Protein kinase C (PKC) is an important signal transduction protein that has been proposed to interact with general anesthetics at its cysteine-rich diacylglycerol/phorbol ester-binding domain C1, a tandem repeat of C1A and C1B subdomains. To test this hypothesis, we expressed, purified, and characterized the high affinity phorbol-binding subdomain, C1B, of mouse protein kinase Cδ, and studied its interaction with general anesthetic alcohols. When the fluorescent phorbol ester, sapindoside-D, bound to PKCδ C1B in buffer at a molar ratio of 1:2, its fluorescence emission maximum, λ_{max}, shifted from 437 to 425 nm. The general anesthetic alcohols, butanol and octanol, further shifted λ_{max} of the PKCδ C1B-bound sapindoside-D in a concentration-dependent, saturable manner to ~415 nm, suggesting that alcohols interact at a discrete allosteric binding site. To identify this site, PKCδ C1B was photolabeled with three photoactivatable diazirine alcohol analogs, 3-azioctanol, 7-aziotanol, and 3-azibutanol. Mass spectrometry showed photoincorporation of all three alcohols in PKCδ C1B at a stoichiometry of 1:1 in the labeled fraction. The photolabeled PKCδ C1B was subjected to tryptic digest, the fragments were separated by online chromatography and sequenced by mass spectrometry. Each azialcohol photoincorporated at Tyr-236. Inspection of the known structure of PKCδ C1B shows that this residue is situated adjacent to the phorbol ester binding pocket, and within ~10 Å of the bound phorbol ester. The present results provide direct evidence for an allosteric anesthetic site on protein kinase Cδ.

The molecular mechanisms of general anesthesia remain poorly understood (1–4). Anesthetics are relatively nonspecific drugs that interact with many transmembrane ion channels and soluble proteins, often causing unwanted side effects.

Received for publication, May 10, 2004, and in revised form, July 1, 2004
Published, JBC Papers in Press, July 2, 2004, DOI 10.1074/jbc.M405137200

Identification of a General Anesthetic Binding Site in the Diacylglycerol-binding Domain of Protein Kinase Cδ*

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Gaining a detailed understanding of the structural motifs governing anesthetic-protein interactions is a critical step in elucidating the molecular mechanisms underlying general anesthesia and in developing more selective anesthetic agents. However, little progress can be made until such sites are unequivocally identified. The physiological sites of anesthetic action remain difficult to define, and some potential targets such as the transmembrane domains of the ligand-gated ion channel superfamily are so poorly characterized compared with soluble proteins that atomic level principles governing their anesthetic binding cannot yet be defined. Consequently, several crystallizable soluble proteins such as myoglobin (5), hemoglobin (6), adenylyl kinase (7), luciferase (8), human serum albumin (9), and insulin (10) have been used as surrogate targets to obtain structural information on protein-anesthetic interactions.

An important signal transduction protein, protein kinase C (PKC), has been proposed as the target of anesthetics such as alcohols, halothane, and enflurane (11, 12); for a review, see Rebecchi and Pentyala (13). The protein kinase C superfamily plays a central role in signal transduction, regulating divergent cellular functions by phosphorylation of target proteins such as ion channels (14, 15). It can be separated into two major categories, the conventional (α, β, βII, and γ) and the novel (δ, ε, θ, and η) kinases, each having four domains, termed C1 though C4, that play distinct roles in the function of the kinase. C1 and C2 are the regulatory domains, C3 is the ATP-binding domain, and C4 is the catalytic domain. The two phorbol-binding sites are in the C1 domain, and consist of a tandem repeat of highly conserved cysteine-rich zinc finger subdomains C1A and C1B (residues 159–208 and 231–280, respectively, in the δ isoform). These domains differ in their binding affinities for phorbol ester and sn-diacylglycerol (16–19). Homology between different members of the superfamily is high within domains but the novel kinases differ in having their C2 domain N-terminal to their C1 domain (Fig. 1) and in associating with membranes in a calcium-independent manner.

