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Alcohol binding in the C1 (C1A + C1B) domain of protein kinase C epsilon

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Abstract

Background—Alcohol regulates the expression and function of protein kinase C epsilon (PKC ϵ). In a previous study we identified an alcohol binding site in the C1B, one of the twin C1 subdomains of PKC ϵ .

Methods—In this study, we investigated alcohol binding in the entire C1 domain (combined C1A and C1B) of PKC ϵ . Fluorescent phorbol ester, SAPD and fluorescent diacylglycerol (DAG) analog, dansyl-DAG were used to study the effect of ethanol, butanol, and octanol on the ligand binding using fluorescence resonance energy transfer (FRET). To identify alcohol binding site(s), PKC ϵ C1 was photolabeled with 3-azibutanol and 3-azioctanol, and analyzed by mass spectrometry. The effects of alcohols and the azialcohols on PKC ϵ were studied in NG108-15 cells.

Results—In the presence of alcohol, SAPD and dansyl-DAG showed different extent of FRET, indicating differential effects of alcohol on the C1A and C1B subdomains. Effects of alcohols and azialcohols on PKC ϵ in NG108-15 cells were comparable. Azialcohols labeled Tyr-176 of C1A and Tyr-250 of C1B. Inspection of the model structure of PKC ϵ C1 reveals that these residues are 40 Å apart from each other indicating that these residues form two different alcohol binding sites.

Conclusions—The present results provide evidence for the presence of multiple alcohol-binding sites on PKC ϵ and underscore the importance of targeting this PKC isoform in developing alcohol antagonists.

Graphical abstract

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The authors have no conflict of interests.

AUTHOR CONTRIBUTION

Joydip Das designed the research. Joydip Das and Satyabrata Pany performed the research, analyzed the data, and wrote the paper.

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Keywords

protein kinase C ϵ (PKC ϵ); alcohol-binding site; alcoholism; fluorescence resonance energy transfer (FRET); mass spectrometry (MS); photoaffinity

1. INTRODUCTION

Protein kinase C ϵ (PKC ϵ) [1] is a member of the serine/threonine kinase superfamily that has been implicated in the neurobiology of drug abuse and alcohol addiction [2-5]. An understanding of the molecular mechanisms underlying the alcohol and PKC ϵ interaction and its functional consequence is crucial for the development of alcohol antagonists.

The PKC superfamily plays a central role in signal transduction, regulating diverse cellular functions by phosphorylation of target proteins [6-8]. PKCs modulate both pre- and postsynaptic neuronal functions, such as synthesis and release of neurotransmitters, regulation of receptors and ion channels, neuronal excitability, and gene expression in the brain [9-11]. The PKC superfamily can be separated into three major categories, the conventional (α , β I, β II, γ), novel (δ , ϵ , θ , η), and atypical (ζ , ι) isoforms. The conventional and novel PKCs have four domains, termed C1 through C4. The C1 domain consists of a tandem repeat of highly conserved cysteine-rich zinc finger subdomains, C1A and C1B, which bind diacylglycerol (DAG)/phorbol ester (Fig.1). These subdomains differ in their binding affinities for phorbol ester and DAG [12-15]. In contrast, the atypical PKCs are non-responsive to DAG/phorbol ester. There is high homology within domains among different members of the superfamily but the novel kinases differ in having the C2 domain at the N-terminal. Whereas the conventional PKCs associate with membranes in a calcium-dependent manner, the novel PKCs are calcium-independent. PKCs are translocated from the cytosol to the plasma membrane upon activation, by binding to isoform-specific RACK proteins [16, 17]. The cellular activation causes translocation of PKC to different subcellular locations in an activator-dependent manner [18]. Each domain plays a distinct role in the activation and subcellular translocation of PKC.

Behavioral tests reveal that PKC ϵ null mice have drastically decreased alcohol consumption. PKC ϵ null mouse consumed approximately 75% less alcohol and showed reduced ethanol preference in a two-bottle choice paradigm [19] and self-administered about 50% less

ethanol in an operant self-administration paradigm compared to the wild-type mice [20]. These findings are associated with enhanced sensitivity to the acute hypnotic effects of ethanol. Effects of ethanol are isoform specific, as PKC γ null mice consumed more alcohol compared to the wild-type mice in the two bottle choice paradigm [21] and displayed reduced sensitivity to ethanol [22]. Furthermore, knock-down of PKC ϵ in different brain regions reduced ethanol intake [23]. Rescuing PKC ϵ expression in different brain regions restores ethanol intake and hypnotic sensitivity to levels observed in wild-type mice [24].

Chronic alcohol exposure up-regulates PKC ϵ expression in the dorsal root ganglion (DRG) neuron [25, 26]. Ethanol stimulates expression and translocation of PKC ϵ in PC12 cells [27, 28]. The prominent effect of ethanol on PKC ϵ was evident in the NG108-15 cell system, in which ethanol altered translocation of PKC ϵ either directly [29, 30] or synergistically with dopamine [31].

In order to identify the site(s) of alcohol action on PKC ϵ , we previously studied the alcohol binding site in its C1B subdomain [32]. We found that ethanol inhibited DAG-stimulated PKC ϵ activity with an EC₅₀ of ~43 mM which is higher than the social drinking range (10-20 mM) but below the range of lethal blood alcohol concentration (50-110 mM)[33]. We also identified His-248 and Tyr-250 as the alcohol binding sites in the C1B subdomain using photolabeling and mass spectrometry [32].

In continuation of our studies on characterizing the alcohol binding site(s) in PKC ϵ , here we used the PKC ϵ C1 (combined C1A and C1B) to study alcohol binding. The effect of ethanol, butanol, and octanol on the FRET between the C1 domain and SAPD and dansyl-DAG (Fig. 2) was determined. Two alcohol binding residues Tyr-176 in C1A and Tyr-250 were identified by photolabeling and mass spectrometry.

