Effects of Ethanol on Protein Kinase C α Activity Induced by Association with Rho GTPases†

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ABSTRACT: Previous studies have shown that *n*-alkanols have biphasic chain length-dependent effects on protein kinase C (PKC) activity induced by association with membranes or with filamentous actin [Slater, S. J., *et al*. (1997) *J. Biol. Chem. 272*, 6167−6173; Slater, S. J., *et al*. (2001) *Biochim. Biophys. Acta 1544*, 207−216]. Recently, we showed that PKCα is also activated by a direct membrane lipid-independent interaction with Rho GTPases. Here, the effects of ethanol and 1-hexanol on Rho GTPase-induced activity were investigated using an *in vitro* assay system to provide further insight into the mechanism of the effects of *n*-alkanols on PKC activity. Both ethanol and 1-hexanol were found to have two competing concentration-dependent effects on the Ca^{2+}- and phorbol ester- or diacylglycerol-dependent activities of PKCα associated with either RhoA or Cdc42, consisting of a potentiation at low alcohol levels and an attenuation of activity at higher levels. Measurements of the Ca^{2+}, phorbol ester, and diacylglycerol concentration−response curves for Cdc42-induced activation indicated that the activating effect corresponded to a shift in the midpoints of each of the curves to lower activator concentrations, while the attenuating effect corresponded to a decrease in the level of activity induced by maximal activator levels. The presence of ethanol enhanced the interaction of PKCα with Cdc42 within a concentration range corresponding to the potentiating effect, whereas the level of binding was unaffected by higher ethanol levels that were found to attenuate activity. Thus, ethanol may either enhance activation of PKCα by Rho GTPases by enhancing the interaction between the two proteins or attenuate the level of activity of Rho GTPase-associated PKCα by inhibiting the ensuing activating conformational change. The results also suggest that the effects of ethanol on Rho GTPase-induced activity may switch between an activation and inhibition depending on the concentration of Ca^{2+} and other activators.

Protein kinase C (PKC)[1] comprises a family of minimally 12 isozymes, each of which plays a pivotal role in signaling networks that regulate cellular processes, including differentiation, proliferation, secretion, and metabolism (*1*−*5*), that are likely to be involved in the deleterious effects of ethanol (*6*). The cellular activation of PKC isozymes is commonly accompanied by a translocation between cellular compartments, the most well-known example of which is the movement from cytosol to membranes containing anionic phospholipids (*7*). Whereas this process results in the colocalization of PKC with target substrates, the subsequent phosphoryltransferase activity requires an activating conformational change that leads to the removal of a pseudosubstrate sequence from the active site which allows substrate binding to occur (*8*−*10*). Both of these processes are regulated by the combined interaction of multiple activators and cofactors with conserved domains within the enzyme molecule (*3*).

A direct effect of *n*-alkanols, including ethanol and volatile anesthetics, on membrane-associated PKC has been demonstrated by *in vitro* experiments in several studies from this laboratory (*11*, *12*) and others (*13*). These studies showed that lipid-independent PKC activity was inhibited by a homologous series of *n*-alkanols and also by volatile anesthetics with a potency that was a linear function of the hydrophobicity of the agent, supporting the existence of a hydrophobic binding site on the PKC molecule itself (*11*, *12*). The possible relevance of these findings to general anesthesia was supported by whole-animal studies in which the presence of the PKC inhibitor, staurosporine, decreased the concentration of *n*-alkanol required to induce a loss of righting reflex in tadpoles, a commonly used model for anesthesia (*14*, *15*).

Recent studies from this laboratory showed that PKCα contains two phorbol ester binding sites with low and high affinity corresponding to the C1A and C1B domains,

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[1] Abbreviations: DiC8, 1,2-dioctanoyl-*sn*-glycerol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GMP-PNP, γ-imidoguanosine 5′-triphosphate; MANT-GDP, 2′-*O*-(*N*-methylanthraniloyl)guanosine 5′-diphosphate; MBP_{4-14}, bovine myelin basic protein peptide substrate; PKC, protein kinase C; RET, resonance energy transfer; SAPD, 4β-12-*O*-(*N*-methylanthraniloyl)phorbol 13-acetate; SD, standard deviation; SPR, surface plasmon resonance; TPA, 4β-12-*O*-tetradecanoylphorbol 13-acetate.

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respectively, and that the low-affinity phorbol ester binding site within the C1A domain contained the hydrophobic site of interaction of alcohols and anesthetics (*12*, *16*, *17*). Importantly, it was shown that the interaction of long chain *n*-alkanols with this site resulted in a potentiation of high-affinity phorbol ester binding, which resulted in an enhanced level of phorbol ester- and Ca^{2+}-induced membrane-associated activity. However, the magnitude of this "cooperative" effect was found to be a nonlinear function of the hydrophobicity of the *n*-alkanol, and was only apparent for alcohols with chain lengths of at least six carbon units.

In addition to membrane association, there has been a growing realization that PKC isoforms may become active by associating with nonmembrane protein targets (*18*−*21*). Several recent studies have shown that PKC isozymes translocate to the F-actin component of the cytoskeleton in intact cells (*22*−*29*), and that this is mediated by a *direct* protein−protein interaction with F-actin (*30*−*34*). Evidence that PKC isozyme activities associated with F-actin may also be targets for ethanol and anesthetic action was provided by a recent study from this laboratory (*35*). As for membrane-associated PKC, our study showed that the level of conventional PKCα and -βI activities induced by interaction with F-actin was also modulated by a series of *n*-alkanols in a phorbol ester- and Ca^{2+}-dependent and *n*-alkanol chain length-dependent manner (*35*). However, contrasting with effects on membrane-associated PKC activity, the mechanism underlying the effects of *n*-alkanols on F-actin-associated activity did not appear to involve competition for activator binding to the C1 domains.

In addition to the association with F-actin, recent studies have provided evidence supporting a close association between PKC isozymes and members of the Rho family of low-molecular weight GTPases, and a convergence of corresponding signaling pathways (*36*−*39*). Each of the Rho GTPases plays a critical regulatory role in several key cell processes, such as the cytoskeletal rearrangements underlying changes in cell shape, motility, and polarization, as well as being involved in the control of gene transcription through various signaling pathways (*40*−*48*). Recently, we showed that PKCα is activated by a direct interaction with RhoA, Cdc42, and to a lesser extent Rac1, in an *in vitro* assay that precluded the possibility of indirect interactions involving intermediary proteins or lipids (*49*). This activation was dependent on the PKCα activators, phorbol ester and Ca^{2+}, and also on the GTP/GDP-bound state of the Rho GTPases, suggesting that the interaction is regulated by conformational changes in both PKCα and Rho GTPases.