Stimulated PKC activity is modulated by anesthetics, and it has been proposed that discrete binding sites for general anesthetics could be present and possibly located near or within the phorbol ester-binding sites (11, 20). The interaction between PKC activators and anesthetics, however, is complex and incompletely understood. The isolated catalytic domain, C4, is unaffected by anesthetics, indicating that the primary anesthetic interaction occurs within the regulatory domains (11, 18, 20).
In intact PKCs, volatile anesthetics and long- and short-chain alcohols modulate PKC activation in vitro and alter activator binding affinities. The enhancement of PKCa activity induced by octanol in the TPA/phospholipid system was attributed to a positive allosteric interaction between it and the high affinity phorbol ester-binding site (20).

To study the interaction between anesthetics and high affinity phorbol ester binding, we expressed the C1B subdomain of mouse PKCδ in Escherichia coli and purified it by affinity chromatography. We chose PKCδ C1B, which is highly homologous with other major isoforms, because it is the best characterized, both structurally and functionally, of the isolated subdomains (16, 22–24). We found that butanol and octanol allosterically enhanced the binding of a fluorescent phorbol ester to PKCδ C1B, and that diazirine derivatives of butanol and octanol photoincorporated into a single residue identified as Tyr-6 of C1B, corresponding to Tyr-236 in intact PKC.

EXPERIMENTAL PROCEDURES

Materials—Sapintoxin D (SAPD) was purchased from LC Laboratories, Woburn, MA; TPA and 4a-TPA from Sigma. N-Tosyl-L-phenylalana- nine chloromethyl ketone-treated trypsin was obtained from Roche Diagnostics; glutathione-Sepharose 4B, from Amersham Biosciences. 3-Aziobutanol (25), 7-aziocetanol (26), and 3-azibutanol (27) were synthesized as described. All other reagents were obtained from Aldrich.

Protein concentration was determined with a BCA protein assay reagent kit (Pierce).

Bacterial Expression and Purification of the PKCδ C1B Subdomain—The PKCδ C1B subdomain fused with glutathione S-transferase was expressed in BL21 gold E. coli as described earlier (28). Briefly, cell pellets were treated with 1% Triton X-100 and lysozyme (1 mg/ml), followed by sonication and centrifugation. The clarified supernatant was applied to a glutathione-Sepharose column. The bound protein was thoroughly washed, released by thrombin cleavage, eluted with ammonium sulfate precipitation. It was further purified by fast performance liquid chromatography (Bio-Rad Biologic Workstation) using a Superdex™ 75 column (Amersham Biosciences), a mobile phase of 50 mM Tris, 100 mM NaCl, pH 7.0, and a flow rate of 0.5 ml/min.

CD Measurements—Circular dichroism spectra were recorded from 260 to 190 nm at 25 °C on an Aviv 62 DS instrument (Aviv Associates, Lakewood, NJ) in a quartz cell of 0.1-cm path length. Data were collected at 0.5-nm intervals and an accumulation time of 20 s. CD spectra were fitted using a CDNN program and the neural network approach of Bohm et al. (29).

Fluorescence Measurements—Fluorescence measurements were performed on a Jobin Yvon Spex (model Fluoromax-2, ISA Instruments S.A., Inc.) fluorimeter equipped with temperature and stirring control systems. A 1.5-ml cuvette (Hellma) with a Teflon stopper was used for fluorescence measurements. PKCδ C1B and SAPD were mixed in a buffer (50 mM Tris, pH 7.2) and stirred gently for 20 min with a small Teflon-coated magnetic stir bar at room temperature. SAPD was excited directly at 355 nm and emission spectra were recorded from 375 to 575 nm. Effects of alcohols on the SAPD binding were studied in a 1-mI mixture containing 4 μM protein and 2 μM SAPD. Alcohols were titrated by adding successive 2-μl aliquots of Me2SO containing either 0.5 M octanol or 10 mM butanol. Spectra were recorded after 30 min incubation with slow stirring. PKCδ C1B was denatured by heating the solution at 90 °C for 20 min, and its fluorescence was monitored after cooling and transferring to the cuvette.