The diazirine analog of ethanol, aziethanol has never been synthesized because of the inherent instability associated with the molecule. Therefore, the azialcohols, such as, 3-azibutanol and 3-azioctanol have been used extensively for studying the alcohol binding sites in enzymes and receptors [34]. However, neither the effect of these azialcohols nor the effects of the parent alcohols, butanol and octanol on PKC ϵ in a cellular environment were previously examined. Studying the effect of these alcohols on the expression/activity of PKC ϵ would therefore add the biological significance of the present binding studies. We found that in NG108-15 cells ethanol and butanol increased the expression of PKC ϵ in cytosol, while octanol decreased the expression.

2. EXPERIMENTAL

2.1. Materials

E.coli BL21 (DE3) competent cells were obtained from Stratagene, La Jolla, CA, USA. pET21d+ was purchased from Novagen, USA. SAPD (sapintoxin D) was purchased from LC laboratories, Woburn, MA, USA. Glutathione–Sephacrose 4B was from GE Healthcare Life Sciences, Piscataway, NJ, USA. Dansyl-DAG was kindly provided by Dr. M. D. Best of the University of Tennessee. 3-Azioctanol [35] and 3-azibutanol[36] were synthesized as described previously. Aldrithiol-4 (4,4'-dithiodipyridine) was purchased from Sigma

chemicals Co., St. Louis, MO, USA. LB (Luria–Bertani) broth medium, TB (terrific broth) medium, and SOC (super optimal culture) were obtained from Invitrogen, Carlsbad, CA, USA. Protein estimations were carried out using the Bradford protein estimation kit from Bio-Rad, Hercules, CA, USA. Primary (PKC ϵ and β -actin) and secondary (anti-rabbit HRP) antibodies were from Cell Signaling Technology, Danvers, USA. All the other reagents were from Sigma and of highest purity available.

2.2. Cell culture, Cell fractionation and Western blot analysis

NG108-15 cells were maintained in DME medium supplemented with 25 mM glucose, 44 mM NaHCO₃, 10% fetal bovine serum (FBS), and 100 unit/ml antibiotics in humidified atmosphere (37 °C, 5% CO₂). Prior to the alcohol treatment, cells were starved in the media devoid of FBS and antibiotics for 12 h at 60–70% confluency. This was done to avoid possible variations induced by serum factors and their interaction with alcohol [37, 38]. Cells were treated with various concentrations of alcohol (ethanol, butanol, octanol, azibutanol, or azioctanol) for 24 h. For measuring the effect of alcohol on the PKC ϵ expression in total cell lysate, alcohol-treated cells were washed PBS and lysed with cell lysis buffer (Cell Signaling, Danvers, MA). Cells were then sonicated briefly (20 s, 5 s pulse, 5 s break, 10% amplitude) and centrifuged at 10,000 rpm (Sorvall Legend Micro 17) for 10 min at 4 °C. Cell lysate (40 μ g/lane) was subjected to SDS-PAGE and immunoblot.

Membrane fractionations were done according to the protocol described earlier [30]. Cell lysis was carried out in lysis buffer (20 mM Tris, protease inhibitor, pH 7.4) with brief sonications (4 times, 5 seconds, and 10% amplitude). Cell debris was removed by centrifuging the sample at 3500 rpm (Sorvall Legend Micro 17) for 10 min at 37 °C. Cell lysate (200 μ g protein/100 μ l) was then centrifuged at 40,000 rpm for 2 h at 4 °C using Beckman TLA 120.2 rotor to separate out the cytosolic fraction. The pellet (membrane fraction) was incubated in lysis buffer (100 μ l) containing 1% Triton X-100 for 1 h in ice and was sonicated again for homogenization. Cytosolic (40 μ g) and membrane fraction (40 μ g) were subjected to SDS-PAGE and immunoblot. Proteins were transferred to nitrocellulose membrane and the membrane was incubated successively with 5% BSA in TBST buffer (50 mM Tris, pH 7.5, containing 0.15 M NaCl and 0.1 % Tween-20) at room temperature for 1 h. The blots were probed with PKC ϵ (1:1000 dilution) and β -actin antibody (1:5000 dilution). The blot was further probed with anti-rabbit HRP (1:5000 dilution). The immunoreactive bands were visualized using ECL (enhanced chemiluminescence) reagent (Pierce, Rockford, IL) and quantitated using Alpha Imager Gel Documentation system (Alpha Innotec, Santa Clara, CA).

2.3. Expression and purification of PKC ϵ C1

The PKC ϵ C1 (residues 170-292 in the full-length protein) was subcloned into pET28a⁺ vector with the carboxy terminus having a His₆ tag [39]. The recombinant plasmid was transformed in *E. coli* BL21 (DE3) for expression and purification of the protein. A single colony from a freshly transformed plate was inoculated in 10 ml LB medium with ampicillin (100 μ g/ml) and the culture was grown overnight at 37 °C. 5 ml of this inoculum was added to 1 liter TB-ampicillin media and allowed to grow until the absorbance at 600 nm reached 0.8. IPTG (0.5 mM) was then added to induce protein expression and cultures were allowed

