The Nature of the Hydrophobic *n*-Alkanol Binding Site within the C1 Domains of Protein Kinase Cα†

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ABSTRACT: The activator-binding sites within the C1 domains of protein kinase C (PKC) are also able to bind alcohols and anesthetics. In this study, the nature of the interaction of these agents with the hydrophobic region within the C1 domains was investigated and a structure−activity relationship for the alcohol effects was obtained. The effects of a series of *n*-alkanols on PKCα activity, determined using an in vitro assay system that lacked lipids, were found to be a nonlinear function of the chain length. In the absence of phorbol ester or diacylglycerol, 1-octanol potently *activated* PKCα in a concentration-dependent manner, while 1-heptanol was completely without effect, despite differing by one methylene unit. The minimal structural requirement for the activating effect corresponded to R−CH(OH)−$(CH_2)_n$−CH_3, where R = H or an alkyl group and $n \geq 6$. Consistent with this, 2-octanol, for which $n = 5$, was without effect on the activity, even though this alcohol is only marginally less hydrophobic than 1-octanol, whereas 2-nonanol, for which $n = 6$, was able to produce activity. Importantly, it was found that PKCα was activated to a greater extent by *R*-2-nonanol than by the *S* enantiomer. The potentiation of phorbol ester-induced, membrane-associated PKCα activity by long-chain *n*-alkanols reported previously (Slater, S. J., Kelly, M. B., Larkin, J. D., Ho, C, Mazurek, A, Taddeo, F. J., Yeager, M. D., Stubbs, C. D. (1997) *J. Biol. Chem. 272*, 6167−6173), was also found here for nonmembrane associated PKC, indicating that this effect is an intrinsic property of the enzyme rather than a result of membrane perturbation. Overall, the results suggest that the alcohol-binding sites within the C1 domains of PKCα contain spatially distinct hydrophilic and hydrophobic regions that impose a high degree of structural specificity on the interactions of alcohols and other anesthetic compounds, as well as diacylglycerols and phorbol esters.

The twelve isozymes that constitute the protein kinase C (PKC)[1] family each play a central role in signaling networks that regulate a diversity of cellular processes, including differentiation, proliferation, apoptosis, secretion, and metabolism (*1*−*6*). In general, PKC becomes active upon translocating to membranes, which is mediated by interaction with anionic lipids, primarily phosphatidylserine (*7*, *8*). The isozymes are distinguished based on the presence of structural motifs that direct cofactor requirements for membrane association and activation (*3*). The "conventional" α, βI, βII, and γ isozymes each contain two conserved C1 domains (C1A and C1B) that harbor the diacylglycerol binding sites, along with a Ca^{2+}-binding C2 domain. The "novel" δ, ε, η, and θ isoforms contain C1 domains but lack a C2 domain capable of binding Ca^{2+}, which results in the membrane association and activation of these isoforms being Ca^{2+}-independent. In the case of "atypical" PKCζ and -ι/λ, neither the C1 nor C2 domains are functional and the translocation and activation of these isoforms is independent of Ca^{2+} and diacylglycerol.

Whereas both diacylglycerol and the tumor-promoting phorbol esters have been shown to interact with the two C1 domains of the conventional and novel PKC isozymes, the interactions of each activator with these domains are not equivalent (*9*−*21*). On the basis of binding studies using the fluorescent phorbol ester, sapintoxin D (SAPD), it has been shown that PKCα and also a fusion peptide corresponding to the C1A and C1B domains of the isozyme, each bind phorbol esters with low and high affinity, respectively (*10*, *11*). Notably, it was observed that diacylglycerol bound to the sites on the holoenzyme with affinities that differed from those of the phorbol ester. Furthermore, a recent study also showed that the C1B domain binds phorbol ester with higher affinity than diacylglycerol, whereas the C1A domain binds diacylglycerol with higher affinity than phorbol ester (*16*). On the basis of this apparent asymmetry in the binding affinities, we suggested that the C1A and C1B domains of PKCα may be *simultaneously* occupied by diacylglycerol and phorbol ester, respectively (*10*). As a result, the occupation of the C1A domain by diacylglycerol was found to result in an enhanced level of phorbol ester binding to the C1B domain, which corresponded to a potentiation of

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[1] Abbreviations: DiC8, 1,2-dioctanoyl-*sn*-glycerol; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; LUV, large unilamellar vesicles; MBP_{4-14}, bovine myelin basic protein peptide substrate; PKC, protein kinase C; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; RET, resonance energy transfer; SAPD, 4β-12-*O*-(2-*N*-methyaminobenzoyl)phorbol-13-acetate; TPA, 4β-12-*O*-tetradecanoylphorbol-13-acetate.

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the level of activity obtained in the presence of both activators compared to that induced by either activator alone.

In a previous study from this laboratory, evidence was provided for the existence of a hydrophobic binding site for *n*-alkanols and anesthetics within the PKC molecule (*12*, *22*), which constituted the first such example of a site on a protein likely to be central in the mechanism of anesthesia (*23*−*25*). Recent studies from this laboratory have shown that in addition to diacylglycerol, alcohols and clinically used general anesthetics displace low-affinity phorbol ester binding (*12*), suggesting that the low-affinity phorbol ester-binding site within the C1A domain (*11*, *16*) may also contain a hydrophobic site(s) for these agents. The effects of *n*-alkanols on the in vitro activity of membrane-associated PKCα were found to be a biphasic function of the chain length (*n*) of the alcohol. Thus, the concentration-dependent effects of short-chain *n*-alkanols ($n < 6$) on activity induced by phorbol ester were largely inhibitory, whereas long-chain *n*-alkanols ($n \geq 6$) were found to potentiate the level of activity. Similar to the mechanism underlying the potentiating effect of diacylglycerol on phorbol ester-induced activity reported previously (*10*), the potentiating effect of the long-chain alcohols corresponded to an increase in the level of high-affinity phorbol ester binding that resulted from competition for the low-affinity phorbol ester interaction (*12*). Short-chain *n*-alkanols also competed for the low-affinity phorbol ester-binding site, but this interaction did not result in a modification of the high-affinity phorbol ester interaction. Thus, it was concluded that the potentiating effect of long-chain *n*-alkanols on phorbol-ester-induced, membrane-associated activity has a minimal hydrophobic requirement, corresponding to an *n*-alkanol chain length of $n \geq 6$, which suggests that the hydrophobic region of the activator-binding site within the C1A domain possesses a high degree of structural specificity.