The wavelength maxima of the emission spectra were determined by fitting the symmetrical top of the peaks to a Gaussian function with slow stirring. PKCδ C1B was denatured by heating the solution at 90 °C for 20 min, and its fluorescence was monitored after cooling and transferring to the cuvette.

For fluorescence quenching experiments, phorbol esters (TPA or 4a-TPA, 1 mM in Me2SO) were titrated by additions of successive 1-μl aliquots into 1 ml of PKCδ C1B (1 μM) in buffer (50 mM Tris, pH 7.2). The sample was excited at 290 nm and intrinsic fluorescence quenching was monitored by recording fluorescence emission from 300 to 500 nm. The average fluorescence intensity between 348 and 350 nm was normalized to that without additions (F0) and used in the analysis of binding curves and plots to calculate the Stern-Volmer constant, KSV (31).

Determination of Stoichiometry of Photolabeled PKCδ C1B by Electrospray Ionization Mass Spectrometry—The stoichiometry of photoincorporation of 3-azioctanol into PKCδ C1B was determined using micropipette liquid chromatography coupled to an ion trap mass spectrometer with electrospray ionization. Samples of PKCδ C1B (95 pmol/sample) were photolabeled at 365 nm for 20 min after 10 min preincubation with 1 mM 3-azioctanol, 7-aziocetanol, or 3-azibutanol using a model UVL-56 W2 Black-Ray handheld lamp (Upland, CA). A 1-μl aliquot of each reaction mixture was diluted 1:20 with 0.1% acetic acid to yield a 1 pmol/μl solution. A 1-μl aliquot of each of these diluted samples was then analyzed by micropipette liquid chromatography coupled to an ion trap mass spectrometer with electrospray ionization (LCQ, Finnigan MAT, San Jose, CA). The column (75 × 360 μm) was packed with POROS 10R2 (Perceptive Biosystems, Framingham, MA) to 15 cm and butt-connected to a fused silica nanospray tip (5 μm). The reaction mixture was eluted from the column with a gradient of 0–80% acetonitrile in 15 min at a flow rate of 0.2 μl/min. Mass spectra were acquired from m/z 300 to 2000 with a maximum ejection time of 400 ms. Molecular weights for both the modified and unmodified forms of PKCδ C1B were calculated from the resulting charge envelopes. Essentially, the charge state of an ion was determined by subtracting two adjacent ions from each other and the reciprocal of this difference multiplied by the ion of lower mass/charge minus one. The result is the charge state of the ion with the higher m/z of the two adjacent peaks. To calculate the unprotonated molecular weight, the determined charge state is multi-

Fig. 1. Photolabeling and protein used in the present study. A, diazirine analogs of butanol and octanol. B, schematic representation of the domains of protein kinase Cδ: the C1 regulatory domain binds to lipids, diacylglycerol, and phorbol esters; the C2 regulatory domain binds to anionic lipids, and thus, the catalytic domain. P denotes the pseudo substrate-binding domain. C1B (residues 231–280) binds to phorbol esters with higher affinity than C1A (residues 159–208). The 50 residues of subdomain C1B used in this study are shown in bold and additional residues used in the construct are shown in regular type.
Anesthetic Binding Site in the Phorbol-binding Domain of PKC

RESULTS

Protein Purification and Characterization—SDS-PAGE (18%) analysis of affinity purified and thrombin-cleaved PKC\(_6\) C1B showed a single band around 7 kDa (data not shown) similar to that previously reported (23). A number of criteria suggest that the purified PKC\(_6\) C1B protein is correctly folded. First, fast performance liquid chromatography on a gel filtration column confirmed that the PKC\(_6\) C1B subdomain migrated as a single symmetrical peak with the expected Stokes radius. Second, analysis of the CD spectrum (Fig. 2) suggests that the secondary structure of PKC\(_6\) C1B has about two times more \(\beta\)-sheet content than \(\alpha\)-helix content and a high content of random coil (\(-50\%) (32). This estimate is consistent with the published crystal structure (23). Third, phorbol ester binding had the required specificity (see below).