In this study, to provide further insight into the mechanism by which *n*-alkanols have an impact on PKC-mediated signaling networks, the effects of ethanol and 1-hexanol on PKCα activity resulting from a direct interaction with the Rho GTPases, RhoA and Cdc42, were investigated. This was achieved by determining the effects of each *n*-alkanol on the *in vitro* activity of purified PKCα induced in the absence of membrane lipids by interaction with purified Rho GTPases. Using this approach, it was shown that in each case the level of phorbol ester- and Ca^{2+}-induced PKCα activity was enhanced by low levels of each *n*-alkanol, which was followed by an inhibitory effect at higher *n*-alkanol levels. The activation was found to correspond to an enhancement of the extent of binding of PKCα to the Rho GTPases, while the inhibitory effect appeared to result from an attenuation of the activating conformational change induced by the interaction. The mechanism underlying these separate and competing effects on the association of PKCα with Rho GTPases and on the ensuing activating conformational change appeared to be similar to that proposed for the effects of alcohols on F-actin-associated PKC activity (*35*), but differed from that described previously for the membrane-associated enzyme (*12*).

EXPERIMENTAL PROCEDURES

Materials. Human RhoA and Cdc42 and Rho-GDI were each obtained from Cytoskeleton, Inc. (Denver, CO), as purified glutathione *S*-transferase (GST) fusion proteins expressed in *Escherichia coli*. These proteins yielded homogeneous single bands on Coomassie blue-stained SDS−PAGE gels and were used without further purification. The GTPases were supplied as a mixture of GTP- and GDP-bound forms in an approximately 1:1 ratio, this being required to stabilize the recombinant proteins. For activity and binding measurements, RhoA and Cdc42 were each initially loaded with the nonhydrolyzable GTP analogue γ-imidoguanosine 5′-triphosphate, GMP-PNP (Sigma, St. Louis, MO), by incubating each GTPase (375 nM) with the nucleotide (10 μM) in a buffer consisting of 10 mM HEPES (pH 7.4) and 0.1 mM EDTA, to form RhoA·GMP-PNP and Cdc42·GMP-PNP complexes, respectively. 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), and the phorbol ester 4β-12-*O*-tetradecanoylphorbol 13-acetate (TPA), GDP, and *n*-alkanols were all obtained from Sigma. The fluorescent GDP analogue 2′-*O*-(*N*-methylanthraniloyl)guanosine 5′-diphosphate (MANT-GDP) was obtained from Molecular Probes (Eugene, OR), and the fluorescent phorbol ester 4β-12-*O*-(*N*-methylanthraniloyl)-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 (*50*) and purified to homogeneity according to previously described procedures (*16*, *51*).

Measurements of Rho GTPase-Induced PKCα Activity. PKCα activity was assayed by measuring the rate of phosphate incorporation into MBP_{4-14}, as described previously (*49*). Briefly, PKCα activity was measured in an assay (75 μL) consisting of 50 mM Tris-HCl (pH 7.40), 50 μM MBP_{4-14}, 0.1 mM $CaCl_2$, 500 nM TPA or 30 μM DiC8, and 50 nM RhoA·GMP-PNP or 100 nM Cdc42·GMP−PNP, unless otherwise indicated. Additions of *n*-alkanols were made from concentrated stock buffer solutions. For experiments in which activity was measured as a function of Ca^{2+} concentration, Ca^{2+} was added to the assay at a level calculated to yield the required concentration when buffered by 0.1 mM EGTA (*52*). After thermal equilibration to 30 °C, 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 of [γ-^{32}P]ATP (3000 Ci/mmol), and terminated after 30 min with 100 μL of 175 mM phosphoric acid. Following this, 100 μL of the mixture was transferred to P81 filter papers, which were washed three times in 75 mM phosphoric acid. The amount of phosphorylated peptide was determined by scintillation counting.

To determine the reversibility of effects of alcohol on PKCα activity induced by Rho GTPases, PKCα activity was initially determined with 30 μM DiC8 and 0.1 mM Ca^{2+} in the presence and absence of 12 mM 1-hexanol, as described above. Prior to termination of these first assays with 100 μL of 175 mM phosphoric acid, 7.5 μL aliquots were transferred into second assays (75 μL) that were identical except that the alcohol was omitted. The effective alcohol concentration was therefore reduced by 10-fold to 1.2 mM. The second assays were terminated after 30 min with 100 μL of 175 mM phosphoric acid and were then processed along with the first assays as described above.

Binding of PKC Isoforms to Rho GTPases. The binding of PKCα to Cdc42 was assessed at 30 °C based on surface plasmon resonance (SPR) measurements using a Biacore 2000 instrument (Biacore, Inc., Piscataway, NJ). Cdc42·GMP-PNP (50 nM) was initially immobilized on the surface of a nickel−NTA sensor chip through an N-terminal His_6 tag by injection at a concentration of 100 nM in elution buffer [10 mM HEPES (pH 7.4) and 150 mM NaCl] and a flow rate of 5 μL/min to give a level of ~100 response units (Ru). The drift in the resultant baseline response was typically >1% of the total signal, indicating a stable Cdc42 surface (results not shown). Elution buffer solutions containing ethanol at the required concentration with PKCα (3 nM) alone, TPA (500 nM), or both TPA and Ca^{2+} (0.1 mM) were then injected over this surface at a flow rate of 50 μL/min, and the response was measured as a function of time. The Cdc42·GMP-PNP surface was regenerated after each PKCα injection by two 10 s injections of a solution containing 50 mM NaOH and 0.1% (v/v) Triton X-100. This detergent solution was completely removed from the flow cell prior to PKCα injections. Separate control experiments indicated that the Cdc42·GMP-PNP surface was stable under the regeneration conditions that were used. After subtraction of the contribution of bulk refractive index changes and nonspecific interactions of PKC isozymes with the nickel−NTA surface (typically <1% of the total signal), the response (binding) at equilibrium was obtained by global fitting of data to a 1:1 Langmuir binding model using BIAevaluation (Biacore, Inc.). The values of the average squared residual (χ^2) were typically ~10 and were not found to be significantly improved by fitting data to models that assumed bivalent or heterogeneous interactions between PKCα and Cdc42.

Assessment of Binding of Phorbol Ester to PKCα. Binding of phorbol ester to PKCα was quantified at 30 °C based on resonance energy transfer (RET) from PKC tryptophans to the *N*-methylanthraniloyl fluorophore of the phorbol ester, SAPD, as previously described (*16*). Briefly, the fluorescence intensities at the emission maxima of PKC tryptophans and SAPD (340 and 425 nm, respectively) were obtained upon excitation of the tryptophan fluorophore at 290 nm. The assay constituents consisted of 50 mM Tris-HCl (pH 7.40), 100 nM Cdc42·GMP-PNP, 5 mM Mg^{2+}, 100 μM ATP, 0.1 mM Ca^{2+}, and 100 nM PKCα, in a total volume of 2 mL. Additions of ethanol were made from a concentrated stock buffer solution. After thermal equilibration was allowed at 30 °C, SAPD was titrated into the assay system, and after equilibrium was attained, the emission fluorescence intensity at 425 nm was measured. The contribution of RET to the observed signal was isolated by subtracting the fluorescence intensity measured in the presence of all assay components, except SAPD. The fluorescence signal resulting from RET between the single tryptophan of Cdc42 and SAPD in the bulk solution was found to be negligible compared to the PKCα minus SAPD RET signal [see Figure 5 (▼)].