On the basis of a crystal structure derived for the C1B domain of PKCδ in complex with phorbol-13-acetate, it has been suggested that the free energy required for high-affinity phorbol ester binding to the C1 domains may, in part, be provided by hydrophobic interactions between the phorbol ester acyl chains and the membrane interior (*26*, *27*). However, phorbol esters can nevertheless bind with low affinity to full-length PKCδ and PKCα in the *absence* of lipids (*11*, *14*, *28*), and with *high* affinity to PKCα in complex with Rho GTPases (*29*) or with filamentous actin (*30*). These observations suggest that the high-affinity interaction of phorbol ester or diacylglycerol may be mediated by a hydrophobic cleft in the C1 domain structure that binds the acyl chains of these compounds *independent* of a requirement for membrane association. Consistent with this, recent mutagenesis studies have indicated that hydrophobic residues surrounding the opening of the phorbol-ester-binding site of the C1B domain of PKCδ may form hydrophobic contacts with bound phorbol ester (*31*, *32*). However, the degree to which the hydrophobic regions of the C1 domains contribute to the specificity of the interaction with the activators and thus to the ensuing effects on activity remains to be determined.

In the present study, a structure−activity relationship for the effects of a series of *n*-alkanols of increasing chain length, along with structural isomers, on PKCα activity was investigated. Using a lipid-free assay system, there was a potentiating effect of long-chain alcohols ($n > 7$) that was dependent on the position of the hydroxyl moiety on the alkyl chain and that was stereospecific for the *S* configuration of the 2-alkanols. The results indicate that the effects of alcohols on PKCα activity are critically dependent on the length and orientation of the alkyl chain within a specific binding pocket(s) of the C1 domains and do not result from membrane perturbation.

EXPERIMENTAL PROCEDURES

Materials. A peptide substrate for PKCα, corresponding to the phosphorylation site domain of myelin basic protein (QKRPSQRSKYL, MBP_{4-14}), was custom-synthesized by the Kimmel Cancer Institute peptide synthesis facility of Thomas Jefferson University. ATP was from Boehringer Mannheim (Indianapolis, IN) and [γ-^{32}P]ATP was from New England Nuclear (Boston, MA). The soluble diacylglycerol, 1,2-dioctanoyl-*sn*-glycerol (DiC8), the phorbol ester, 4β-12-*O*-tetradecanoylphorbol-13-acetate (TPA), and alcohols were all obtained from Sigma−Aldrich (St. Louis, MO). The optical purities of the *R* and *S* enantiomers of 2-octanol and 2-nonanol were in each case ≥99.5%. The fluorescent phorbol ester, 4β-12-*O*-(2-*N*-methyaminobenzoyl)phorbol-13-acetate (SAPD), was from Calbiochem (San Diego, CA). All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA). Recombinant PKCα (rat brain) was prepared using the baculovirus *Spodoptera frugiperda* (Sf9) insect cell expression system (*33*) and purified to homogeneity according to previously described procedures (*10*, *34*).

Measurements of PKCα Activity. PKCα activity was assayed by measuring the rate of phosphate incorporation into MBP_{4-14}, as described previously (*29*). Briefly, PKCα activity was measured in the absence of lipids using an assay (75 μL) consisting of 10 mM HEPES (pH 7.40), 50 μM MBP_{4-14}, and 1 μM TPA or 30 μM DiC8, unless otherwise indicated. Where added, POPS and POPC were present at a total concentration of 150 μM as large unilamellar vesicles (1:4, molar), prepared as described previously (*35*). Additions of alcohols were made from concentrated stock buffer solutions. The alcohol concentration ranges used were chosen to compensate for differences in hydrophobicity, estimated from calculations of the respective octanol/water partition coefficients, log *P*, using ALOGPS version 2.0 (*36*), the values of which are summarized in Table 1. After equilibration to 30 °C in a water bath, assays were initiated by the simultaneous addition of PKCα (0.1 nM), along with 5 mM Mg^{2+}, 100 μM ATP, and 0.3 μCi [γ-^{32}P]ATP (3000 Ci/mmol), and terminated after 30 min with 100 μL of 175 mM phosphoric acid. After this, 100 μL was transferred to Whatman P81 filter papers, which were washed three times in 75 mM phosphoric acid. Phosphorylated MBP_{4-14} peptide was determined by scintillation counting.

Determination of Phorbol Ester Binding to PKCα. Phorbol ester binding to PKCα was quantified at 30 °C based on resonance energy transfer (RET) from PKC tryptophans to the 2-*N*-methyaminobenzoyl-fluorophore attached at the 12 position of the phorbol ester, SAPD, as previously described (*10*). Briefly, the fluorescence intensity at the emission maxima of SAPD (425 nm) was measured upon excitation of the tryptophan fluorophore at 290 nm using a SpectraMax Gemini fluorescence plate reader in 96-well format (Molec-

Table 1: Summary of the Effects of Alcohols on Membrane-Lipid-Independent PKCα Activity

alcohol	log P^a	percent activation (% increase over basal)^b	C_{eq} (mM)^c
ethanol	−0.17	*d*	*d*
1-butanol	0.84	*d*	*d*
1-hexanol	1.83	*d*	*d*
1-heptanol	2.31	*d*	*d*
1-octanol	2.62	136 ± 17	0.4
R,*S*-2-octanol	2.58	*d*	*d*
R-2-octanol	2.58	*d*	*d*
S-2-octanol	2.58	*d*	*d*
1-nonanol	3.06	197 ± 10	0.2
R,*S*-2-nonanol	3.02	194 ± 14	0.2
R-2-nonanol	3.02	275 ± 37	0.2
S-2-nonanol	3.02	139 ± 23	0.2
3-nonanol	3	*d*	*d*
1-decanol	3.44	149 ± 7	0.05
2-decanol	3.42	153 ± 17	0.05
4-decanol	3.4	*d*	*d*
1-undecanol	3.87	111 ± 17	0.02