Phorbol Ester Binding—In 50 mM Tris buffer, pH 7.4, SAPD showed an emission maximum at 437 nm that was unaffected by addition of Me\(_2\)SO, anesthetics, or the combination of both in the concentrations used in the present studies. Addition of a 10-fold excess of PKC\(_6\) C1B to SAPD caused a broad blue-shifted emission spectrum that could be deconvoluted into two components with maxima at 404 and 437 nm representing bound and free SAPD, respectively (Fig. 3). When the spectrum of the same solution was recorded at 90°C for 20 min, the spectrum narrowed to a single component centered on 437 nm, indicating release of SAPD from PKC\(_6\) C1B. Consistent with this interpretation, when PKC\(_6\) C1B, preheated at 90°C for 20 min, was added to SAPD, no wavelength shift was observed.

PKC\(_6\) C1B contains a single tryptophan (Trp-252) whose fluorescence was quenched in a concentration-dependent manner when TPA was added (Fig. 4, inset). Quenching of the intrinsic fluorescence of PKC\(_6\) C1B depended in a sigmoidal manner on the concentration of the phorbol ester TPA (Fig. 4, trace a). By contrast, the inactive isomer, 4a-TPA (33), quenched significantly less efficiently and with linear dependence on concentration (Fig. 4, trace b). Assuming that the 4a-TPA data points represent nonspecific quenching, they were subtracted from the TPA quenching data to yield corrected data points that fitted well to the mass action equation (Fig. 4, trace c) with an \(EC_{50}\) of 0.31 \(\pm\) 0.072 \(\mu\)M (total concentration). Controls showed that addition of TPA (0.1–1.2 \(\mu\)M) to 1 \(\mu\)M l-tryptophan in buffer did not cause any quenching of the tryptophan fluorescence, nor did addition of Me\(_2\)SO in the
concentration range used caused significant changes in the fluo-
rescence emission spectra of PKCβ C1B.

When the TPA fluorescence quenching data were plotted ac-
gording to the Stern-Volmer equation, 4α-TPA quenching was
linear with a slope \( K_{sv} \) of 0.54 × 10^6 m^−1. On the other
hand, TPA quenching was linear up to 0.5 μM TPA with a much
higher \( K_{sv} \) of 2.9 × 10^6 m^−1. At higher concentrations, howe-
ever, the plot curved upwards suggesting additional quenching
mechanisms.

Consistent with the TPA study, SAPD also quenched intrin-
sic PKCβ C1B fluorescence. With increasing concentration of
SAPD, intrinsic fluorescence decreased and bound SAPD fluo-
rescence increased in parallel (data not shown).

Phorbol Ester-Anesthetic Interactions—As shown above (Fig.
3), the emission spectrum of SAPD in the presence of 10–50-
fold excess protein consists of overlapping free and bound con-
tributions with \( \lambda_{\text{max}} \) of 437 and 404 nm, respectively. The
addition of butanol (100–200 mM) or octanol (1–2 mM) caused
no significant change in the composite spectrum under these
conditions. However, when the protein to SAPD molar ratio
was reduced from 10:1 to 2:1 the composite peak was more
symmetrical and exhibited a maximum at 425 nm (Fig. 5).

Titration of alcohols caused more SAPD to bind resulting in a
blue shift that reached a plateau of −415 nm at 80 mM butanol
or 1 mM octanol (Fig. 6). These concentration-dependent emis-
sion shifts were fitted to logistic curves yielding EC_{50} values of
34 ± 16 mM for butanol and 410 ± 150 μM for octanol and Hih-
coefficients that did not differ significantly from one (2 ± 1.6 for
butanol and 2 ± 1.4 for octanol). Control experiments in which
identical additions of butanol, octanol, or MeSO were made to
SAPD in buffer did not cause any blue shift.