Assessments of GTP−GDP Exchange on Cdc42. The effects of alcohols on binding of GTP and GDP to Cdc42 were investigated by determining the corresponding effects on the kinetics of nucleotide exchange. The rates of exchange were determined at 30 °C by measuring the rate of displacement of the fluorescent GDP analogue MANT-GDP, which was initially bound to Cdc42, by the unlabeled GTP analogue GMP-PNP, as described previously (*53*). Briefly, the assay system (200 μL) consisted of 1 μM Cdc42 and 2 μM GMP-PNP, which were initially incubated for 20 min in a buffer containing Mg^{2+} [10 mM HEPES (pH 7.4), 150 mM NaCl, and 10 mM $MgCl_2$], the presence of which inhibits exchange (*53*). The loading of MANT-GDP was then initiated by decreasing the free Mg^{2+} concentration by the addition of excess EDTA (11.5 mM). The binding of MANT-GDP was monitored by measuring the resultant increase in emission fluorescence intensity, obtained at 440 nm upon excitation at 360 nm, using a Spectramax Gemini plate reader (Molecular Devices, Sunnyvale, CA). Once a maximal level of MANT-GDP binding had been achieved, exchange was then initiated by the addition of 25 μM GMP-PNP. Since the initial concentration of GMP-PNP that was used was >20-fold greater than that of Cdc42 and MANT-GDP, this allowed the fluorescence as a function of time data to be fitted to a pseudo-first-order rate equation to yield an observed pseudo-first-order rate constant (k_{obs}).

RESULTS

Previous studies from this laboratory have shown that the level of PKC activity is modified by *n*-alkanols in a manner that differs according to the *n*-alkanol chain length and whether the enzyme is associated with membranes (*11*, *12*) or with nonmembrane targets, including F-actin (*35*) or protamine sulfate (*11*, *12*). To provide further insight into the mechanism by which *n*-alkanols have an impact on PKC activity, this study examines the effects of ethanol and 1-hexanol on the activation of PKCα induced by the direct membrane lipid-independent interaction with Rho GTPases, reported in a previous study from this laboratory (*49*). To achieve this, activity and binding measurements were taken using *in vitro* assays that contained highly purified PKCα and Rho GTPases, together with the required substrates, activators, and cofactors, thus avoiding potential complications resulting from alcohol effects on indirect PKCα−Rho GTPase interactions mediated by intermediary proteins or lipid structures.

Concentration-Dependent Effects of n-Alkanols on Rho GTPase-Induced Activity. The effects of ethanol and 1-hexanol on the activity of PKCα induced by RhoA·GMP-PNP and Cdc42·GMP-PNP are shown in Figure 1. First, PKCα

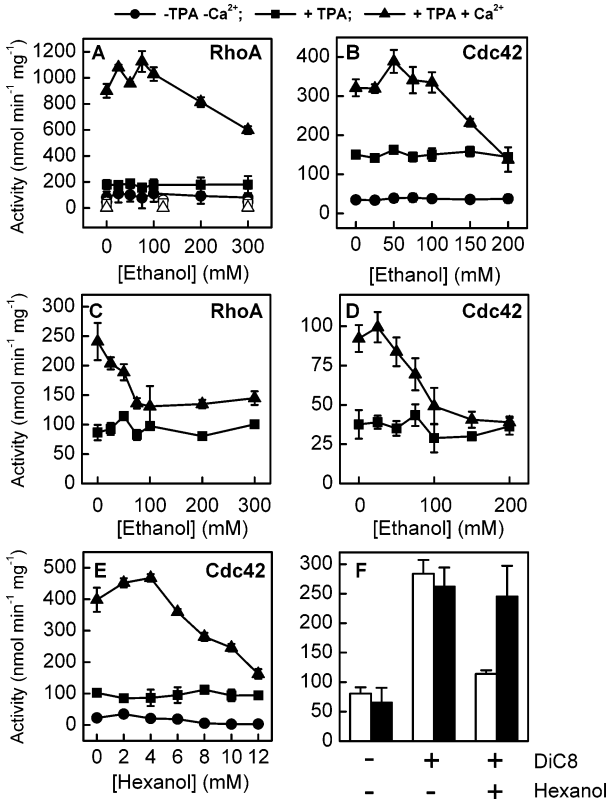

FIGURE 1: (A and B) Concentration-dependent effects of ethanol on PKCα activity induced by interaction with either RhoA·GMP-PNP (50 nM) or Cdc42·GMP-PNP (100 nM) in the presence of the GTPases alone (●), with 500 nM TPA (■), or with TPA and 0.1 mM Ca^{2+} (▲). Also shown in panel A is the effect of ethanol (0, 120, and 300 mM) on activity of PKCα measured in the absence of Rho GTPases, either alone (○), with 500 nM TPA (□), or with TPA and 0.1 mM Ca^{2+} together (△). The concentration-dependent effects of ethanol on Rho GTPase-associated activity induced by the diacylglycerol, DiC8, in place of TPA, are shown in panels C and D. PKCα activities induced by either RhoA·GMP-PNP (C) or by 100 nM Cdc42·GMP-PNP (D) were determined with 30 μM DiC8 alone (■) or with DiC8 and 0.1 mM Ca^{2+} together (▲). Panel E shows the concentration-dependent effects of 1-hexanol on PKCα activity induced by 100 nM Cdc42·GMP-PNP in the presence of Cdc42·GMP-PNP alone (●), with 500 nM TPA (■), or with TPA and 0.1 mM Ca^{2+} (▲). (F) To test whether the inhibitory effect of 1-hexanol on RhoA-induced PKCα activity was reversible, PKCα activity was initially determined with 30 μM DiC8 and 0.1 mM Ca^{2+}, in the presence and absence of 12 mM 1-hexanol (white bars). Aliquots were then transferred into a second assay that was identical except for the omission of the alcohol, effectively diluting the alcohol by 10-fold, and the resultant PKCα activity was again measured (black bars). Data represent means of triplicate determinations ± SD from three independent experiments. Other details are described in Experimental Procedures.

activity was found to be unaffected by each Rho GTPase in the absence of ethanol and without TPA and Ca^{2+} [panels A and B of Figure 1 (●)]. Whereas the presence of a fixed concentration of TPA (500 nM) resulted in a small increase in both RhoA·GMP-PNP- and Cdc42·GMP-PNP-associated PKCα activity [panels A and B of Figure 1, respectively (■)], which is consistent with the results of our previous study (*49*). Also consistent with previous findings, the level of PKCα activity induced by TPA and each GTPase was further enhanced by the addition of 0.1 mM Ca^{2+} [panels A and B of Figure 1 (▲)]. In addition, it is shown here that the soluble diacylglycerol, DiC8, can substitute for phorbol ester in inducing RhoA·GMP-PNP- and Cdc42·GMP-PNP-associated PKCα activation [panels C and D of Figure 1 (■)], although with a reduced potency, and that the level of this activity is again enhanced in the presence of Ca^{2+} [panels C and D of Figure 1 (▲)].