to grow for an additional 4 h at 37 °C. The bacterial cells were harvested and suspended in 20 ml of Buffer A (50 mM Tris, pH 10, 100 mM NaCl, 50 μ M ZnSO₄, 8 M Urea) and kept at room temperature for 1 h. The cells were then sonicated 20 times for 5 s at 30% amplitude using a Branson digital sonifier model 250 and the insoluble cellular debris was removed by centrifugation (17,000g for 30 min at 22 °C). The supernatant constituted the denatured protein fraction which was incubated with 1 ml bed volume of Ni-NTA agarose column. The column was washed with Buffer B (Buffer A plus 10 mM imidazole) until maximum amounts of impurity were removed. Protein was eluted using elution buffer (Buffer A plus 500 mM L-arginine, 10 mM cysteine protease inhibitor and 300 mM imidazole). Protein refolding was initiated by dilution of the denatured protein suspension up to 0.5 M urea with refolding buffer (Buffer A with 250 mM L-arginine, 10 mM cysteine protease inhibitor, and without 8 M urea). Refolded protein was concentrated and dialyzed with above refolding buffer. Purity of the protein was checked by SDS-PAGE (15%) using Coomassie blue staining. Molecular weight of the purified protein was verified by MALDI-TOF mass spectrometry (Applied Biosciences Voyager System 4160). Protein (10nmole/ μ l) was mixed properly in 1: 10 ratio with saturated solution (50:50 water/aceonitrile with 0.1% trifluoroacetic acid) of α -cyano-4-hydroxycinnamic acid followed by ZipTip (Milipore Corp. GA) purification. The functionality of the refolded protein was examined by intrinsic fluorescence and ligand binding assays.

2.4. Reaction of PKC ϵ C1 with aldrithiol-4 (4,4'-dithiodipyridine) (PDS)

PKC ϵ C1 contains 13 cysteine residues. The reactivity of these cysteine residues with water soluble thiol reagent could be an indicator of the number of cysteine residues exposed to water or buried inside the protein. To study this, PDS was used as the thiol reactive agent [40]. The assay mixture (500 μ l) contained 5 μ M PKC ϵ C1 and 25 μ M of aldrithiol-4 in 5 mM phosphate buffer, pH 6. Reaction of cysteine with PDS generates dithiopyridine, which has the absorption maximum at 323 nm. Right after the addition of PDS to the protein, absorbance at 323 nm was measured at regular time intervals. Background absorption at 323 nm was recorded by using the same concentration of PDS as a reference. Stoichiometry of labeling was determined using the extinction co-efficient value of 19,000 M⁻¹cm⁻¹ for dithiopyridone [41]. The denatured protein (in 5 mM phosphate buffer, pH 6 and 8M urea) was also labeled with PDS for comparing with the refolded PKC ϵ C1.

2.5. Fluorescence measurements

Fluorescence measurements were carried out using PTI fluorimeter (Photon Technology Instruments, Princeton, NJ). FRET between the tryptophan residues of PKC ϵ C1 and SAPD/dansyl-DAG (Fig.2) and the effect of alcohol on FRET were determined by steady state fluorescence spectroscopy. Briefly, the assay system (1 ml) consisted of buffer (50 mM Tris, 100 mM NaCl and 50 μ M ZnSO₄, pH 8), 1 μ M SAPD, or dansyl-DAG and purified PKC ϵ C1 (4 μ M for SAPD and 1 μ M for DAG). After 60 min incubation at 25 °C, the mixture was excited at 290 nm and emission spectra were recorded from 300 to 550 nm. Fluorescence emission maxima of tryptophan, SAPD, and dansyl-DAG were found to be at 336, 437, and 505 nm, respectively. The relative FRET value was corrected by subtracting the background FRET value following the formula as described earlier [42]: $[F_{i, +protein} - F_{i, -protein}] - [F_{0, +protein} - F_{0, -protein}]$, where $F_{i, +protein}$ and $F_{i, -protein}$ are the intensities of

SAPD or dansyl-DAG in the presence and absence of PKC ϵ C1, respectively, and $F_{0,+protein}$ and $F_{0,-protein}$ are fluorescence intensities of the buffer solution in the presence or absence of PKC ϵ C, respectively. For FRET involving SAPD and dansyl-DAG, intensities at 425 nm and 485 nm, respectively, were recorded. To study the effects of alcohol, increasing concentrations of alcohol were added to the protein and ligand mixture and spectra were recorded after 30 min incubation with slow stirring. The change in fluorescence intensities for each concentration of alcohol was normalized using the equation: $(F_i - F_0)/F_0$, where F_i and F_0 are intensities of SAPD or dansyl-DAG plus PKC ϵ C1 in the presence or in the absence of alcohol.

2.6. Photoincorporation of 3-azioctanol and 3-azibutanol into PKC ϵ C1 and identification of the site of photoincorporation by mass spectrometry

Photolabeling experiments were done according to the methods described earlier [32, 43]. Briefly, 50 μ l of freshly purified PKC ϵ C1 protein (1 μ g/ μ l) and an azialcohol (1 mM) was incubated for 30 min. Photo-labeling was carried out at 365 nm for 30 min using a model UVL-56 20 W hand-held lamp (Black-Ray, Upland, CA, U.S.A.). Alkylation, reduction by DTT, trypsin digestion, and protein sequencing with LTQ instrument (Thermo-Fisher, San Jose, CA, USA) were done as described earlier [32, 44, 45].

2.7. Molecular modeling

Robetta [46, 47], the structure prediction server which models domains either by homology modeling or by *ab initio* modeling, was used to predict PKC ϵ C1 domain structure. Sequence of rat PKC ϵ C1 (Fig.1) was used as the input sequence. Robetta modeled the five lowest energy structures on the basis of the structure of homology models using the reference structures with PDB codes 2e73 and 2enZ for C1A and C1B domain, respectively. These two subdomains are connected to each other with a flexible linker. Orientation of C1A and C1B subdomains in the predicted structure with respect to membrane is consistent with a previously reported structure [48, 49], which shows that both the DAG binding domains are on the same face but with varied proximity. The Ramachandran plot showed that all of the residues were within permissible region for all models. Overall quality factor for homology models, such as covalent bond distances, bond angles, stereo chemical validation, atom nomenclature, and non-bonded interactions between different atoms types were validated using SAVS server (http://nihserver.mbi.ucla.edu/SAVES_3). Predicted structure was further energy minimized using Swiss pdb viewer [50].

