inhibition by short chain n-alkanols. Furthermore, the present activity assay has also been modified in a number of respects, principally in the use of myelin basic protein peptide substrate instead of histone as a phosphate acceptor and in the use of large unilamellar vesicles instead of small unilamellar vesicles.

By contrast to the inhibitory effect of the short chain n-alkanol n-butyl alcohol, the representative long chain n-alkanol n-octanol enhanced TPA-induced activity (Fig. 4A). This was observed as both a shift in the TPA dose-response curve to lower activator concentrations and also as an increase in the maximal activity attained. Furthermore, the TPA-induced activity dose-response curve observed in the presence of n-octanol
FIG. 3. Dependence of the concentration of n-alkanol required to increase TPA-induced, membrane-associated PKCa activity by 50% (EC$_{1.50}$) on n-alkanol chain length (n-heptanol to n-decanol). Values of EC$_{1.50}$ were derived from the anaesthetic or n-alkanol dose-response curves shown in Fig. 2 and are representative of triplicate determinations. Inset, EC$_{1.50}$ values for the n-alkanols plotted as a function of the membrane-buffer partition coefficient ($P_{	ext{M/B}}$, see Refs. 42 and 47) along with those for enflurane (△) and propofol (●). Other details are described under “Experimental Procedures.”

was single sigmoidal, contrasting with the dual sigmoidal curve obtained in its absence. This suggests that long chain n-alkanols compete with low affinity phorbol ester binding (see below). The increase in TPA-induced activity by n-octanol is similar to that previously observed to occur on co-addition of DAG together with TPA (Fig. 4A; see Ref. 31). The data presented in Fig. 4A also show that “basal” membrane-associated PKCa activity induced by Ca$_{2+}$ in the absence of activators was unaffected by short chain n-alkanols, whereas long chain n-alkanols had a small activating effect. This was, however, negligible compared with the level of activity induced by phorbol esters or DAG.

Competition between n-alkanols and phorbol esters for binding to PKCa was determined using a binding assay based on measurements of resonance energy transfer from the PKCa tryptophans to the fluorescent phorbol ester SAPD, as described previously (31). SAPD has a structure similar to TPA and differs only in the presence of an N-methylaminobenzoylfluorophore at the C-12 position, and the two phorbol esters have similar activation dose-response curves (data not shown).

This method for measuring phorbol ester binding has several advantages over the commonly used [H]phorbol dibutyrate binding assay. First, the latter has much lower binding affinity for PKC than SAPD (41), so that low affinity PKC-phorbol ester interactions are not detected. Second, corrections of the data for nonspecific binding to the membrane do not need to be made, since the fluorescence resonance energy transfer signal is derived only from the PKC-bound SAPD. Third, the binding assay is highly sensitive. The SAPD binding isotherm obtained in the absence of n-alkanols was double sigmoidal (Fig. 4B), indicating high and low affinity SAPD binding to PKCa, as previously shown (31). It was necessary to use a logarithmic abscissa in these plots to reveal the effects of n-alkanols on high affinity binding. However, this results in a distortion of the binding isotherm, making it appear that the low affinity binding event has not plateaued. The binding data are therefore replotted on a linear abscissa in Fig. 4B, inset, to clarify that both high and low affinity interactions of SAPD with the enzyme are saturable.

The effects of n-butyl alcohol and n-octanol on SAPD binding were found to closely correspond to the chain length-dependent effects on TPA-induced activity. Thus, n-butyl alcohol inhibited low affinity SAPD binding, consistent with the inhibition of TPA-induced activity over a concentration range corresponding to this low affinity SAPD interaction (Fig. 4B). Similarly, low affinity SAPD binding was also inhibited by the long chain n-alkanol n-octanol (Fig. 4B). However, the long and short chain n-alkanols had markedly different effects on high affinity SAPD binding, which was found to be unaffected by n-butyl alcohol but enhanced by n-octanol (Fig. 4B). This enhancement of high affinity SAPD binding by n-octanol is consistent with the enhancement of TPA-induced activity (Fig. 4A) and again is similar to the effect of DAG on phorbol ester binding (Fig. 4B; see Ref. 31).

The finding that n-butyl alcohol and n-octanol inhibited low affinity SAPD binding suggested that these n-alkanols may share a common site(s) of action. Consistent with this, the dose-dependent amplification of TPA activation induced by n-octanol was inhibited by a fixed concentration of n-butyl alcohol (Fig. 5A). A similar inhibition of the n-octanol-induced amplification of phorbol ester activity was obtained when the concentration of n-octanol was fixed and the level of n-butyl alcohol varied (Fig. 5B).