The concentration-dependent effects of ethanol on the enhanced levels of PKCα activity induced by fixed levels of TPA (500 nM) and Ca^{2+} (0.1 mM) were found to be biphasic for each Rho GTPase [panels A and B of Figure 1 (▲)], consisting of a potentiation induced within a low alcohol concentration range (from 0 to 100 mM), and a competing inhibitory effect that became dominant at higher alcohol levels (>100 mM). In contrast, the activity of PKCα obtained with each Rho GTPase in the absence of activators [panels A and B of Figure 1 (●)] or with TPA alone [panels A and B of Figure 1 (■)] was unaffected by ethanol. Similar to the effects on TPA-induced activity, Rho GTPase-induced activity obtained in the presence of DiC8 alone was found to be unaffected by ethanol [panels C and D of Figure 1 (■)], and the effects of ethanol on activity induced by DiC8 and Ca^{2+} together again appeared to be biphasic [panels C and D of Figure 1 (▲)]. However, the alcohol concentration ranges within which the potentiating and inhibitory effects occurred appeared to differ from those observed for TPA- and Ca^{2+}-induced activity [panels A and B of Figure 1 (▲)]. Thus, by contrast to the effects on TPA- and Ca^{2+}-induced PKCα activity where ethanol concentrations in excess of 100 mM were required for the inhibitory effect, the concentration of the alcohol required for a half-maximal decrease in activity (EC_{50}) was ~50 mM for each Rho GTPase [panels C and D of Figure 1 (▲)].

To address the question of whether the inhibitory effect of alcohols on Rho GTPase-induced PKCα activity is reversible, PKCα activity induced by RhoA was initially determined with 30 μM DiC8 and 0.1 mM Ca^{2+} in the presence and absence of 12 mM 1-hexanol (Figure 1F), a level of alcohol sufficient for a maximal inhibitory effect [see Figure 1E (▲)]. This first assay was then diluted into a second one with an identical composition, except for the presence of the alcohol, so that the alcohol concentration was reduced by 10-fold. It was found that RhoA-induced PKCα activity was inhibited by hexanol in the first assay, as expected (Figure 1F, white bars). However, the potency of this inhibitory effect was decreased upon dilution of the alcohol concentration in the second assay, indicating that the inhibitory effect of the alcohol is reversible (Figure 1F, black bars).

The possibility that the activity of PKCα alone may be affected by alcohols, independent of its interaction with Rho GTPases, was ruled out because it was found that the activities of the enzyme either alone [Figure 1A (○)], with 500 nM TPA [Figure 1A (□)], or with TPA and Ca^{2+} [Figure 1A (△)], measured in the absence of Rho GTPases, were each unaffected by levels of ethanol that induced a potentiating (120 mM) and inhibitory (300 mM) effect on Rho GTPase-, TPA-, and Ca^{2+}-induced activity [Figure 1A (▲)].

Similar to the effects on Rho GTPase activity, previous studies have shown that *n*-alkanols also have biphasic activating/inhibitory effects on the activity of PKCα induced by membrane association, the relative potencies of which varied as a function of the chain length of the alcohol (*11*, *12*, *54*). It was therefore of interest to compare the effects of ethanol on PKCα activity induced by Rho GTPases with

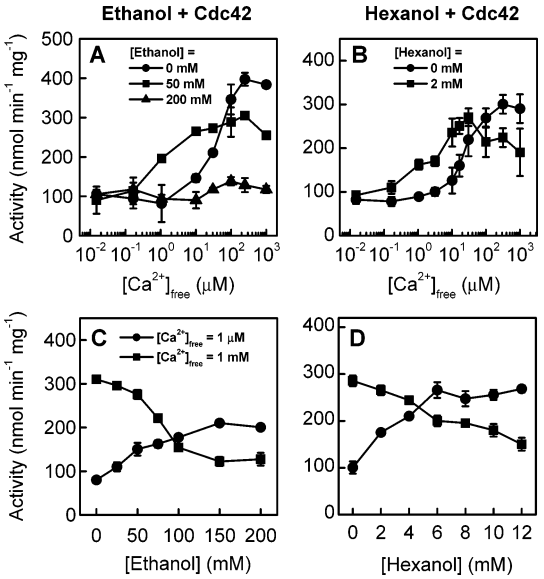

FIGURE 2: Effects of *n*-alkanols on the Ca^{2+} concentration dependences of Cdc42-associated PKCα activity. (A) The activity of PKCα associated with Cdc42·GMP-PNP (100 nM) was measured as a function of the concentration of Ca^{2+} with a fixed level of 500 nM TPA in the absence of ethanol (●), with 50 mM ethanol (■), or with 200 mM ethanol (▲). (B) The activity of PKCα associated with Cdc42·GMP-PNP (50 nM) was measured as a function of the concentration of Ca^{2+} with a fixed level of 500 nM TPA either in the absence of 1-hexanol (●) or with 2 mM 1-hexanol (■). (C) PKCα activity induced by 100 nM Cdc42·GMP-PNP was measured as a function of ethanol concentration with 500 nM TPA and Ca^{2+} present at either a submaximal level [1 μM (●)] or a maximal level [1 mM (■)]. (D) Concentration-dependent effects of 1-hexanol on PKCα activity induced by 100 nM Cdc42·GMP-PNP with 500 nM TPA and Ca^{2+} present at either a submaximal level [1 μM (●)] or a maximal level [1 mM (■)]. Data represent means of triplicate determinations ± SD from three independent experiments. Other details are described in Experimental Procedures.

those of the long chain *n*-alkanol, 1-hexanol (Figure 1E). It was found that the concentration-dependent effects of 1-hexanol on Cdc42·GMP-PNP-associated PKCα activity induced by TPA together with Ca^{2+} were similar to those of ethanol, consisting of an activating effect within a low 1-hexanol concentration range followed by a competing inhibitory effect at higher levels [Figure 1E (▲)]. Furthermore, the effects of 1-hexanol were again confined to PKCα activity induced by Ca^{2+} and TPA, and the activities induced by association with Cdc42·GMP-PNP alone [Figure 1E (●)] or with TPA [Figure 1E (■)] were again found to be unaffected.