2.8. Statistical Analysis

Statistical analyses were performed using Sigma Plot 11. Statistical analysis in figures 3 and 6 was based on three independent experiments. The results were expressed as the mean $\pm$ SEM. Statistical significance was established using one-way ANOVA, followed by Bonferroni *post hoc* test. A value of $P < 0.05$ was considered significant.

3. RESULTS

3.1. Effect of alcohol and azialcohol on PKC ϵ in NG108-15 cells

To find out the effect of butanol, octanol, and their diazirine analogs on PKC ϵ , we measured the expression of endogenous PKC ϵ in NG108-15 cells by Western blot analysis of the total cell lysate (Fig. 3A). Incubation of ethanol (100 mM) for 24 h increased the total PKC ϵ expression by 55% compared to control cells. This increase was also observed for butanol and azibutanol. In contrast, octanol and azioctanol decreased the expression by 60-65% compared to untreated cells. We also found a consistent increase in expression of PKC ϵ in the presence of butanol or 3-azibutanol, like ethanol. We also measured the amounts of PKC ϵ in the cytosol (Fig. 3B) and membrane fraction (Fig. 3C) of the alcohol-treated cells. Ethanol and butanol increased and octanol decreased the amounts of PKC ϵ in the cytosol. Overall, the effect of the alcohols on PKC ϵ in the cytosolic fraction was similar to that of the total cell lysate (Fig. 3A). On the other hand, the levels of PKC ϵ in the membrane fractions did not change.

3.2. Protein purification and characterization

The C1 (C1A+C1B) domain of PKC ϵ has been tagged with a His₆ at its C-terminus for the purposes of easy purification. This type of modification did not affect the overall folding and ligand property in PKC ϵ C1 as reported earlier [39]. Because of the presence of 13 cysteine residues in this domain that may cause intrinsic instability and insolubility, we decided to characterize the protein thoroughly before using it for alcohol binding studies. The characterization was done by several methods. The purity of the protein was checked by SDS-PAGE and the Coomassie blue staining of the band revealed that the protein was about 95% pure (Figure 4A). The molecular mass was confirmed by MALDI-TOF analysis (Figure 4B), which showed the main peak is at around 15986 Da as expected. In the quantitation experiment of the water-exposed cysteine residues of PKC ϵ C1 with thiol reactive agent aldrithiol-4, it was found that while in the denatured protein about 10 cysteine residues reacted with thiol reactive agent aldrithiol-4 (PDS), no cysteine was reactive in the renatured protein. The presence of these shielded cysteines indicates the folded state of the renatured protein (Fig. 4C). In addition, the fluorescence emission maximum of the renatured PKC ϵ C1 was at 336 nm, the typical emission maximum of a buried tryptophan, again indicating the folded state of the protein (Fig. 5A).

3.3. Effect of alcohol on the fluorescence resonance energy transfer between PKC ϵ C1 and SAPD/dansyl-DAG

For studying energy transfer in PKC ϵ C1, we used two different fluorescent PKC ligands, SAPD (a fluorescent phorbol ester analog) and dansyl-DAG (a fluorescent DAG analog).

The emission maxima of SAPD and dansyl-DAG are 437 nm and 505 nm in buffer, respectively, when excited at 355 nm. In the presence of PKC ϵ C1, these emission maxima shifted to 425 nm and 485 nm, respectively, indicating binding of the fluorophores to the protein. When excited at 290 nm the fluorescence intensities of these two fluorophores at the corresponding emission maxima were much less in the absence of PKC ϵ C1 than the intensities in the presence of PKC ϵ C1 (Fig. 5). This indicates the energy transfer between

the tryptophan and the fluorescence ligands, where the former acts as a donor and the latter acts as an acceptor. Next, we measured the effect of alcohol on the FRET between PKC ϵ C1 and these ligands. The effect of butanol (5 mM) on the FRET between PKC ϵ C1 and SAPD and the effect of octanol (1mM) on the FRET between PKC ϵ C1 and dansyl-DAG are shown on Fig. 5A and Fig. 5B, respectively.

The effect of varying concentrations of ethanol, butanol, and octanol for both SAPD and dansyl-DAG is shown in Fig. 6. Overall, with increasing concentrations of either ethanol, butanol, or octanol, the FRET with SAPD increased although the extent of this increment varied among the alcohols studied here. The increase in FRET indicated that with the increasing concentrations of alcohol, there was increased binding of SAPD to the C1B. Similar results were also observed in our previous studies, where alcohols increased the SAPD affinity for truncated δ C1B [44] and truncated ϵ C1B [32]. In the case of PKC α , Slater et al also showed that alcohol increased the SAPD binding to the high affinity phorbol site, i.e., α C1B [51]. The changes in FRET between bound SAPD and tryptophan in the presence of alcohol could also be due to the conformational change of the protein caused by alcohol. However, the recent 1.3Å structure of the cyclopropylmethanol-bound δ C1B [51] revealed no significant conformational changes between the native and the alcohol-bound structure, thereby reducing this possibility.

Effect of varying concentrations of alcohol on the energy transfer between PKC ϵ C1 and dansyl-DAG was biphasic. At lower concentration range, ethanol (0-65 mM) and octanol (0-1 mM) caused an increase in FRET, but at higher concentration ranges, ethanol (65-130 mM), butanol (13-54 mM) and octanol (1-3 mM) caused decrease in FRET. This decrease in DAG binding in the presence of alcohol indicates that alcohol binding either allosterically or competitively occludes DAG from its binding site.