[a] Values of log *P* were calculated according to Tetko et al. (*36*). [b] PKCα activation measured in the presence of 1 μM TPA and 0.1 mM Ca^{2+}, induced by a level of alcohol equivalent to 0.4 mM 1-octanol (C_{eq}), was expressed as a percent of the basal level of activity obtained in the absence of alcohol according to $[(v - v_0)/v_0]\cdot 100\%$, where v_0 and v are the basal activity and activity obtained at C_{eq}, respectively. [c] C_{eq} was estimated from $(P_{1-octanol}/P_x)\cdot 0.4$ mM, where $P_{1-octanol}$ and P_x are the octanol−water partition coefficients for 1-octanol and each alcohol (x), respectively. [d] No effect. Estimated errors were obtained by multiplying percent activation values by $\pm[(SD_0/v_0)^2 + (SD/v)^2]^{1/2}$, where SD_0 and SD are the values of standard deviation for v_0 and v, respectively.

ular Devices, Sunnyville, CA). For determinations of SAPD binding, the assay (200 μL) consisted of 10 mM HEPES (pH 7.40), 5 mM Mg^{2+}, 100 μM ATP, 0.1 mM Ca^{2+}, 100 nM PKCα, and the required concentration of SAPD. Additions of 1-octanol or 1-heptanol were made as required from concentrated stock buffer solutions to yield final concentrations of 0.6 and 2 mM, respectively. After equilibration to 30 °C for 30 min, the emission fluorescence intensity at 425 nm was measured. The contribution of RET to the observed signal was isolated by normalizing the intensities for the contribution from the direct excitation of the SAPD fluorophore, according to $RET = (F_{i,+PKC} - F_{i,-PKC}) - (F_{0,+PKC} - F_{0,-PKC})$, where $F_{i,+PKC}$ and $F_{i,-PKC}$ are the fluorescence intensities measured for each SAPD concentration in the presence and absence of PKCα, respectively, and $F_{0,+PKC}$ and $F_{0,-PKC}$ are the fluorescence intensities measured in the absence of SAPD in the presence and absence of PKCα, respectively. RET as a function of the SAPD concentration data was fitted either by nonlinear least-squares analysis to a biphasic Hill equation based on the assumption that SAPD interacts with two independent binding sites, as described previously (*10*, *11*), or to a single-site Hill equation, as appropriate. An identical assay was used for measurements of SAPD binding as a function of 1-octanol or 1-heptanol concentrations, except that SAPD was present at fixed concentrations of either 1 or 100 μM.

RESULTS

To delineate the nature of the hydrophobic sites within the C1 domains of PKCα that mediate effects on activity, a series of *n*-alkanols along with some structural isomers were used to probe this region. The effects of alcohols of differing structure on enzyme activity were determined using an in vitro assay system that lacked membrane lipids or protein-interacting partners, thus removing possible contributions arising from a perturbation of membrane structure or protein−protein interactions occurring in a PKC membrane or protein complex.

*Structural Specificity of the Interaction of Alcohols with the C1 Domains of PKC*α. The concentration-dependent effects of a homologous series of *n*-alkanols of increasing chain length on PKCα activity were measured in the absence of lipids and in the presence of Ca^{2+} (0.1 mM), with (■) or without (●) 1 μM TPA (parts A−H of Figure 1). The specific activities of PKCα induced by association with POPC/POPS lipid vesicles (4:1, molar) in the presence of Ca^{2+} (0.1 mM), with (▲) or without (△) 1 μM TPA are shown for comparison. The effects of *n*-alkanols on PKCα activity, determined in the absence of TPA, were found to be a nonlinear function of *n*-alkanol chain length. Thus, whereas PKCα activity was found to be unaffected by *n*-alkanols with chain lengths from $n = 2$ (ethanol) to $n = 7$ (1-heptanol), 1-octanol was found to activate the enzyme in a concentration-dependent manner, even though this alcohol differs from 1-heptanol by only a single methylene unit ($n = 8$). Alcohols with longer chain lengths ($n > 8$) also activated PKCα, although the maximal level of activation attained was found to decrease with increasing chain length (parts E−H of Figure 1).

The presence of TPA was found to result in a slight increase in PKCα activity (parts A−H of Figure 1), consistent with previous observations indicating that membrane association is not an absolute requirement for phorbol ester binding or activation (*28*, *29*, *37*, *38*). The chain-length-dependent effects of the *n*-alkanol series on this TPA-induced activity were found to follow a similar trend to that obtained in the absence of the phorbol ester (see Table 1). Thus, the long-chain *n*-alkanols ($n > 7$) further enhanced TPA-induced activity (parts E−H of Figure 1), which corresponded to a shift in the dose−response curve to the left, whereas the short-chain *n*-alkanols ($n < 8$) had negligible effects on activity (parts A−D of Figure 1). Also similar to the effects of long-chain *n*-alkanols on activity observed in the absence of TPA, the potency of the potentiating effect of these alcohols on TPA-induced activity was found to decrease with increasing chain length (parts E−H of Figure 1).