Effects of Ethanol on the Ca^{2+} Concentration Dependence of Cdc42-Induced PKCα Activity. The finding that neither of the *n*-alkanols affected the activity of Rho GTPase-associated PKCα in the absence of Ca^{2+} suggests that both the potentiating and inhibitory effects on the activity of the enzyme, observed in Figure 1, may be Ca^{2+}-dependent. To investigate this, the effects of ethanol and 1-hexanol on the Ca^{2+} concentration−response curves for Cdc42·GMP-PNP-induced PKCα activity were determined (Figure 2). Consistent with the results of our previous study (*49*), Cdc42·GMP-PNP-induced activity measured in the presence of a fixed level of TPA (500 nM) increased as a function of the concentration of Ca^{2+}, with a midpoint value of ∼20 μM [panels A and B of Figure 2 (●)]. Further, consistent with the observed biphasic concentration-dependent effects of ethanol and 1-hexanol on Rho GTPase-associated PKCα activity induced by either TPA or DiC8 and Ca^{2+} shown in Figure 1, it was found that the presence of increasing levels of ethanol (Figure 2A), and 1-hexanol (Figure 2B), each resulted in dual effects on the Ca^{2+} concentration dependences for Cdc42·GMP-PNP-induced PKCα activation. Thus, the activity of PKCα induced within a low Ca^{2+} concentration range (from 0.1 to 10 μM) was potentiated in the presence of low levels of ethanol (50 mM) or 1-hexanol (2 mM), which was observed as a leftward shift in the midpoint of the curve [panels A and B of Figure 2 (■)]. In contrast, the level of activity induced at maximal Ca^{2+} levels (>100 μM) was attenuated [panels A and B of Figure 2 (■)]. Consistent with this, PKCα activity measured in the presence of TPA and a fixed, low level of 1 μM Ca^{2+} was found to increase as a function of ethanol and 1-hexanol concentration [panels C and D of Figure 2 (●)], whereas activity measured in the presence of TPA and a fixed high concentration of 1 mM Ca^{2+} decreased as a function of alcohol concentration [panels C and D of Figure 2 (■)]. Importantly, each curve described a monophasic-type relationship between PKCα activity and alcohol concentration. This contrasts with the biphasic alcohol concentration−response curves shown in Figure 1, which appear to result from the potentiating and inhibitory effects being relatively more balanced at the fixed Ca^{2+} level of 100 μM used in those experiments.

Effects of Ethanol on the TPA and DiC8 Concentration Dependences of Cdc42-Induced PKCα Activity. Consistent with the results of our previous study (*49*), it was found that in the presence of a fixed level of Ca^{2+} (100 μM), Cdc42·GMP-PNP-associated PKCα activity increased as a function of the concentration of TPA, and the level of the phorbol ester required to induce a 50% increase in activity was ∼10 nM [Figure 3A (●)]. Like that of TPA, Cdc42·GMP-PNP-associated PKCα activity was also potentiated in a concentration-dependent manner by DiC8 [Figure 3B (●)]. However, the midpoint value of DiC8 concentration was ∼150-fold higher than that obtained for TPA, suggesting that the affinity of the binding of DiC8 to Cdc42·GMP-PNP-associated PKCα is lower than that of TPA. This observation is consistent with the previously reported lower affinity of PKC for membranes containing diacylglycerol compared to those containing phorbol esters (*55*). The effects of ethanol on both the TPA and DiC8 concentration dependences of Cdc42·GMP-PNP-associated PKCα activity were similar to those on the Ca^{2+} concentration−response curves, consisting of a potentiating effect at low activator levels and an inhibitory effect at maximal activator concentrations (panels A and B of Figure 3).

Effects of Ethanol on the Binding of Cdc42 to PKCα. The possibility that the activation and/or inhibitory effects of ethanol might have resulted from corresponding effects on the interaction between PKCα and the Rho GTPases was investigated by measuring the extent of binding of PKCα to GMP-PNP-bound Cdc42 in the presence of increasing levels of the alcohol using SPR. It was found that the level of binding of PKCα to Cdc42·GMP-PNP at equilibrium (R_{eq}) was negligible in the absence of TPA and Ca^{2+} [Figure 4 (●)], whereas the presence of a fixed level of 500 nM TPA resulted in a low level of binding [Figure 4 (■)] that was further increased upon addition of 100 μM Ca^{2+} [Figure 4 (▲)]. These results are consistent with the observed TPA

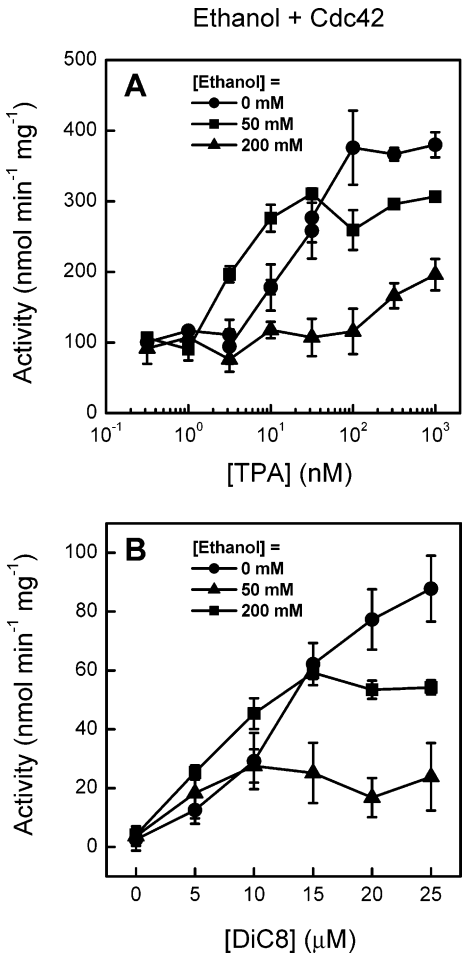

FIGURE 3: Effects of ethanol on PKCα activity associated with Cdc42·GMP-PNP (50 nM) were measured as a function of the concentration of TPA (A) or 30 μM DiC8 (B) with a fixed level of 0.1 mM Ca^{2+}, either in the absence of ethanol (●), with 50 mM ethanol (■), or with 200 mM ethanol (▲). Data represent means of triplicate determinations ± SD from three independent experiments. Other details are described in Experimental Procedures.

and Ca^{2+} dependences of the activation of PKCα by Cdc42·GMP-PNP shown in Figure 1, and also with the results of our previous study (*49*). The level of binding measured in the absence of activators [Figure 4 (●)] or with TPA alone [Figure 4 (■)] was unaffected by ethanol, which is in keeping with the lack of an effect of ethanol on activity under the same conditions shown in Figure 1. Also consistent with the effects on TPA- and Ca^{2+}-induced activity (Figure 1), the level of binding of PKCα to Cdc42·GMP-PNP induced by TPA and Ca^{2+} was further potentiated by ethanol in a concentration-dependent manner [Figure 4 (▲)]. Importantly, the concentration dependence of this increase in binding was found to coincide with that observed for the activating effect of the alcohol (see Figure 1). In contrast, the extent of binding was unaffected by the higher ethanol levels that were found to inhibit PKCα activity.