3.4. Identification of the site of azialcohol photoincorporation in PKC ϵ C1

To identify possible alcohol binding site(s), PKC ϵ C1 was photolabeled with azialcohols 3-azibutanol and 3-azioctanol. After photoincorporation, alkylation, and trypsin digestion, the samples were eluted directly from the online HPLC column into the mass spectrometer, and MS/MS spectra were acquired under automatic acquisition of the most intense ion from each MS scan. About 97% of the peptide sequence for the PKC ϵ C1 was detected by this method. For an unlabeled PKC ϵ C1 sample, the identified peptides, their sequences, charge states, accuracies, and the related peptide match parameter, such as delta correlation (dCn) and X-correlation (XCorr), are listed in Table 1. The collision-activated dissociation MS/MS spectrum of 3-azibutanol modified doubly charged peptide FMATYLR with m/z 487.3, obtained from the digest of 3-azibutanol (1mM)-labeled PKC ϵ C1, is shown in Fig. 7A. All the b ions except the b1 were detected and b5-b7 had extra mass of 72 Da indicating modification at position 5. In the y ion series all the ions were detected and y3-y7 had extra mass of 72 Da. This confirmed that the tyrosine at position 5 (Tyr-176 in the full length protein) in the peptide is modified with azibutanol. The delta correlation (dCn) and the cross correlation (XCorr) values for this peptide are 0.236 and 2.8, respectively. The unmodified peptide with the m/z of 451.2 was also detected. In the 3-azibutanol photolabeled sample, the peptide FGIHNYK was also found to be labeled. Fig. 7B shows the collision-activated

dissociation MS/MS spectrum of the doubly charged peptide ion with m/z 476.3 for the peptide FGIHNYK. In the b ion series, b1 through b6 were observed of which b6 ion has the extra mass of 72 Da for the addition of a molecule of 3-azibutanol. In the y ion series, y2-y5 were observed and have extra mass of 72 Da for the addition of one molecule 3-azibutanol. Although the y6 was not observed, the $y6^{++}$ at 402.4 was very distinct in the MS/MS spectrum. Another doubly charged fragment ion $y5^{++}$ was also observed at m/z 374. This indicates that position 6 of the peptide is the site of modification which is Tyr-250 of the full length PKC ϵ . The unmodified doubly charged peptide was detected at m/z of 440 having all the assigned b and y ions.

In the 3-azioctanol (1mM) photolabeled sample, the same doubly charged heptamer peptide FMATYLR at m/z 515.3 was found to be modified (Fig. 7C). In this case all the b ions except the b1 were detected and b5-b7 have extra mass of 128 Da indicating 3-azioctanol modification. In the y ion series all the ions from y1 to y7 were detected and the y3 through y7 were found to be modified. This indicated that tyrosine at position 5 is the site of modification. In this case, the unmodified peptide also has been detected and showed similar fragmentation pattern. In this digest, another peptide FSVNMPHKFGIHNKYK corresponding to the C1B subdomain was also found to be modified with 3-azioctanol (XCorr, 2.08; dCn, 0.04). However, the exact site of modification could not be assigned because only 22 of the 84 possible fragment ions were observed in the MS/MS spectrum.

In summary, the photolabeling experiments identified Tyr-176 in the C1A and Tyr-250 in the C1B subdomain of PKC ϵ .

4. DISCUSSION

Regulation of alcohol action by PKC, especially PKC ϵ , is well studied [2, 23, 24] although the mechanism of this regulation is still not well-established. We hypothesized that alcohol molecules directly interact with PKC ϵ and identified an alcohol binding site in the C1B subdomain of PKC ϵ . In view of the observation by Slater et al that in PKC α , alcohol binding sites are present in both C1A and C1B [51], we asked the question whether such alcohol binding sites could also be present in PKC ϵ . To address this we expressed and purified the C1(combined C1A and C1B) domain of PKC ϵ and studied its interactions with alcohols. PKC is a complex kinase which is active when tightly associated with the membrane (hence limited solubility) and susceptible to proteolysis when activated. PKC also undergoes massive conformational changes during its activation. To circumvent these issues we decided to study the truncated C1 domain of PKC ϵ for pinpointing the alcohol binding sites. Such a piecemeal approach is not uncommon in studying alcohol-protein interactions [52] [43]. The C1 domains of PKC possess a highly conserved structure that retains all the structural elements whether studied in isolation [53] or in the full length protein [54]. For example, binding affinity of PDBu (phorbol-12, 13 dibutyrate) to PKC ϵ C1B (K_d = 1.5 nM) and full-length PKC ϵ (K_d = 0.63 nM) are not significantly different [55].

Our results show that azialcohols behave similarly to their precursor alcohols on the expression of PKC ϵ in NG108-15 cells (Fig. 3). Ethanol, butanol and azibutanol increased expression of PKC ϵ in a manner similar to what was reported earlier for ethanol (200 mM,

48h) by Gordon et al [29]. This data for ethanol is consistent with cytoplasmic increases as noted by Gordon et al.[29]. This is likely indicative of transcription/translation effects. Decrease in protein expression by octanol and 3-azioctanol could be due to octanol-induced cell death [56] as we observed that a significant number of cells were not viable and were detached from the plates when the octanol concentration was higher than 1 mM. Other possibilities could be the inhibition of cell adhesion and/or alcohol-dependent cell apoptosis at higher alcohol concentrations [57]. Another objective of this study was to find out if alcohol and azialcohol could induce translocation of PKC ϵ from cytosol to other cellular compartments. The results shown in Fig.3 demonstrated that there was no change in the PKC ϵ in the membrane fraction, suggesting that there was no significant translocation occurred from cytosol to plasma membrane. However, this does not rule out the possibility of translocation of PKC ϵ to the perinuclear region, which the present study could not resolve. Previous studies on the effect of ethanol on PKC ϵ in cells and brain tissues suggested that alcohol-induced increase of PKC ϵ in cytosol was a combination of both increased expression and translocation to the membrane or perinuclear region [29, 58].