To further investigate the requirement of the activating effect for a chain length greater than 8 carbon units, PKCα activity was measured in the presence of a series of *n*-alkanols in which the position of the −OH group along the alkyl chain was varied (Figure 2). Consistent with the results shown in Figure 1E, 1-octanol activated PKCα in a concentration-dependent manner (Figure 2A, ●), with this activation being additive with that induced by TPA (■). However, 2-octanol was completely without effect on activity, with or without TPA (Figure 2B), despite the fact that the calculated log *P* value for the 2-hydroxy isomer, a measure of hydrophobicity, was only slightly less than that calculated for the 1-hydroxy isomer (Table 1). This apparent structural specificity for the position of the −OH moiety was also observed for the nonanol series (parts C−E of Figure 2), for which the 1- and 2-hydroxy isomers induced activation, whereas the 3-hydroxy isomer did not, and for the decanol series (parts F−H of Figure 2), where the 1- and

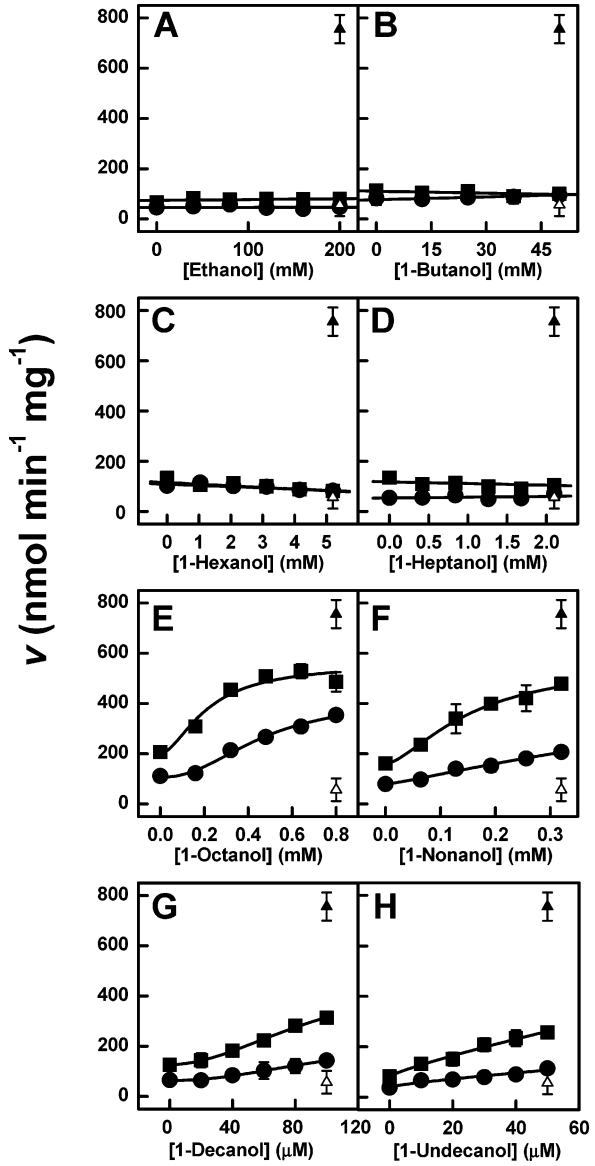

FIGURE 1: Chain-length-dependent effects of a homologous series of *n*-alkanols on nonmembrane PKCα activity. PKCα activity (υ) was measured as a function of the concentration of (A) ethanol, (B) 1-butanol, (C) 1-hexanol, (D) 1-heptanol, (E) 1-octanol, (F) 1-nonanol, (G) 1-decanol, and (H) 1-undecanol, in the absence of lipids, with 0.1 mM Ca^{2+}, in the presence (■) or absence (●) of 1 μM TPA. The specific activities of PKCα, measured in the presence of POPC/POPS membranes (1:4, molar) and 0.1 mM Ca^{2+}, with (▲) or without (△) 1 μM TPA, are shown for comparison. The solid curves are included to show trends and are of no mathematical significance. Data represent the means of triplicate determinations (± standard deviation) from three independent experiments. Other details are described in the Experimental Procedures.

2-hydroxy isomers were found to activate, whereas the 4-hydroxy isomer did not. Similar to 1-octanol (Figure 2A), the potentiating effects of the nonanol and decanol isomers were additive with respect to TPA-induced activation (parts C−H of Figure 2). The differences in the log *P* values calculated for these alcohols were again relatively small, indicating that the divergent effects of the alcohols on activity dependent on the position of the −OH group did not arise from differences in hydrophobicity (Table 1).

The stereospecificity of the interaction of alcohols with the hydrophobic site(s) within the C1 domains of PKCα was investigated by comparing the concentration-dependent effects of the optical isomers of 2-octanol and 2-nonanol on membrane-independent PKCα activity measured in the presence of TPA (Figure 3). In contrast to the potentiating effect of 1-octanol, which served as a positive control (Figure 3A, ●), neither the *R* nor the *S* enantiomer of 2-octanol was found to impact PKCα activity, consistent with the observed lack of an effect of racemic 2-octanol on activity shown in Figure 2B. In contrast, it was found that PKCα activity was potentiated with a greater potency by *R*-2-nonanol (Figure 3B, ▲), compared to the *S* enantiomer (Figure 3B, ▼). The potency of the activation induced by racemic 2-nonanol was intermediate between those of the *R* and *S* isomers, as expected (Figure 3B, ●).