Effects of Ethanol on Binding of Phorbol Ester to Cdc42-Associated PKCα. Binding of phorbol ester to PKCα associated with Cdc42·GMP-PNP, and the effects of ethanol on this interaction, were determined on the basis of assessments of RET from PKCα tryptophans to the *N*-methylanthraniloyl (MANT) fluorophore of SAPD (*16*). Consistent with the results of our previous studies (*12*, *16*, *17*, *56*, *57*), the SAPD binding isotherm obtained for PKCα with Ca^{2+}

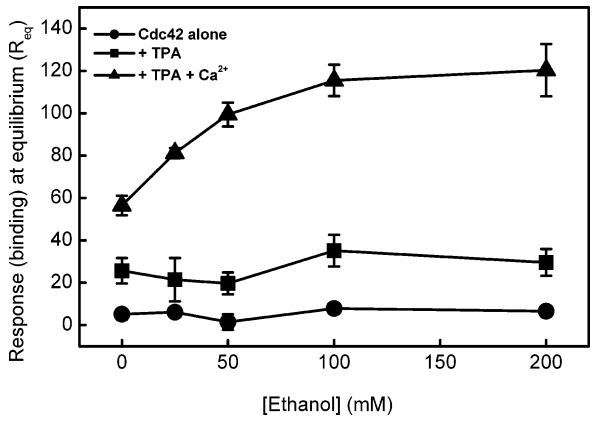

FIGURE 4: Effects of ethanol on the interaction of PKCα with Cdc42. The binding of PKCα to Cdc42·GMP-PNP was determined from SPR measurements. Cdc42·GMP-PNP was initially immobilized on the surface of a nickel−NTA chip via a His_6 tag. PKCα (3 nM) was then injected, either alone (●), with 500 nM TPA (■), or with TPA and 0.1 mM Ca^{2+} (▲). The response (binding) at equilibrium (R_{eq}), obtained from fits of response vs time data to a Langmuir binding model that assumed a 1:1 binding stiochiometry, was then plotted as a function of the concentration of ethanol. The data are representative of four independent experiments. Other details are given in Experimental Procedures.

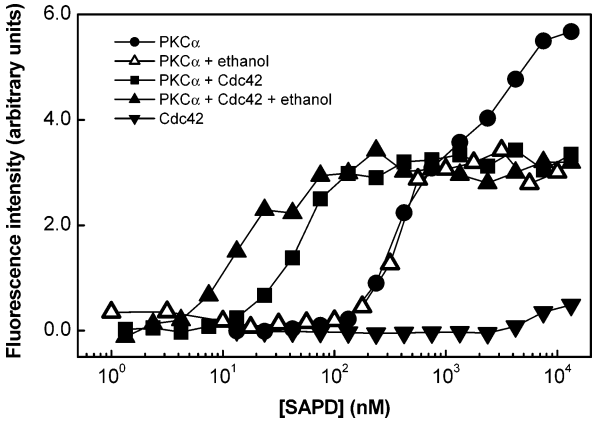

FIGURE 5: Effects of ethanol on phorbol ester binding to Cdc42-associated PKCα. Phorbol ester binding to 50 nM PKCα alone (●), or to PKCα bound to 50 nM Cdc42·GMP-PNP (■), was quantified in the presence of 0.1 mM Ca^{2+} based on the increase in fluorescence intensity that results from resonance energy transfer (RET) from PKCα tryptophans to the 2-(*N*-methylanthraniloyl) fluorophore of SAPD, according to a previously described method (*16*). The extent of phorbol ester binding to PKCα alone (△), or associated with Cdc42·GMP-PNP (▲), was also determined in the presence of 200 mM ethanol. Also shown are data corresponding to RET from the tryptophans of Cdc42·GMP-PNP to the SAPD fluorophore (▼) obtained from measurements of fluorescence intensity in the absence of PKCα. The data are means obtained from independent experiments carried out three times. Values of SD for fluorescence intensity measurements used in determinations of SAPD binding were typically within ±1% of the mean. Other details are as described in Experimental Procedures.

alone, in the absence of Cdc42·GMP-PNP and ethanol, was found to be "dual-sigmoidal" [Figure 5 (●)], indicating the existence of low- and high-affinity phorbol ester binding sites on the PKCα molecule. It was found that the presence of ethanol (200 mM) resulted in a decrease in the level of RET within a SAPD concentration range corresponding to the low-affinity interaction, whereas the RET signal corresponding to high-affinity SAPD binding was unaffected [Figure 5 (△)]. This observation is similar to that reported in our previous

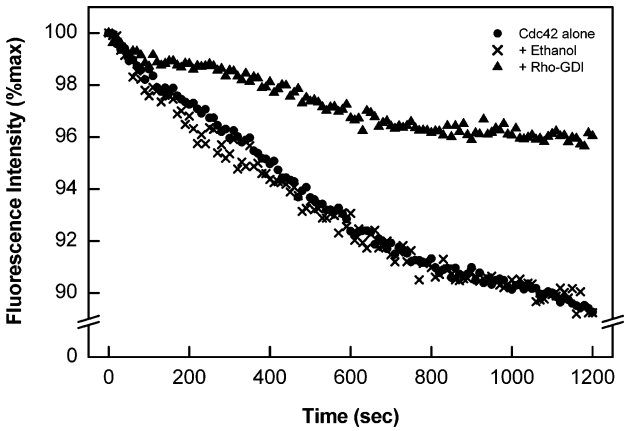

FIGURE 6: Effects of ethanol on the rates of GTP−GDP exchange on Cdc42. Cdc42 (1 μM) was initially loaded with MANT-GDP (2 μM), the fluorescence of which increases upon binding to the nucleotide binding site on the GTPase (results not shown). The decrease in fluorescence due to the displacement of MANT-GDP by the addition of unlabeled GMP-PNP was then measured as a function of time, with either Cdc42 alone (●) or Cdc42 with 200 mM ethanol (×). To confirm the validity of the assay, Rho-GDI (1 μM) was added to MANT-GDP-loaded Cdc42, prior to the initiation of exchange by the addition of GMP-PNP (▲). The data are representative of three independent experiments carried out in triplicate. Other details are given in Experimental Procedures.

study of the effects of alcohols on membrane-associated PKCα (*12*), and suggests that the alcohol may target the low-affinity phorbol ester binding site on PKCα independent of its association with membranes.