To test the stability of the diazire group during the binding
assay, the absorbance change of a 2 mM aqueous 3-azioctanol
solution at 350 nm was recorded before and after a single
emission scan. About 10% of the chromophore was photo-
decomposed. Because of the photosensitive nature of the diazire
group we did not titrate the azioctohols. However, 2 mM 3-azi-
octanol caused a substantial, near maximum blue shift to 417
nm under the above conditions.

Determination of the Mole Ratio of Alcohol Photoincorpora-
tion into PKCβ C1B by LC/MS—The photolabeled samples
were diluted (1 pmol/μl) and analyzed using online microcap-
illary liquid chromatography coupled to an ion trap mass spec-
trometer with electrospray ionization. An ion chromatogram
for the photo reaction mixture of PKCβ C1B and 1 mM 3-azi-
octanol can be seen in Fig. 7A. Complete separation of unla-
beled and labeled PKCβ C1B was not achieved. However, in
LC/MS experiments, peak heterogeneity does not prevent mo-
lecular weight determination of the eluted proteins because of
their unique charge envelopes. Thus, it was unnecessary to
refine chromatographic conditions to achieve resolution of the
components. Fig. 7B shows the mass/charge envelope for sam-
pies eluting between 10.8 and 13.5 min obtained by summing
the spectra obtained every second during this time period.

Deconvolution of the mass/charge envelope yielded two major
peaks with molecular masses that did not differ significantly
from the expected ones of 7,363 Da for PKCβ C1B and 7,491 Da
for PKCβ C1B modified by photoincorporation of azioctanol
(Fig. 7C). The observed difference between the two major peaks
was 128 ± 1 Da, corresponding to phosphoincorporation of a
single 3-azioctanol molecule in PKCβ C1B. No higher molecular
weight peak corresponding to the photoincorporation of two
azioctanols (7,619 Da) was observed. Nonetheless, it is hard to
true this out because the probability of such labeling is likely to
be low (26). A major unidentified contaminant occurred at 7458
Da, but only in this particular preparation.

Similarly, samples of PKCβ C1B photolabeled by 3-azibuta-
ol and 7-azioctanol (1 mM) were analyzed, and their deconvo-
luted charge spectra are shown in Fig. 8A and B, respectively.
The deconvoluted charge envelope of the 3-azibutanol sample
appears quite complex at first sight. The largest peak is ob-
served at 7,363 Da, corresponding to the unmodified protein.
The two satellites marked a and b in Fig. 8A are offset from the
main peak by 16 and 32 Da and most likely correspond to
oxidized methionines, of which PKCβ C1B has two, Met-9 and
Met-36. The second largest peak is observed at 7,435 Da and
corresponds to the protein modified by phosphoincorporation of
a single 3-azibutanol (+72). Interestingly, both oxidized states
are also photolabeled. A small peak in the vicinity of, but not
centered on, 7,507 Da makes it difficult to rule out that a
second azibutanol photocorporated into PKCβ C1B.

The charge envelope derived from spectra of 7-azioctanol-
labeled PKCβ C1B eluting between 10.2 and 13.1 min was
deconvoluted to yield molecular masses corresponding to the
expected values of 7,363 and 7,491 Da. The observed molecular
mass difference of 127 ± 1 Da being consistent with photoin-

Fig. 5. Enhancement of SAPD binding to PKCβ C1B by buta-
hol. Emission spectra of SAPD (2 μM): a, in buffer alone; b, in buffer
containing 80 mM butanol; c, in the presence of 4 μM PKCβ C1B alone;
and d, in the presence of 4 μM PKCβ C1B plus 80 mM butanol. SAPD
was added in 2 μl of MeSO and incubated for 25 min. Butanol was then
added from a stock of 10 mM in Me2SO and incubated for 15 min. \( \lambda_{\text{ex}} \)
355 nm.