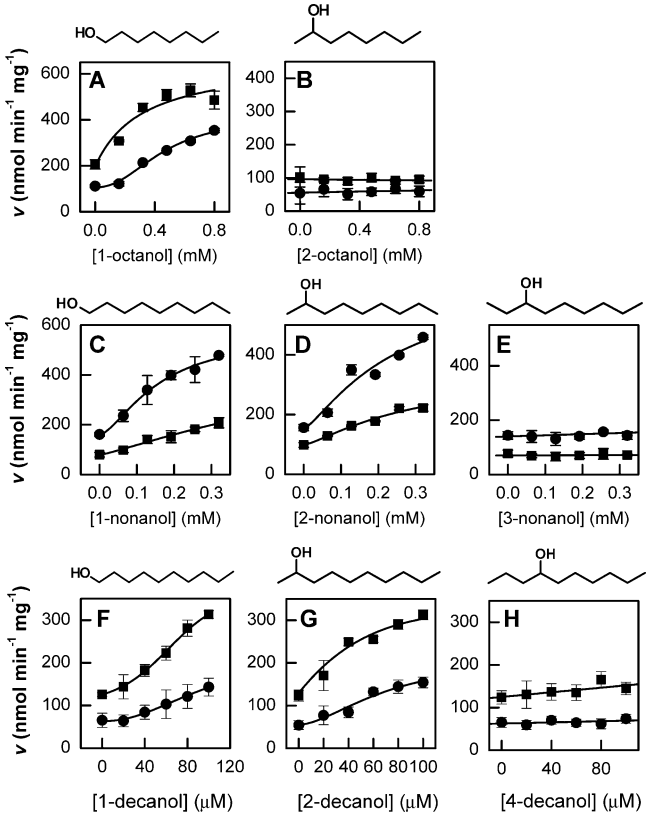

FIGURE 2: Effects of varying the position of the hydroxyl moiety along the alkyl chain of long-chain *n*-alkanols on the activation of PKCα. PKCα activity (υ) was measured with 0.1 mM Ca^{2+}, in the presence (■) or absence (●) of 1 μM TPA as a function of the concentration of (A) 1-octanol, (B) 2-octanol, (C) 1-nonanol, (D) 2-nonanol, (E) 3-nonanol, (F) 1-decanol, (G) 2-decanol, or (H) 4-decanol. Data are representative of the means of triplicate determinations (± standard deviation) from three independent experiments. The solid curves are included to show trends and are of no mathematical significance. Other details are described in the Experimental Procedures.

While the data shown in Figure 1 indicate that the short-chain *n*-alkanols lack the structural determinants for inducing PKCα activation, the possibility remains that these agents still bind to the enzyme and could therefore impact the effects of other agents on enzyme activity. It was found that the activation of PKCα by 1-octanol was inhibited by 1-butanol in a concentration-dependent manner (Figure 4A). Plots of $\log(\upsilon_{[1-\text{octanol}]}/V_{max} - \upsilon_{[1-\text{octanol}]})$ as a function of log[1-octanol] for each fixed 1-butanol concentration, where $\upsilon_{[1-\text{octanol}]}$ is the activity of PKCα at each 1-octanol concentration and V_{max} is the maximal activity, were linear (inset of Figure 4A), with slopes ~1, equivalent to the Hill coefficient (*39*). A

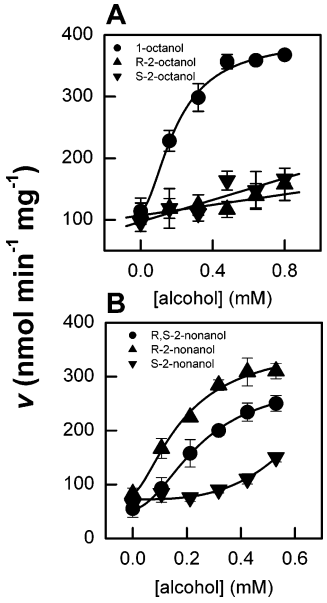

FIGURE 3: Stereospecific activation of PKCα by long-chain *n*-alkanols. (A) PKCα activities (v) were measured in the presence of 0.1 mM Ca^{2+} and 1 μM TPA, as a function of the concentration of either 1-octanol (●) or the *R* (▲) or *S* (▼) enantiomers of 2-octanol. (B) The concentration-dependent effects of the *R* (▲) or *S* (▼) enantiomers of 2-nonanol or the racemate on PKCα activity measured in the presence of 0.1 mM Ca^{2+} and 1 μM TPA. The solid curves are included to show trends and are of no mathematical significance. Data are representative of the means of triplicate determinations (± standard deviation) from three independent experiments. Other details are described in the Experimental Procedures.

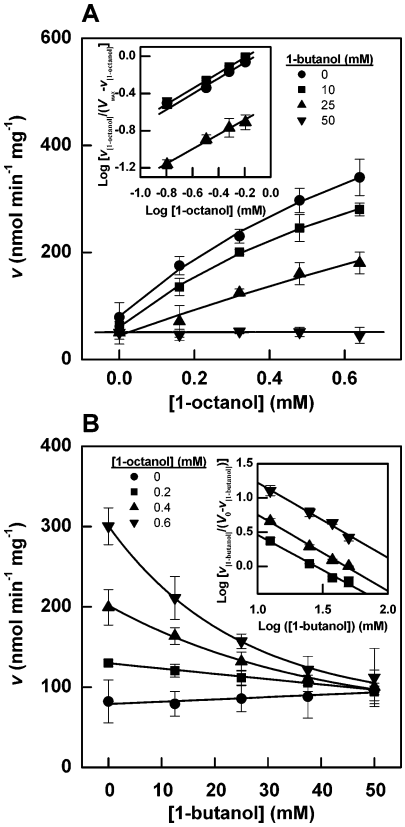

FIGURE 4: Inhibition of the activation of PKCα induced by 1-octanol in combination with TPA by 1-butanol. (A) PKCα activity (v) was measured in the presence of 0.1 mM Ca^{2+}, as a function of the concentration of 1-octanol, either alone (●) or in the presence of 1-butanol at fixed levels of 10 mM (■), 25 mM (▲), or 50 mM (▼). Solid curves represent fits of the data to a Hill equation using nonlinear least-squares analysis. (B) PKCα activity (v) was measured in the presence of 0.1 mM Ca^{2+}, as a function of the concentration of 1-butanol, either alone (●) or in the presence of levels of 0.2 mM (■), 0.4 mM (▲), or 0.8 mM (▼) 1-octanol. Solid curves represent fits of the data to a dose−response equation assuming competition for a single site using nonlinear least-squares analysis. Data were also plotted either in the form $\log(v_{[1-\text{octanol}]}/v_{\max} - v_{[1-\text{octanol}]})$ as a function of log[1-octanol] for each fixed 1-butanol concentration, where $v_{[1-\text{octanol}]}$ is the activity of PKCα at each 1-octanol concentration and $v_{\max}$ is the maximal activity (inset of A) or as $\log(v_{[1-\text{butanol}]}/v_0 - v_{[1-\text{butanol}]})$ as a function of log[1-butanol] for each fixed 1-octanol concentration, where $v_{[1-\text{butanol}]}$ is the activity of PKCα at each 1-butanol concentration and v_0 is the activity in the absence of 1-butanol (inset of B). This allowed determination of the Hill coefficients from the slopes of each line (*39*), obtained from linear regression analysis. Data represent the means of triplicate determinations (± standard deviation) from at least three independent experiments. Other details are described in the Experimental Procedures.