In contrast to the dual-sigmoidal binding isotherm obtained for PKCα alone [Figure 5 (●)], the binding curve obtained in the presence of Cdc42·GMP-PNP only contained a single increase in fluorescence due to RET that corresponded to a high-affinity phorbol ester interaction, and lacked the second increase that accompanied the low-affinity interaction [Figure 5 (■)]. The midpoint of the SAPD binding isotherm obtained in the presence of Cdc42·GMP-PNP was ~10-fold lower than that observed for the high-affinity interaction of SAPD with PKCα alone, suggesting that the PKCα−Cdc42·GMP-PNP interaction resulted in an increase in phorbol ester binding affinity. The addition of ethanol (100 mM) was found to result in a further shift in the SAPD binding curve to lower phorbol ester levels, suggesting an increase in the phorbol ester binding affinity, whereas the maximal level of RET attained in the presence of Cdc42·GMP-PNP was unaffected [Figure 5 (▲)].

Effects of Ethanol on GDP-GTP exchange on Cdc42. Since the activation of PKCα by interaction with Rho GTPases results from conformational changes in both participating proteins (*49*), we investigated the possibility that alcohols may affect activity by modulating the conformation of the Rho GTPase. To achieve this, the effects of ethanol on the equilibrium between active, GTP-bound, and inactive, GDP-bound, forms of Cdc42 were determined. Consistent with the results of previous studies (*53*), initially loading Cdc42 with the fluorescent GDP analogue MANT-GDP resulted in an increase in emission fluorescence intensity (results not shown). The addition of unlabeled GMP-PNP then resulted in a time-dependent decrease in fluorescence due to the displacement of MANT-GDP [Figure 6 (●)]. Fitting the fluorescence as a function of time data to a pseudo-first-order rate equation yielded an observed rate constant (k_{obs}) of 0.011 ± 0.001 s^{-1}. This value was not significantly changed by the presence of ethanol (k_{obs} = 0.012 ± 0.002 s^{-1}), indicating a lack of an effect of the alcohol on the kinetics of nucleotide exchange [Figure 6 (×)] and ruling out the possibility that the effects of ethanol on PKCα activity might have been due to an impact on nucleotide binding to the GTPases. To validate the assay system that was used, the effects of Rho-GDI on the exchange rate were determined [Figure 6 (▲)]. Consistent with the results of previous studies (*58*), the addition of Rho-GDI at a concentration equal to that of the Cdc42·MANT-GDP complex resulted in a potent inhibition of the rate of exchange of MANT-GDP for GMP-PNP.

DISCUSSION

In this study, the effects of *n*-alkanols on the activity of PKCα induced by interaction with the Rho GTPases, RhoA and Cdc42, were investigated in an effort to provide further insight into the mechanism by which these agents affect PKC. It was found that both ethanol and 1-hexanol had biphasic concentration-dependent effects on PKCα activity associated with RhoA or Cdc42, which consisted of separate *competing* potentiation and inhibitory effects. The relative potencies of each effect were found to be a function of the concentration of Ca^{2+}, phorbol ester, and diacylglycerol so that the net effect of the alcohols on PKCα activity observed at a particular activator concentration was determined by the relative contribution of the two competing effects at that concentration. Thus, the effects of each alcohol switched between a potentiation, apparent at low activator levels, and an inhibition, apparent at higher activator levels. The observed dual effects of *n*-alkanols on PKCα activation suggest an impact of these agents on at least two distinct steps in the mechanism by which the enzyme becomes active upon association with Rho GTPases. Comparison of the effects of ethanol on the binding of PKCα to Cdc42 with those on activity revealed that the potentiation of activity corresponded to an increase in the extent of the interaction, whereas the inhibition appeared to be result from a reduction in the activity of PKCα associated with the GTPase. The results provide evidence that Rho GTPase-associated PKCα activity is a target for *n*-alkanols, which can have a plurality of effects on this activity depending on the presence and concentrations of PKC activators and the level of the alcohol.

The effects of ethanol on Rho GTPase-induced PKCα activity shared some features with those observed for PKC activity induced by association with membranes, or with the nonmembrane targets, F-actin and protamine sulfate, to the extent that the level of activity induced under each condition was attenuated in a concentration-dependent manner (*12*, *35*). The effects of the *n*-alkanols on PKCα activity induced under each activating condition were also similar in that the "basal" level of activity obtained in the absence of activators was unaffected by each alcohol. However, biphasic concentration-dependent effects of ethanol and 1-hexanol on TPA- and Ca^{2+}-induced Rho GTPase-associated activity observed here clearly differ from those observed previously for membrane- and F-actin-associated PKCα activity, which only displayed the inhibitory effect (*12*, *35*). The biphasic effects of each alcohol on the Ca^{2+} and phorbol ester concentration dependences of Cdc42-induced PKCα activation also reflected this

difference. Thus, whereas ethanol was found to increase Cdc42-associated activity at low activator levels, while decreasing activity at higher activator levels, the corresponding effects on membrane- and F-actin-associated PKCα activity consisted of only an inhibition of activity obtained at maximal activator levels (S. J. Slater and C. D. Stubbs, unpublished observations; see ref *35*). Consistent with this, it was observed here that the potentiation of the level of activity by ethanol, in the presence of saturating levels of Ca^{2+} and phorbol ester, corresponded to an increased extent of binding of PKCα to Cdc42, whereas our previous results indicated that the binding of PKCα to membranes or F-actin was unaffected by the alcohol. A further distinction is that the effects of ethanol on Rho GTPase-associated PKC activity are likely to be primarily on PKCα, based on our previous study that showed that the potency of activation by Rho GTPases was markedly higher for this isozyme than for PKC*β*I, -*β*II, -*γ*, −*δ*, -*ϵ*, and -*ζ* (*49*). In contrast, each of these isozymes is activated by interaction with F-actin (*34*) and by membrane association, and in each case, ethanol inhibited the activation of PKCα, -*β*I, and -*γ* but not PKC*δ*, -*ϵ*, or -*ζ* (S. J. Slater and C. D. Stubbs, unpublished observations; see refs *11* and *35*).