Fig. 6. Allosteric enhancement of SAPD binding to PKCβ C1B
by alcohols. The alcohol-induced emission maximum blue shift (\( \lambda_{\text{em}}-\lambda_{\text{em0}} \))
of SAPD, where \( \lambda_{\text{em}} \) and \( \lambda_{\text{em0}} \) represent emission maximum in the presence
and absence of alcohols, respectively, is plotted against the alcohol
concentrations for octanol (filled square, dashed line) and butanol (filled
triangle, solid line). Lines represent nonlinear least square fits to the
logistic equation (see “Experimental Procedures”). At the protein (4 μM)
and ligand (2 μM) ratio of 2:1, the emission maximum of SAPD, \( \lambda_{\text{em}} \), is 425
nm. Upon addition of varying amounts of octanol or butanol, the emis-
sion maximum shifted toward the blue. Protein and SAPD were in-
cubated for 25 min in buffer (50 mM Tris, pH 7.4). Alcohol was then added
in aliquots as described in the legend to Fig. 5 and spectra were
recorded after 15 min.
corporation of 7-azioctanol at a molar ratio of 1:1 (Fig. 8B). No peak that would correspond to double photoincorporation was found at 7,619 Da (not shown). Once again, both peaks exhibited satellites consistent with two oxidized methionines. In addition, an unidentified contaminant was observed around 7,455–7,475 Da.

Identification of Photolabeled Amino Acid Residues by LC/MS/MS—All peptides generated by trypsin digestion of photolabeled PKC6 C1B were analyzed by LC/MS/MS. In this type of experiment, eluted peptides are ionized and randomly fragmented along the peptide backbone, resulting in several observed ion fragments. Fragment ions containing the C terminus are called smaller observed ion fragments. Fragment ions containing the N terminus of the peptide are called "a," "b," and "c" ions; fragment ions containing the C terminus are called "x," "y," and "z" ions. Under our experimental conditions, b and y ions predominate. Conventionally, numbers following these letters denote the number of residues in the fragment.

Digested samples were eluted directly from the online high performance liquid chromatography column into the mass spectrometer as described above, and MS/MS spectra were acquired under automatic acquisition of the most intense ion from each MS scan. Fig. 9 shows the collision-activated dissociation MS/MS spectrum of the triply charged peptide ion with m/z 887.5 that presented in the tryptic digest of 3-azioctanol-labeled PKC6 C1B. The 22-amino acid sequence of the peptide, VYNYSPTFCDHCSGLWLGGLV, and the amino acid modified with 3-azioctanol were deduced from this spectrum. Photoincorporated azioctanol caused a mass shift of 128 Da for all fragment ions containing the modified amino acid. The b2 (m/z 391) and a2 (m/z 363; loss of 28 Da from carbon monoxide) ions and all subsequent observed b ions (b3 through b5) contained a mass shift of 128 Da, indicating that either Tyr-6 or Val-5 is photolabeled. This ambiguity was resolved in favor of Tyr-6 by the y ions. The y ions with 4, 5, 6, 9, and 13 through 20 amino acids were all unmodified, whereas a mass shift of 128 Da was observed for the doubly charged y21 ion (m/z 1280). Although the latter peak appears small in Fig. 9, this is an artifact of the different scales employed in the three panels. Furthermore, its position is secured by being a member of the complete doubly charged sequence from y17 to y21.

MS/MS spectrum of the triply charged peptide with m/z of 887.5, derived from a digest of 7-azioctanol-photolabeled PKC6 C1B showed b2 (m/z 391) and a2 (m/z 363; loss of 28 Da from carbon monoxide) ions (data not shown). All subsequent observed b ions (b3 through b5) contained a mass shift of 128 Da, indicating that either Tyr-6 or Val-5 is photolabeled. This ambiguity was resolved in favor of Tyr-6 by the y ions. The y ions with 4, 5, 6, 9, and 13 through 20 amino acids were all unmodified, whereas a mass shift of 128 Da was observed for the doubly charged y21 ion (m/z 1280). Although the latter peak appears small in Fig. 9, this is an artifact of the different scales employed in the three panels. Furthermore, its position is secured by being a member of the complete doubly charged sequence from y17 to y21.