The possibility has to be considered that the low affinity phorbol ester binding region might be a nonspecific “site” resulting from a perturbation of the membrane by the high SAPD (and/or TPA) concentrations used. Based on this, the observed inhibition of low affinity SAPD binding by the n-alkanols may have resulted from a “stabilization” of this perturbation. How-

FIG. 4. Effects of n-butyl alcohol and n-octanol, chosen as representative n-alkanols, on the binding of the fluorescent phorbol ester SAPD to PKCa and on TPA-induced activation. A, activity dose-response curves for TPA alone (●) and with n-butyl alcohol (■), n-octanol (△), or 4 mol % DAG (△). Specific activities are averages of triplicate determinations ± S.D. (bars). Other details are described under “Experimental Procedures.” B, binding isotherms obtained for SAPD alone (●) and with 50 mM n-butylo alcohol (■), 0.8 mM n-octanol (△), or 4 mol % DAG (△). Values of ($F - F_0$)/$F_0$ represent the fluorescence signal resulting from resonance energy transfer, as described under “Experimental Procedures,” and are representative of triplicate determinations.
ever, we are able to discount this hypothesis for several reasons. 1) The low affinity SAPD binding region is saturable (Fig. 4B, inset). 2) The resonance energy transfer signal observed between 1 and 10 \( \mu M \) SAPD (the low affinity binding range) was found in a previous study to be inhibited by TPA (31). This is inconsistent with a perturbation of the membrane, since both TPA and SAPD would have been expected to induce a similar nonspecific perturbing effect, which would have resulted in an enhanced resonance energy transfer signal, contrasting with the decrease observed. 3) It was previously shown that, in contrast to TPA, its “nonactivating” epimer 4a-TPA inhibited PKC activity induced by levels of SAPD corresponding to its low affinity binding interaction (31). This isomer of TPA differs only in the orientation of the 4-hydroxy moiety. Therefore, again, had the low affinity phorbol ester binding site corresponded to a nonspecific event, then both TPA and 4a-TPA would have been expected to have similar effects on SAPD-induced activity. 4) The effects of SAPD or TPA at concentrations between 1 and 10 \( \mu M \) on the head group motional properties of the fluorescent phospholipid \( C_{16}-\text{NBD-PC} \) were negligible, based on fluorescence anisotropy data (results not shown). This indicates that the extent of nonspecific perturbation of this membrane region by the phorbol esters is negligible. 5) SAPD partitioning into the membrane was determined using \( C_{16}-\text{NBD-PC} \) as a probe (Fig. 6). Since the level of quenching of the NBD fluorescence is proportional to the concentration of SAPD in the vicinity of the NBD fluorophore within the membrane, measurement of \( C_{16}-\text{NBD-PC} \) fluorescence intensity as a function of SAPD concentration (Stern-Volmer plot) allows SAPD partitioning into the membrane to be determined. The results show a linear dependence of SAPD partitioning into bovine brain phosphatidylserine/POPC bilayers on added SAPD concentration (Fig. 6). If the membrane were perturbed by SAPD (or TPA) at the higher phorbol ester concentrations in such a manner as to increase SAPD binding to a nonspecific membrane site, then a nonlinear Stern-Volmer plot should have resulted. We conclude, therefore, that the low affinity SAPD binding site does not result from a perturbation of membrane structure by the phorbol ester itself. 6) The possibility that the inhibitory effect of \( n \)-alkanols (and DAG) on low affinity SAPD binding results from a stabilization of a nonspecific perturbation of the membrane by the phorbol ester is ruled out by the method of data analysis used; the resonance energy transfer signal, corresponding to SAPD binding, was isolated from the raw observed fluorescence signal by subtraction of the fluorescence intensities obtained in the absence of PKC but in the presence of all other assay components, including SAPD and \( n \)-alkanols. This therefore cancels out any nonspecific effects on SAPD fluorescence arising from the (minimal) perturbation of the membrane by either the phorbol ester itself or by the \( n \)-alkanols.

It is also possible that the low affinity interaction of SAPD with PKC\( \alpha \) may have resulted from an increase in membrane association of the enzyme due to a perturbation of the membrane structure at high SAPD concentrations. This possibility was investigated by determining the level of membrane association based on measurements of resonance energy transfer from PKC\( \alpha \) tryptophans to dansyl-PE, which was incorporated into membranes used in this study. It was found that the enzyme was fully associated with the membranes even at SAPD concentrations below that corresponding to low affinity binding (results not shown). Therefore, the low affinity binding event could not have resulted from increased membrane association due to a perturbation of the membrane structure by the phorbol ester. Similarly, the enhanced level of phorbol ester-induced activity in the presence of long chain \( n \)-alkanols (or anesthetic) may also have resulted from an enhanced level of membrane association. This possibility was ruled out by the finding that the level of membrane association induced by maximally activating concentrations of SAPD or TPA was unaffected by long chain \( n \)-alkanols and DAG (results not shown).