The observed biphasic effects of *n*-alkanols on Rho GTPase-associated PKCα activation suggest that these agents may have an impact on at least two distinct steps in the mechanism by which this interaction results in activation. In connection with this, recent studies have provided evidence that the mechanism by which PKC becomes active by membrane association also involves two discrete steps, consisting of an initial translocation to the membrane followed by an activating conformational change (*7*, *59*−*64*). It was suggested that prior to membrane association, PKCα is initially retained in an inactive "closed" conformation by an intramolecular interaction that occurs between the two cysteine-rich domains (C1A and C1B) and the C2 domains (*59*). In this state, the PKCα molecule is folded so that the active site is blocked by the N-terminal pseudosubstrate (*8*−*10*), and activator interaction with the C1 domains may be at least partially blocked by virtue of the C1−C2 interactions. Membrane association, mediated by parallel but independent binding of phorbol ester or diacylglycerol to one or both of the C1 domains, together with Ca^{2+} and anionic phospholipid binding to the C2 domain, then results in the disruption of these C1−C2 interactions, facilitating the formation of an active "open" conformation in which the pseudosubstrate is released from the active site (*59*). Recent studies from this laboratory have suggested that PKCα activation, resulting from binding to F-actin (*35*) and to Rho GTPases (*49*), may also in each case be mediated by phorbol ester binding to the C1 domains together with Ca^{2+} binding to the C2 domain. The possibility therefore exists that by analogy to the activation of PKCα by membrane association, the combined interactions of phorbol ester with the C1 domains, Ca^{2+} binding to the C2 domain, together with protein−protein interactions with Rho GTPases may together stabilize the open active conformation of the enzyme.

Previous studies from this laboratory have provided evidence indicating that the C1A and C1B domains within the PKCα molecule each contain functional activator binding sites that bind phorbol esters with low and high affinity, respectively (*16*, *17*, *65*). Importantly, it was found that the C1A domain of membrane-associated PKCα also contains a hydrophobic binding site for *n*-alkanols and anesthetics, based on the observation that these agents competed for the low-affinity phorbol ester interaction with this domain (*12*). The effects on the activity of membrane-associated PKCα mediated by this site were found to vary as a function of *n*-alkanol chain length. Thus, the interaction of long chain *n*-alkanols ($n < 6$) with the low-affinity phorbol ester binding site was found to enhance the level of high-affinity phorbol ester binding and the level of phorbol ester-induced activation, whereas short chain *n*-alkanols lacked such effects. Importantly, the finding here that ethanol displaced the low-affinity phorbol ester interaction with PKCα alone in the absence of membranes [see Figure 5 (△)] provides evidence that alcohol binding to the low-affinity phorbol ester binding site is an intrinsic property of the PKCα molecule itself and does not require membrane association.

The observation that ethanol had biphasic effects on Rho GTPase-associated PKCα activation contrasts with the lack of an effect of short chain *n*-alkanols on phorbol ester-induced membrane-associated PKCα activity. Furthermore, the finding that both ethanol and 1-hexanol had similar biphasic effects on Rho GTPase-induced activation also contrasts with the divergent effects of long and short chain *n*-alkanols on membrane-associated activity. These differences suggest that the mechanism underlying the effects of *n*-alkanols on Rho GTPase-induced activity may differ, although the finding that the effects of 1-hexanol on Rho GTPase-associated PKCα activity occurred within a concentration range lower than that of ethanol suggests that the site(s) on the Rho GTPase-associated enzyme is hydrophobic in nature, as shown previously for membrane-associated PKCα (*12*).

The finding that concentration−response curves for both phorbol ester-induced activation and phorbol ester binding to Cdc42-associated PKCα were each monophasic [see Figure 3A (●) and Figure 5 (■)], contrasting with the dual-sigmoidal curves obtained for membrane-associated PKCα (*16*), suggests that the activation of the isozyme induced by association with Cdc42 may be mediated by a single high-affinity phorbol ester interaction, rather than two phorbol ester binding sites of low and high affinity, respectively. Interestingly, a similar observation was made for the phorbol ester- and Ca^{2+}-induced activation of PKCα and PKC*β*I by association with F-actin, for which monophasic phorbol ester concentration−response curves for activation and phorbol ester binding isotherms were also obtained (*34*). The observation that low-affinity binding of phorbol ester to PKCα associated with Rho GTPase was not apparent further indicates that the mechanism underlying the effects of *n*-alkanols on the activity induced by this interaction differs from that involving competition for activator binding derived previously for the membrane-associated enzyme (*12*). However, even though the low-affinity phorbol ester interaction with Cdc42-associated PKCα was not detected within the phorbol ester concentration range that was used in our binding assays, the possibility remains that this site, while having a reduced affinity for phorbol ester binding, may still retain the ability to bind *n*-alkanols, and thus may mediate one or both of the processes that comprise the biphasic effects on activity.

The activating effect induced by low levels of the *n*-alkanols appears to result from an enhancement of the extent of binding of PKCα to Cdc42, based on the observation that the two processes share similar concentration dependencies. The finding that the activating effect of ethanol corresponded to a shift in the Ca^{2+}, TPA, and DiC8 concentration−response curves for Cdc42-induced activation to lower activator levels suggests that the interaction of the alcohol results in a decrease in the concentration of activators required to induce the PKCα−Cdc42 interaction. This was also apparent from the observation that the presence of ethanol resulted in an increase in phorbol ester binding affinity (see Figure 5). A possible explanation for this would be that the interaction of *n*-alkanols with a site within the C1 domains may interfere with the C1−C2 domain interactions that otherwise keep the enzyme in the closed inactive state, which would shift in the conformational equilibrium toward the open active state leading to the exposure of the Rho GTPase-binding site. In contrast, the inhibitory effect observed at high activator levels appeared to be "noncompetitive", with respect to the activator concentrations, and did not appear to result from an effect on PKCα−Rho GTPase binding. The question of whether both the potentiating and inhibitory effects on Rho GTPase-associated activity are mediated by the same site requires further study.

Studies of the association between proteins in the cellular environment are commonly based on methodologies that include immunoprecipitation, and indeed, the use of this technique has provided substantial evidence supporting a close association between PKC isoforms and Rho GTPases in cell lysates (*36*−*39*, *66*−*68*). It is clear, however, that along with the direct interaction demonstrated in our previous study, the PKC−Rho GTPase interaction in cells will also be mediated by indirect interactions involving intermediary complexes (*49*). Thus, separating effects of alcohols on PKC activity induced by the direct interaction with Rho GTPases from those resulting from a perturbation of indirect interactions in an immunoprecipitated complex derived from a cell lysate is not trivial, and requires the use of *in vitro* assays as utilized here.

In conclusion, the results identify the direct Rho GTPase−PKCα interaction as a novel target for ethanol and potentially volatile anesthetics. The results also suggest that the effects of alcohols on the ensuing PKCα activity will differ according to the cellular localization of the isozyme, the alcohol concentration, and the levels of activators. Furthermore, because of the centrality of both Rho and PKC in signaling networks, it is likely that the interaction between the two is critical for a wide range of associated cellular functions. An impact of ethanol upon such interactions could therefore underlie some of its deleterious effects on cell functioning.

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