There are few discrepancies in the literature on the magnitude of the DAG/phorbol ester affinity for the C1A and the C1B subdomains of PKC ϵ . For example, whereas Irie et al first reported much higher affinity of ϵ C1B ($K_d=1.5$ nM) than ϵ C1A ($K_d > 10000$ nM) for phorbol ester [55], Stahelin et al reported only 3 times higher affinity for ϵ C1B than ϵ C1A [59]. In another publication, Irie et al. reported only 7 times higher phorbol ester affinity for ϵ C1B ($K_d=0.81$ nM) than ϵ C1A ($K_d=5.6$ nM) [60]. For DAG, ϵ C1A binds to DiC18 (a DAG analog) with about five times higher affinity than ϵ C1B [59]. Taken together, the ϵ C1B has higher affinity than ϵ C1A for phorbol ester and ϵ C1A has higher affinity than ϵ C1B for DAG. That SAPD (a phorbol ester) and dansyl-DAG (a DAG analog) have different affinities for C1A and C1B, and show different FRET sensitivities for different alcohols, it is logical to conclude that alcohols exert differential effects on C1A and C1B subdomains of PKC ϵ .

In addition to the fluorescent phorbol ester SAPD, which was used previously for studying the C1B subdomain [32], here we introduced a new fluorescent DAG analog, dansyl-DAG. The present result is closely similar to the previous results involving the isolated C1B subdomain in that ethanol, butanol, and octanol increased the SAPD binding to the C1B subdomain and the azialcohol labeled the protein in the vicinity of His-248 and Tyr-250. The only difference is that in the photolabeling experiment, we did not detect the His-248 unambiguously, although a peptide, FSVNMPHKFGIHNYK, containing this residue was photolabeled. This is most likely due to the presence of the linker and C1A subdomain which fine-tunes the access of this residue to the photo reagents and/or the protease trypsin. In addition to the Tyr-250 in C1B we identified another site Tyr-176 in the C1A. The photoincorporation of the tyrosine residues in both C1A and C1B subdomains of PKC ϵ photolabeling cannot be viewed as azialcohols being solely tyrosine-selective agents because azialcohols react with variety of amino acids [34, 43] and other tyrosine residues (Tyr-182 and Tyr-199) in the C1 domain were not labeled. Interestingly, the double dissociative effects of n-chain alcohols on phorbol/DAG binding closely resembles to the recent *in vivo* data reported by the Morrow group where DAG-dependent PKC ϵ activity was

evident at moderate alcohol doses [58], but completely absent at higher doses [61]. However, more studies are required to establish direct correlation between the *in vitro* binding and the *in vivo* data.

The modeled structure of the C1 domain indicates that the distance between the photolabeled residues, Tyr-176 and Tyr-250 (C β of Tyr-176 and C β of Tyr-250) is 40.07 Å, (Fig. 8) meaning that these residues may not be part of a single alcohol binding site but most likely form two different alcohol binding sites, one in the C1A and the other in the C1B. In the C1A, the Trp-191 (involved in FRET) and Tyr-176 are 10.38 Å apart from each other and both are located close (3-5 Å) to the ligand binding site of C1A, indicating that binding of alcohol molecules in the vicinity of Tyr-176 could influence the energy transfer between dansyl-DAG and Trp-191. The structure also reveals that the side chain of Tyr-176 and Trp-191 are oriented in the same direction forming a well-defined binding site in which both butanol and octanol could fit. Leu-177, Arg-178, Gln-179, and Gln-197 also contribute to the formation of this binding site. In the C1B, the photolabeled residue Tyr-250 is located 13.25 Å from Trp-264 (involved in FRET) and both are located about 5-7 Å from the ligand binding site of C1B. However, their side chains are oriented opposite to one another indicating that Trp-264 may not contribute to the formation of the binding site. His-248, Val-252, Thr-254, and Gln-272 are close to Tyr-250 and form the putative alcohol binding site in C1B. While it is not clear whether these differences in orientation could affect the alcohol binding of these domains, this will certainly affect their membrane affinity [59].

A comparison of the alcohol binding site residues of PKC δ , PKC α , and PKC ϵ from the available literature data reveals that, like PKC δ and PKC α , PKC ϵ has alcohol binding sites in the C1A and the C1B domains. While the alcohol binding residues have not been determined for the α C1A, the alcohol binding residue in the δ C1A is Tyr-187, which is homologous to Tyr-199 of PKC ϵ . Tyr-199 is 11.18 Å apart from Tyr-176 and orientation of their side chains is in opposite directions, indicating that they perhaps do not belong to the same alcohol binding site. On the other hand the Tyr-236 of δ C1B, which is homologous to His-248 of PKC ϵ , is close to Tyr-250, the site identified in this study. These data indicate that the alcohol-binding site for δ C1A and ϵ C1A are different, but could be similar for δ C1B ϵ C1B. Furthermore, the residues present within 3 Å of the alcohol binding residues of C1A and C1B are different. Interestingly, our recent 1.3 Å data for the PKC δ C1B-cyclopropylmethanol revealed that the Tyr-236, homologous to His-248 in epsilon, formed a hydrogen bond (2.8-3 Å) with the hydroxyl group of the alcohol and the methylene group of Met-239, homologous to Lys-251 in PKC ϵ , which undergoes van der Waals interactions with the cyclopropane ring of the alcohol [52]. The homologous residues in PKC α and PKC ϵ isoforms are different (Fig.9). All of these indicated that the microenvironments of the alcohol binding sites in C1A and C1B are different for different PKC isoforms.