Fig. 10 shows the MS/MS spectrum of the triply charged peptide ion with m/z of 874.6 that presented in the tryptic digest of 3-azibutanol-labeled PKC6 C1B. The presence of photoincorporated 3-azibutanol caused a mass shift of 72 Da for all fragment ions containing the modified amino acid. The b2 (m/z 335) and a2 (m/z 307; loss of 28 Da from carbon
monoxide) ions and all subsequent observed b ions (3, 4, and 6) contained a mass shift of 72 Da or 88 (72/16; mono-oxygenated version), indicating that either Tyr-6 or Val-5 are labeled with the alcohol. Confirmation of Tyr-6 as the site of labeling was again provided by the observed mass shift of 72 Da in the doubly charged y21 ion (m/z 1261) and not in the doubly charged y20.

**DISCUSSION**

The present work provides strong evidence in favor of the hypothesis proposed by Slater et al. (20) that discrete alcohol-binding sites exist in the proximity of the phorbol ester binding sites within the regulatory C1 domain of PKC. Specifically, we used a highly simplified model consisting of only the second phorbol-binding domain C1B of PKCβ, which contains the high

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**Fig. 9. Identification of Tyr-6 as the site for 3-azioctanol by LC/MS/MS.** In a tryptic digest of PKCβ C1B the amino acid sequence VYNMSPTFCDHCGSLL-WGLVK was indicated, and the site of attachment for 3-azioctanol was deduced from this spectrum. At the top of the figure, the predicted charge/mass 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 128 Da added to them. Observed values are shown in bold.
affinity copy of the two phorbol sites in PKC. Surprisingly, in the isolated C1B subdomain, which contains only 50 of the 674 amino acid residues from the whole protein (Fig. 1), we observed the major features of alcohol-PKC interactions that have been reported in the intact kinase (20). Whereas we would not expect all features of alcohol action on PKC to be rationalized by actions within a single subdomain, nonetheless this provides a further indication of the modular nature of this kinase. That the general anesthetic site is intact is also consistent with studies on individual human serum albumin subdomains, which bind general anesthetics in much the same way as does the intact protein (34).

Our work shows that there is a positive heterotropic interaction between alcohols and phorbol-binding sites because both butanol and octanol enhance the binding of the fluorescent phorbol ester, SAPD, to PKC₁B (Figs. 5 and 6). Our finding for octanol is remarkably similar to that reported for membrane-bound intact PKC₁A, where SAPD titrations indicate that octanol in the 100 μM range increases the affinity of high affinity SAPD binding, an observation that has been confirmed in a membrane-free assay (35). However, in those studies, unlike in ours, butanol was not observed to enhance high affinity SAPD binding. Whether this reflects subtle differences in the structure of the two isoforms, or a limitation of our simple model, remains to be determined.

Having established that the effect of alcohols on PKC₁B resembled those in intact PKC₁A, we went on to more directly test the hypothesis that an alcohol-binding site is responsible for this action. To do so, we employed diazirine derivatives of butanol and octanol to photolabel PKC₁B. Each of these alcohols, 3-azibutanol, 3-azioctanol, and 7-azioctanol, photoincorporated into PKC₁B with a stoichiometry of 1:1 in the labeled fraction (Figs. 7 and 8). It is not possible from mass spectrometry alone to establish the percentage of PKC₁B that was photolabeled, but, based on previous experience with adenylate kinase, it is unlikely to be high (26). Consequently, as discussed previously (26), it is difficult to rule out the existence of fractions photoincorporating two alcohols per PKC₁B because of the predicted low probability of such species occurring. However, two observations make this unlikely. First, in adenylate kinase, 3-azibutanol photoincorporated with a stoichiometry up to at least 5, whereas in PKC₁B, only 1:1 was observed unequivocally. Second, no second site of photoincorporation was observed in the 22 positively identified amino acid residues, whereas in adenylate kinase three additional sites were identified for 3-azibutanol that were not ob-