similar inhibition of the 1-octanol-induced activity was obtained when the level of 1-butanol was varied in the presence of a series of fixed levels of 1-octanol (Figure 4B). Furthermore, plots of $\log(v_{[1-\text{butanol}]}/v_0 - v_{[1-\text{butanol}]})$ as a function of log[1-butanol] for each fixed 1-octanol concentration (inset of Figure 4B), where $v_{[1-\text{butanol}]}$ is the activity of PKCα at each 1-octanol concentration and v_0 is the activity in the absence of 1-butanol, were again linear with Hill coefficients ~1 for each curve. Overall, these findings are consistent with the inhibitory effects of 1-butanol on 1-octanol-induced activation involving competition for at least one equivalent site(s) located within the C1 domains of PKCα.

*Effects of Long-Chain n-Alkanols on Phorbol Ester Binding to PKC*α. To investigate whether the additive effects of long-chain *n*-alkanols on TPA-induced PKCα activity observed here also involve an impact on high- and low-affinity phorbol ester binding, which was observed previously for membrane-associated PKCα (*12*), we employed a phorbol ester-binding assay based on measurements of RET from PKCα tryptophans to the 2-*N*-methyaminobenzoyl-fluorophore of the phorbol ester, SAPD (*10*). Consistent with the results of a previous study from this laboratory (*11*), the SAPD-binding isotherm obtained for lipid-independent PKCα in the presence of Ca^{2+} was found to be "dual-sigmoidal" (parts A and B of Figure 5, ●), supporting the existence of low- and high-affinity phorbol ester-binding sites within the C1A and C1B domains. The values of the association constants for SAPD binding to the high- and low-affinity-binding sites (K_H and K_L) and the corresponding Hill coefficients (n_H and n_L), obtained from fits of the data to a biphasic Hill equation (*10*), are shown in Table 2. The presence of 2 mM 1-heptanol ($n = 7$) resulted in a decrease in the level of RET within a SAPD concentration range corresponding to the low-affinity phorbol ester interaction with PKCα, whereas the RET signal corresponding to high-affinity SAPD binding was relatively unaffected (Figure 5A, ■), as reflected by a negligible effect on the values of K_H and n_H (Table 2). This is consistent with the observed lack of an effect of this *n*-alkanol on the activity of lipid-independent PKCα, either alone or in the presence of TPA (Figure 1D). The level of low-affinity phorbol ester binding to PKCα was also attenuated by 0.6 mM 1-octanol ($n = 8$). However, in this case, the reduction in low-affinity phorbol

Table 2: Effects of 1-Heptanol and 1-Octanol on the Association Constants and Hill Coefficients for SAPD Binding to Lipid-Independent PKCα

	K_H (nM)	n_H	K_L (nM)	n_L	χ^2
PKCα alone[a]	874 ± 117	1.4 ± 0.4	25000 ± 1000	1.7 ± 0.3	3.12
+2 mM 1-heptanol[b]	850 ± 196	1.7 ± 0.3	*c*	*c*	2.41
+0.6 mM 1-octanol[b]	303 ± 23	2.4 ± 0.4	*c*	*c*	2.36

[a] Binding constants were obtained from fits of RET as a function of SAPD concentration data for PKCα in the presence of Ca^{2+} from Figure 5A (●) to a biphasic Hill equation based on the existence of two independent phorbol-ester-binding sites on the enzyme (*10*). [b] Binding constants obtained from fits of RET as a function of SAPD concentration data for PKCα in the presence of Ca^{2+} with 2 mM 1-heptanol (Figure 5A, ■) or with 0.6 mM 1-octanol (Figure 5B, ■), to a Hill equation assuming a single SAPD-binding site. [c] No binding. Errors in the binding parameters are ± standard deviation. Goodness of fit was assessed from values of χ^2.

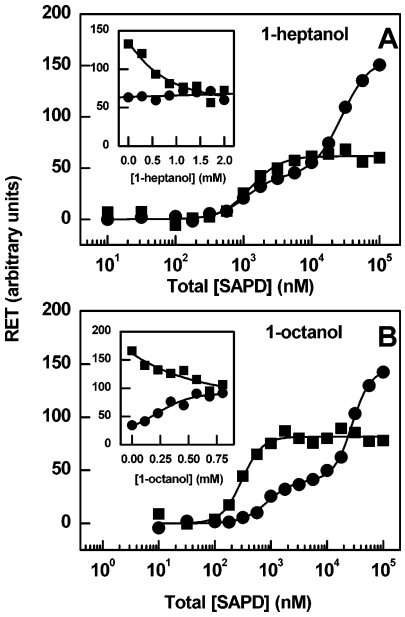

FIGURE 5: Effects of long- and short-chain *n*-alkanols on phorbol ester binding to PKCα. Phorbol ester binding to 100 nM PKCα was quantified in the presence of 0.1 mM Ca^{2+}, either alone (A and B, ●), in the presence of 2 mM 1-heptanol (A, ■), or with 0.6 mM 1-octanol (B, ■) based on the increase in fluorescence intensity that results from resonance energy transfer (RET) from PKCα tryptophans to the 2-*N*-methyaminobenzoyl-fluorophore of SAPD, according to a previously described method (*10*). Data obtained in the absence of alcohols was fitted to a biphasic Hill equation, as described previously (*10*), whereas data obtained in the presence of alcohols was fitted to a Hill equation that assumed a single site. Also shown are the concentration-dependent effects of 1-heptanol (inset of A) and 1-octanol (inset of B) on RET between PKCα tryptophans and fixed levels of 1 or 100 μM SAPD, corresponding to the high- (●) or low- (■) affinity phorbol ester interaction. For effects on low-affinity binding, the solid curves represent fits of the data to a dose−response equation based on competition for a single site. For effects on high-affinity binding, the curves represent fits either to a linear curve for 1-heptanol or to the Hill equation for 1-octanol. The data represent the means obtained from independent experiments carried out three times. Values of standard deviation for fluorescence intensity measurements used in determinations of SAPD binding were typically within ±1% of the mean. Other details are described in the Experimental Procedures.