Our results that alcohol regulates the activity of PKC ϵ [32] and binds to it, validate it as a potential target of alcohol, as regulation and binding are two of the four criteria suggested by Harris et al [62] for a protein to be considered as a target of alcohol, and underscore the importance of this PKC isoform in developing an alcohol antagonist.

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Abbreviations

PKC	protein kinase C
FRET	fluorescence resonance energy transfer
DAG	<i>sn</i> -1,2-diacylglycerol
HRP	horseradish peroxidase
LC-MS/MS	liquid chromatography/ tandem MS
PDB	Protein Data Bank
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
POPS	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphoserine
SAPD	sapintoxin-D
dansyl-DAG	(2S,3S)-1-(4-((5-(dimethylamino)naphthalene-1-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)-4-hydroxybutane-2,3-diyl distearate
GABA	gamma amino butyric acid
SDS-PAGE	sodium dodecyl sulfonate-polyacrylamide gel electrophoresis
MALDI-TOF	matrix-assisted laser-desorption ionization–time-of-flight
WT	wild-type
PBS	phosphate buffered saline
RACK	receptor for activated C-kinase
DiC18	<i>sn</i> -1,2-dioleoylglycerol
PDBu	phorbol 12,13-dibutyrate

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Highlights

- Alcohol and azialcohol affect PKC ϵ expression in NG108-15 cells
- Alcohols bind to both C1A and C1B subdomains of PKC ϵ
- Azialcohols photolabel Tyr-176 in C1A and Tyr-250 in C1B of PKC ϵ

Figure 1.

(**A**) Schematic representation of the domains of protein kinase Cε: the C1 regulatory domain binds to lipids, diacylglycerol, and phorbol esters; the C2 regulatory domain binds to anionic lipids but does not bind to Ca^{2+} ; C3 is the ATP-binding domain and C4 the catalytic domain. PS denotes the pseudo-substrate-binding domain. (**B**) Amino acid sequence of PKCεC1. PKCεC1 with 123 amino acid residues (C1A, 170-220 and C1B, 242-292) was used in the present study. Underlined extra residues were included for cloning and purification purposes.

Figure 2.
SAPD (left) and dansyl-DAG (right).

Figure 3.

Effect of alcohols and azialocohols on PKC ϵ in NG108-15 cells. Upper panels, Western blot analysis of total cell lysate (A), cytosolic fractions (B), and membrane fraction (C) of PKC ϵ after cells were treated with different alcohols for 24 h. 1) control, 2) 100 mM ethanol, 3) 50 mM butanol, 4) 5 mM 3-azibutanol, 5) 0.5 mM octanol and 6) 0.1 mM 3-azioctanol. Lower panel, corresponding bar graph of densitometry analysis of upper panel immunoblots. Values represent means $\pm$ SEM of three measurements. Statistical analysis was performed using one-way ANOVA with a *post hoc* Bonferroni test. * $P < 0.05$ compared to control; # $P < 0.05$ compared to ethanol; & $P < 0.05$ compared to butanol; \$ $P < 0.001$ compared to control and % $P < 0.05$ compared to ethanol and ** $P < 0.05$ compared to butanol. There were no significant differences in membrane fractions ($P > 0.05$).

Figure 4.

Characterization of PKCεC1. **(A)** SDS-PAGE (15%) of the purified PKCεC1. M, molecular weight marker and P, purified PKCεC1. **(B)** MALDI-TOF mass spectrum of purified PKCεC1. The observed peak at 15986.47 Da matches with the expected molecular mass of the protein. **(C)** Reaction of PDS with denatured (○) and refolded (▲) PKCεC1. The degree of labeling was presented as the ratio of moles of 4-thiopyridone released to the moles of protein used and plotted as a function of time by measuring the absorbance of 4-thiopyridone at 323 nm, as described in the methods section.

Figure 5.

Effect of alcohol on energy transfer between tryptophan of PKCεC1 and fluorescent PKC ligands SAPD and dansyl-DAG. **(A)** Emission spectra of a, SAPD (1 μM); b, PKCεC1 (4 μM); c, PKCεC1 (4 μM) + SAPD (1 μM); and d, PKCεC1 (4 μM) + SAPD (1 μM) + butanol (5 mM). **(B)** Emission spectra of a, dansyl-DAG (1 μM); b, PKCεC1 (1 μM); c, PKCεC1 (1 μM) + dansyl-DAG (1 μM); and d, PKCεC1 (1 μM) + dansyl-DAG (1 μM) + octanol (1 mM). Samples were excited at 290 nm and emission spectra were recorded from 300 nm to 600 nm. Alcohols were incubated for 30 min before the spectra were recorded.

Figure 6.

Plot of normalized energy transfer between tryptophan of PKC ϵ C1 and SAPD (▲) or dansyl-DAG (○) in the presence of (A) ethanol; (B) butanol and (C) octanol. Energy transfer efficiency values were calculated using the data from Figure 5 and using the equation described in the methods section. For SAPD (1 μ M), protein concentration was 4 μ M and for dansyl-DAG (1 μ M) the protein concentration was 1 μ M. Samples were excited at 290 nm. Results are means $\pm$ SE from 3 independent experiments. Statistical analysis was performed using one-way ANOVA with a *post hoc* Bonferroni test. * $P < 0.05$ and ** $P < 0.01$ compared to 0 concentration of alcohol.

Figure 7.