**DISCUSSION**

In the present study, it is shown that \( n \)-alkanols interfere directly with phorbol ester binding to membrane-associated PKC\( \alpha \), providing evidence that a hydrophobic binding region for these agents exists on both the membrane-associated and lipid-independent (proamine sulfate-activated) forms of the enzyme. However, the nature of the effects of \( n \)-alkanols and anesthetics on membrane-associated PKC\( \alpha \) activity mediated by this interaction is shown to differ according to activator type and \( n \)-alkanol chain length. Although short chain \( n \)-alkanols have an inhibitory effect, long chain \( n \)-alkanol and general anesthetic interaction results in an enhanced level of high affinity phorbol ester binding and enzyme activity. These differing effects on phorbol ester binding to membrane-associated PKC\( \alpha \) and on the induced level of activity are summarized in Table I.
The observation that \( n \)-alkanols compete for low affinity SAPD binding would be consistent with the binding site for these agents being situated within the cys1 subdomain, if the low affinity phorbol ester binding site is contained within this subdomain, as previously proposed (31). Persuasive evidence that this may be the case has recently been presented by Blumberg and co-workers (29) based on the finding of differing dose-response curves for the phorbol ester-induced translocation in NIH 3T3 cells of PKC\( \alpha \) mutants modified in either the cys1 or cys2 subdomain. Evidence of nonequivalent binding to the two cys subdomains was also presented in another study, which showed that deleting either the first or second zinc finger of PKC\( \alpha \) expressed in yeast resulted in differing dose-dependent effects of phorbol esters on yeast growth (30). It would therefore appear to be unlikely that low affinity phorbol ester binding is a nonspecific event. Furthermore, the possibility that the observation of low affinity phorbol ester binding resulted from a perturbation of the membrane by the activators or the \( n \)-alkanols and anesthetics was ruled out by the finding that low affinity SAPD binding was saturable. Also, the observation of our previous study that TPA and the isomer 4\( \alpha \)-TPA inhibits SAPD binding to this site is contrary to the enhancement expected had the low affinity binding event been nonspecific in nature. Finally, the finding that the quenching of \( C_\text{a} \)-NBD-PC fluorescence intensity by SAPD is a linear function of phorbol ester concentration over a range corresponding to low affinity binding argues against perturbation of the membrane in such a manner as to increase SAPD binding to a nonspecific membrane site. It is worth pointing out that although the phorbol ester concentration required for interaction with the low affinity binding site is relatively high compared with that used in most experimental paradigms, the same cannot be said of the DAG and \( n \)-alkanol levels, which are at physiologically and pharmacologically relevant concentrations, respectively, and would be expected to occupy the low affinity site and modulate activity accordingly.

The activator- and chain length-dependent effects of the \( n \)-alkanols on membrane-associated PKC\( \alpha \) activity contrast starkly with the inhibitory effect of the entire range of \( n \)-alkanols on lipid-independent PKC\( \alpha \) activity. The latter inhibitory effect confirms the original observation made for a mixed rat brain PKC isofrom mixture (17). In the case of the membrane-associated enzyme, the \( n \)-alkanols appear to have two competing effects: an inhibitory effect and a distinct “amplifying” effect on activity that is only revealed for longer chain length \( n \)-alkanols. The observation that \( n \)-butyl alcohol and \( n \)-octanol inhibited SAPD binding and the level of TPA-induced activity over a concentration range corresponding to low affinity SAPD binding provides direct evidence that these agents compete for the low affinity phorbol ester interaction. That both long and short chain \( n \)-alkanols interact with this low affinity phorbol ester binding region is further indicated by the observation that the amplifying effect of \( n \)-octanol on TPA-induced activity could be reduced by the addition of an equipotent level of \( n \)-butyl alcohol (Fig. 5).