ester binding was accompanied by an increase in the level of high-affinity SAPD binding (Figure 5B, ■), which was reflected by a ~3-fold decrease in the value of K_H and an increase in n_H (Table 2), despite the fact that 1-octanol differs from 1-heptanol by only a single methylene unit. The disparate effects of 1-heptanol and 1-octanol on high-affinity SAPD binding are consistent with the apparent critical chain length dependence of the additive effect of *n*-alkanols on TPA-induced PKCα activity (Figure 1E). This is also shown

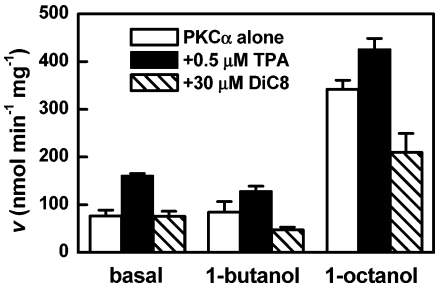

FIGURE 6: Activator-dependent differences in the effects of short- and long-chain *n*-alkanols on PKCα activity. PKCα activity was measured in the absence of *n*-alkanols (basal) or in the presence of 1-butanol (50 mM) or 1-octanol (0.6 mM), chosen as representative short- and long-chain *n*-alkanols, respectively, either with 0.1 mM Ca^{2+} alone (open bars), with 0.1 mM Ca^{2+} and 1 μM TPA (solid bars), or with 0.1 mM Ca^{2+} and 30 μM DiC8 (hatched bars), Data are representative of the means of triplicate determinations (± standard deviation) from at least three independent experiments. Other details are described in the Experimental Procedures.

by plots of RET as a function of the concentration of either 1-heptanol (inset of Figure 5A) or 1-octanol (inset of Figure 5B), in the presence of fixed levels of 1 and 100 μM SAPD, which correspond to high- and low-affinity binding, respectively. Thus, the level of RET corresponding to the high-affinity interaction was unaffected by 1-heptanol, independent of the alcohol concentration, whereas RET corresponding to low-affinity binding was attenuated in a saturable, dose-dependent manner (inset of Figure 5A). 1-Octanol again inhibited the low-affinity interaction in a dose-dependent manner while enhancing the level of high-affinity binding, each effect attaining a saturating level (inset of Figure 5B).

Effects of n-Alkanols on Diacylglycerol-Induced PKCα Activity. To investigate the effects of *n*-alkanols on PKCα activity induced by diacylglycerols, the effects of 1-butanol and 1-octanol on activity induced by TPA were compared with those on activity induced by the soluble diacylglycerol analogue, DiC8 (Figure 6). A low "basal" level of PKCα activity was obtained in the absence of the alcohols (open bars), which was slightly elevated by the addition of 1 μM TPA (solid bars), but unaffected by the addition of 30 μM DiC8 (hatched bars). PKCα activity measured in the absence of activators was unaffected by 1-butanol (50 mM), while being increased in the presence of 1-octanol (0.6 mM), consistent with the data shown in parts B and E of Figure 1. In the presence of TPA, PKCα activity was again unaffected by 1-butanol, while being enhanced by 1-octanol. PKCα activity induced by DiC8 was again only slightly decreased in the presence of 1-butanol, while 1-octanol again enhanced the level of activity. However, whereas the level of PKCα activity obtained in the presence of TPA and 1-octanol was

greater than that induced by 1-octanol alone, the level of activity obtained in the presence of DiC8 and 1-octanol together was found to be *reduced* compared to that induced by the alcohol alone.

DISCUSSION

In this study, the specificity of the hydrophobic interaction of alcohols with activator-binding C1 domains of PKCα was investigated. There was an activation of PKCα by *n*-alkanols that displayed a high degree of structural specificity, being critically dependent on the chain length of the alcohol ($n \geq 8$), the substitution position of the hydroxyl group on the alkyl chain, and the stereoconfiguration of the alcohol, as summarized in Table 1. These findings suggest that the activator binding sites within the C1 domains of PKCα contain both hydrophilic (hydroxyl binding) and hydrophobic (alkyl chain binding) regions that confer a high degree of specificity on ligand binding. Thus, the activation induced by *n*-alkanols requires occupation of both of these regions. This activating effect was additive with respect to phorbol ester-induced activation, which corresponded to an elevated level of high-affinity phorbol ester binding, resulting from binding of the long-chain *n*-alkanol to the low-affinity phorbol ester-binding site, with the mechanism therefore being similar to that proposed for membrane-associated PKCα (*12*).