MS/MS data for the photolabeled peptides. At the top of the figure the predicted m/z ratio of N-terminal ions (b ions) and C-terminal ions (y ions) are shown above and below the sequence, respectively. They are singly charged unless noted otherwise. The horizontal arrows show which m/z values for the b ions (above) and y ions (below) have a mass of 72 Da for 3-azibutanol or 128 Da for 3-azioctanol added to them. Observed values are shown in bold. **(A)** Identification of the Tyr-176 as the site for 3-azioctanol in the PKC ϵ C1 domain. MS/MS data for the 3-azibutanol (1 mM) modified heptamer peptide FMATYLR. The delta correlation (dCn) and the cross correlation (XCrr) values for this peptide are 0.226 and 2.2, respectively. **(B)** Identification of the Tyr-250 as the site for 3-azibutanol in the PKC ϵ C1 domain. MS/MS data for the 3-azibutanol (1 mM) modified heptamer peptide FGIHNYK. In addition to the singly charged ions, a doubly charged y5⁺⁺ (m/z 374) and y6⁺⁺ (m/z 402.4) ion is also observed. The delta correlation (dCn) and the cross correlation (XCrr) values for this peptide are 0.128 and 2.4, respectively. **(C)** Identification of the Tyr-176 as the site for 3-azioctanol in the PKC ϵ C1 domain. MS/MS data for the 3-azioctanol (1 mM) modified heptamer peptide FMATYLR. The delta correlation (dCn) and the cross correlation (XCrr) values for this peptide are 0.226 and 2.2, respectively.

Figure 8.

Location of alcohol binding residues in PKCεC1 structure. Surface diagram of PKCεC1 showing the alcohol-binding tyrosine residues. 3-azialcohol labeled residues (Y176 and Y250) and the tryptophan residues that are involved in FRET with SAPD and dansyl-DAG are shown in red and blue respectively. The linker region between C1A and C1B is shown in purple. Molecular-graphics images were produced by using the UCSF Chimera package from the Computer Graphics Laboratory, University of California San Francisco, San Francisco, CA, U.S.A.

PKC α C1A	36	HKFIARFFKQPTFCSHCTDFIWGFGK-QGFQCQVCCFVVHKRCHEFVTFSC
PKC δ C1A	159	HEFIATFFGQPTFCSVCKDFVWGLNK-QGFKCRQCNAAIHKKCIDKIIGRC
PKC ϵ C1A	170	HKFMATVLRQPTYCSHCRDFIWGVIGKQGYQCQVCTCVVHKRCHELIITKC
PKC α C1B	101	HKFKIHTYGSPTFCDHCGSLLYGLIH-QGMKCDTCDMNVHKQCVINVPSLC
PKC δ C1B	231	HRFKVINYMSPTFCDHCGSLLWGLVK-QGLKCEDCGMNVHKKCREKVANLC
PKC ϵ C1B	243	HKFGIINIKVPTFCDHCGSLLWGLLR-QGLQCKVKMNVHRRCEITNVAENC

Figure 9.

Comparison of the primary sequences of the C1A and C1B subdomains of PKC α , PKC δ , and PKC ϵ . The photolabeled residues are boxed.

Table 1Identification of the PKC ϵ C1 tryptic peptides^a by LTQ-FT mass spectrometry

Position ^b	Sequence	Charge state	Predicted mass ^c	Observed mass ^c	Accuracy ^d (ppm)	dCn	XCorr
172-178	FMATYLR	2	451.231962	451.233658	3.15	.7835	1.81
179-187	QPTYCSHCR	3	403.503095	403.502698	1.44	.4370	1.59
179-196	QPTYCSHCRDFIWGVIGK	3	742.018752	742.019065	.18	.503	2.46
188-196	DFIWGVIGK	2	517.787319	517.787111	.94	.753	2.71
197-210	QGYQCQVCTCVVHK	3	589.596590	589.597221	.76	.495	3.36
197-211	QGYQCQVCTCVVHKR	3	641.631665	641.630925	1.44	.54	3.91
211-219	RCHELIITK	3	390.553295	390.553498	.05	.556	4.09
212-219	CHELIITK	2	478.764124	478.765321	1.92	.618	2.42
220-224	CAGLK	1	548.285830	548.286096	.53	.625	1.12
220-225	CAGLKK	1	676.382810	676.381059	3.40	.405	1.48
225-236	KQETPDEVGSQR	3	458.559339	458.560035	1.12	.613	3.79
212-236	CHELIITKCAGLKKQETPDEVGSQR	3	966.491673	966.491781	.08	.498	6.21
220-236	CAGLKKQETPDEVGSQR	2	951.970914	951.970539	.68	.772	5.31
226-236	QETPDEVGSQR	2	623.288414	623.288932	.39	.636	2.17
237-244	FSVNMMPHK	2	480.241754	480.242014	.03	.766	2.5
237-251	FSVNMMPHKFGIHNKY	3	606.974135	606.975126	1.33	.538	3.31
245-251	FGIHNKY	2	439.729484	439.729597	.38	.747	2.77
252-268	VPTFCDHCGSLLWGLLR	3	677.670302	677.669485	1.48	.569	4.57
269-274	QGLQCK	1	733.366760	733.366137	1.60	.708	1.21
269-277	QGLQCKVCK	2	560.783289	560.783720	.28	.552	1.22
269-282	QGLQCKVCKMNVHR	3	586.628432	586.628855	.41	.613	3.84
278-282	MNVHR	1	656.330200	656.329692	1.62	.707	.99
278-283	MNVHRR	2	406.719324	406.719040	1.38	.728	1.63
275-283	VCKMNVHRR	3	400.546492	400.546461	.53	.52	1.60
269-283	QGLQCKVCKMNVHRR	4	479.248176	479.248739	.89	.535	3.07
284-292	CETNVAPNCGLEHHHHHH	3	729.310397	729.309901	.93	.620	3.80

^aThe protein was digested with trypsin and infused into the mass spectrometer.^bNumbering is according to the position of the peptides in the full-length PKC ϵ as shown in Figure 1.^cMonoisotopic mass that includes iodoacetamide modified (additional mass of 57 Da) cysteines.^dAccuracy is the fractional difference between the predicted and observed mass expressed in ppm.

dCn and Xcorr represents delta correlation and cross correlation respectively.