In addition to competing for low affinity phorbol ester binding, the long chain \( n \)-alkanol \( n \)-octanol, but not the short chain \( n \)-alkanol \( n \)-butyl alcohol, increased the magnitude of high affinity phorbol ester binding, which is consistent with the observed enhancement of phorbol ester-induced PKC\( \alpha \) activity. This closely resembles the effect of DAG, which also competes for low affinity and enhances high affinity phorbol ester binding (Fig. 4B; see Ref. 31). Therefore, the amplification of phorbol ester-induced activity by DAG, \( n \)-alkanols, and anesthetics appears to proceed by a similar mechanism, involving competition for low affinity phorbol ester binding along with both an increase in high affinity phorbol ester binding and enzyme activity. The observation that \( n \)-alkanols do not compete effectively with phorbol esters for the high affinity phorbol ester enzyme binding site does not preclude the possibility that these agents might be capable of competing with DAG for this region. Competition for DAG binding to both activator sites might provide an explanation for the divergent effects of short and long chain alcohols on DAG-activated PKC activity, producing the inhibitory and biphasic effects observed. Thus, long chain alcohols could compete for DAG binding to the low affinity phorbol ester binding site and enhance DAG binding to the high affinity site. This would lead to enhanced activity, as found for TPA. However, at higher concentrations, the long chain \( n \)-alkanols may compete for DAG binding to the high affinity phorbol ester site, which would lead to a reduction in the (potentiated) level of activity that results from \( n \)-alkanol interaction with the low affinity phorbol ester binding site. Both these effects competing together could produce the observed biphasic effect on DAG-induced activity. By contrast, displacement of DAG from either site by short chain alcohols would result in inhibition, as observed. To test for competition between alcohols and DAG for binding to the high and low affinity phorbol ester binding sites, fluorophore-labeled DAG could be used, which, although currently not available, might resolve this point.

The finding of a linear relationship between the potency of the amplification of phorbol ester-induced activity by long chain \( n \)-alkanols (\( n \)-heptanol to \( n \)-decanol) and chain length (Fig. 5) suggests that the low affinity phorbol ester interaction has a hydrophobic component. However, the observation that high affinity phorbol ester binding and the resultant level of activation was unaffected by the short chain \( n \)-alkanol \( n \)-butyl alcohol, even though this agent inhibited low affinity phorbol ester binding, suggests that for increased high affinity binding there is a minimum hydrophobic requirement that is only met by the longer chain \( n \)-alkanols.

Although the existence of a discrete binding site for \( n \)-alkanols and anesthetics within PKC\( \alpha \) provides strong support for the protein theory of anesthesia, the significance of the magnitude of the effects of the \( n \)-alkanols on PKC activity mediated by this site, obtained using a model system, cannot easily be extrapolated to an impact of these agents on a PKC-regulated cellular function. However, the 20–40% change in PKC activity observed in the present study may lead to a much amplified effect on a function (e.g., ion channel opening) that is downstream in a signaling cascade controlled by the enzyme. Furthermore, these effects of long chain \( n \)-alkanols and anes-

### Table I

Summary of the effects of DAG and \( n \)-alkanols on phorbol ester binding and induced activity

| Ligand       | Activity | Low affinity phorbol ester binding | High affinity phorbol ester binding | Activity, when co-added with phorbol ester |
|--------------|----------|-----------------------------------|-------------------------------------|-------------------------------------------|
| DAG*         | Enhanced | Inhibited                         | Enhanced                            | Enhanced                                  |
| \( n \)-Octanol | No effect | Inhibited                         | Enhanced                            | Enhanced                                  |
| \( n \)-Butyl alcohol | No effect | Inhibited                         | Enhanced                            | Enhanced                                  |

* From Slater et al. (31).
the inhibitory effect of n-alkanols and anesthetics on lipid-independent PKC activity obeys a Meyer-Overton type linear relationship between anesthetic hydrophobicity and potency, whereas this was not found for membrane-associated DAG- or TPA-induced enzyme activity. However, this does not preclude a role for membrane-associated PKC in the anesthetic process if one accepts that there may be more than one cellular anesthetic target. The results presented here also suggest that the effects of n-alkanols and anesthetics on PKC activity will differ according to the subcellular distribution of the enzyme. For example, PKC associated with cytoskeletal or other nonmembrane protein elements, as modeled by protamine sulfate-activated PKC, will be inhibited, whereas membrane-associated activity induced by DAG or phorbol ester may be potentiated. The relevance of this should be seen in the context of the importance of cytoskeleton-associated PKC in neuronal function, which has only recently been appreciated.

The results of this study provide evidence of a discrete hydrophobic site on PKC that may have broader consequences for its regulation. Thus there is the intriguing prospect of more highly specific ligands, either naturally occurring or therapeutically intended synthetic compounds, that may provide control over PKC-mediated signal transduction pathways, an aspect we are currently pursuing. At present, the exact location of the low affinity phorbol ester binding domain on PKC is unclear, but alcohols and general anesthetics would appear to interact competitively with DAG and phorbol ester at this region.

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