The observation that PKCα was activated by 1-octanol while being unaffected by 1-heptanol, despite differing by only a single methylene group, suggests that the site(s) of interaction of *n*-alkanols within the C1 domains of PKCα consists of a hydrophilic region that binds the hydroxyl moiety, which is spatially separated from the hydrophobic region that binds the alkyl chain. Consistent with this was the finding that PKCα activity was unaffected by 2-octanol for which the distance separating the hydroxyl and terminal methyl group is effectively shortened by one methylene unit compared to 1-octanol, whereas the isozyme *was* activated by 2-nonanol, for which the required separation between the hydroxyl and terminal methyl group of $n = 8$ is restored. Also consistent with this requirement was the finding that PKCα was unaffected by 4-decanol, which has an effective chain length of $n = 7$, which is less than the minimum required for the activating effect, whereas the enzyme was activated by 1-decanol and 2-decanol, each of which have chain lengths greater than the minimal value. Furthermore, the observation that the potency of the activating effect of *R*-2-nonanol was greater than that of the *S* enantiomer provides further evidence supporting the notion of spatially distinct hydrophilic and hydrophobic regions within C1 domains of PKCα and suggests that *n*-alkanol binding to the site(s) requires not only that the alkyl chain have a minimum length of $n = 8$, but also that it be in the correct orientation. It should be noted that the large differences in the effects of these *n*-alkanol isomers on PKCα activity are not consistent with the relatively small differences in the values of log *P* for each alcohol (see Table 1). This, along with the observed high degree of structural specificity of the activating effects of the alcohols on PKCα activity, suggests that the structure of the alcohol-binding sites within the C1 domains of PKCα is not a simple hydrophobic surface or groove.

On the basis of evidence provided by previous studies that indicate that the low-affinity phorbol ester-binding site is located within the C1A domain of PKCα (*11*, *16*), the observation here that 1-heptanol and 1-octanol inhibited SAPD binding to the low-affinity phorbol ester-binding site (see Figure 5) is consistent with the hydrophobic alcohol-binding site(s) being contained within this domain. However, the possibility that alcohols might modulate low-affinity phorbol ester binding by interacting with separate allosteric sites cannot be ruled out based on the binding data shown in Figure 5. Similarly, whereas the data shown in Figure 4 are consistent with a model in which the inhibitory effect of 1-butanol on 1-octanol-induced PKCα activation results from competition between the two alcohols for the same hydrophobic site(s), the possibility that the alcohols interact with separate allosteric sites cannot be ruled out. Further studies are required to resolve the exact details of the location of the alcohol-binding site(s).

The fact that alcohols interact with a discrete site on PKCα is indicated by the observation that the dose−response curves obtained for the activation of the enzyme by long-chain alcohols in the presence of TPA reached a saturating level. Nevertheless, nonspecific effects of alcohols on PKCα activation cannot be ruled out where conditions are such that saturation was unattainable (e.g., in the absence of TPA). However, the finding that the activating effect displayed an *absolute* requirement for an alkyl chain of length $n > 7$ suggests that such nonspecific effects may be minimal, because if this had been the case one would have expected the short-chain alcohols ($n < 8$) to have had similar concentration-dependent activating effects. The observation that the activating effects of long-chain *n*-alkanols are ablated by moving the −OH group along the alkyl chain (Figure 2) and the *complete* inhibition of the activation induced by the long-chain *n*-alkanol, 1-octanol, and by the short chain *n*-alkanol, 1-butanol (Figure 4), also argues against a significant contribution from nonspecific effects.

The observation that the low- and high-affinity phorbol ester-binding sites exist on the PKCα molecule, *in the absence* of membranes, and that the effects of long- and short-chain *n*-alkanols on these interactions were similar to those reported previously for the membrane-associated enzyme (*12*), suggests that the additive activation of PKCα by *n*-alkanols in combination with phorbol ester is an *intrinsic* property of the enzyme. In addition, it is also shown here that the potentiated level of high-affinity phorbol ester binding resulting from interaction of a long-chain alcohol ($n > 7$) with the low-affinity phorbol ester-binding site corresponds to a decrease in the association constant and an increase in the Hill coefficient for the high-affinity interaction (Table 2). These observations show that the interplay between *n*-alkanol binding to the low-affinity phorbol ester-binding site (C1A domain) and phorbol ester binding to the high-affinity site (C1B domain), which results in the activation of PKCα, involves a conformational response of the PKCα molecule itself that does not require membrane association.

It was suggested elsewhere that the mechanism underlying the activating effects of *n*-alkanols on membrane-associated PKCα activity may involve the alcohol-induced formation of diacylglycerol-rich lateral domains that favor the membrane binding/insertion of PKCα and the subsequent formation of active PKC−substrate or PKC−PKC aggregates (*40*).

This clearly differs from the effects of *n*-alkanols on PKCα activity as observed in the present study, which involves a different mechanism because membrane lipids were absent from the assay system. Interestingly, whereas a minimal *n*-alkanol chain length of $n = 8$ was found here to be required for the activation of membrane-independent PKCα, the optimal chain length for the activation of the membrane-associated enzyme was found previously to be $n > 5$ (*12*), showing that membrane association can modulate the effect. Also, while it was found here that the maximal level of PKCα activation by the long-chain *n*-alkanols *decreased* as a function of the chain length, the activation of the membrane-associated enzyme was found in our previous study to *increase* as a function of the chain length (*12*).

Recent studies have provided evidence that PKCα is initially retained in an inactive "closed" conformation prior to membrane association by an intramolecular interaction that occurs between the C1A and C1B domains and the C2 domain (*14*, *16*, *19*−*21*). In this state, the PKCα molecule is folded so that the active site is blocked by the N-terminal pseudosubstrate (*41*−*43*). The possibility therefore exists that the interaction of *n*-alkanols with the hydrophobic regions within the C1 domains might interfere with C1−C2 domain interactions that otherwise retain the enzyme in the "closed" inactive state, which would shift the conformational equilibrium toward the "open" active state. In this regard, the combined interaction of long-chain *n*-alkanols and phorbol ester with the C1A and C1B domains would be expected to disrupt both C1A−C2 *and* C1B−C2 interactions, which would result in the activation of PKCα observed, although further studies are required to resolve this.

In conclusion, the results show that the activator binding sites within the C1 domains of PKCα contain spatially distinct hydrophilic and hydrophobic regions, the latter containing a critical region that mediates a conformational change that results in activation. The amphiphilic nature of the binding sites imparts a high degree of structural specificity on the interaction of alcohols and potentially anesthetics with the C1 domains of PKCα, offering the possibility that compounds may be designed to optimally bind to the hydrophilic and hydrophobic regions.

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