**Fig. 10. Identification of Tyr-6 as the site for 3-azibutanol by LC/MS.** The amino acid sequence VYNYMSPTFCDHCGSLLWGLVK and the site of attachment for 3-azibutanol were deduced from this spectrum. Notation is as in Fig. 9. The mass shift because of modification of Tyr-6 with 3-azibutanol is clearly observed by the mass shift of 72 Da for C-terminal ions containing this same residue beginning with the doubly charged y21 ion (m/z 1261). Observed values are shown in bold.
Fig. 11. Structure of the PKCδ C1B domain and comparison to PKCa. A, ribbon diagram of PKCδ C1B, (23) with selected features shown in ball-and-stick representation. The side chain of Tyr-6, which was photolabeled in this study, is shown in orange. The side chain of Trp-22 (blue) and the bound phorbol ester (orange) are also shown. Two bound zinc ions are represented with dark blue spheres. B, surface representation of PKCδ C1B, viewed from two opposite sides. The orientation on the left is similar to that shown in panel A. Tyr-6 is shown in red, and the bound phorbol ester is represented as a ball-and-stick model. Residues colored in green are not conserved in PKCa, with light green representing conservative substitutions (e.g. Val → Ile) and dark green representing more drastic substitutions (e.g. Met → Gly). Residues shown in white are identical in PKCa and PKCd. Panels A and B were produced with RIBBONS (37) and GRASP (38), respectively.

A major rationale for choosing to study the δ isoform of C1B is that it is the only isoform for which a high-resolution crystal structure has been reported (23). To aid in the discussion, this structure, which was solved in a complex with a phorbol ester, is reproduced in Fig. 11. The protein contains two zinc ions, which are coordinated by conserved cysteine and histidine residues. The phorbol ester binds in a groove between two loops at the top end of the structure (23) and the photolabeled Tyr-6 is located on the right of the structure as shown and about halfway between the N terminus and the beginning of the phorbol binding loop. It is part of the first β strand and is exposed on the surface of the protein, thus being readily accessible to alcohols from the aqueous phase.

Inspection of the surface close to Tyr-6 reveals no deep cavities but some plausible binding grooves. Tyr-6, shown in red in Fig. 11B, forms an isolated hump on the surface of PKCδ C1B, and there is a wide surface groove between it and Glu-32 and a narrower one between it and Met-9. Either groove is large enough to accommodate octanol. However, the failure of the two azoicotanols to delineate the site means that it would be unwise to draw further conclusions. It is clear, however, by inspection that both putative surface grooves contain polar residues, which is in keeping with observations in other proteins reviewed in Ref. 3. Furthermore, it seems likely that butanol has a relatively tighter interaction with PKCδ C1B than does octanol because the ratios of their EC50 values for their allosteric effects on SAPD binding to their general anesthetic potency are −3 and −9, respectively.

A comparison of unliganded and phorbol-bound C1B structures has shown that phorbol binding results in a subtle widening of the binding site (23) with the two loops forming the groove moving farther apart. Thus the groove has a certain plasticity. It seems plausible that anesthetics can influence the shape of the phorbol-binding site, and therefore the affinity of the protein for phorbol, by interacting with nearby regions. We note that Tyr-6 is only three residues away from residue Met-9, and close to Ser-10 and Pro-11, all of which are in direct contact with the bound phorbol and form one of the two loops that constitute the phorbol-binding site (Fig. 11A). The closest contact between the Tyr-6 side chain and the Met-9 main chain amide is only 5.3 Å, which is almost close enough to be classified as a van der Waals contact. The tyrosine is also only 7.7 Å away from the Ser-10 main chain carbonyl atom. The close proximity of Tyr-6 to a phorbol-contacting loop can thus help to explain the observed allosteric interaction between the alcohol- and phorbol-binding sites. Binding of an alcohol molecule could easily perturb the location of this loop, thereby affecting the affinity of C1B for phorbol esters.

A priori, a possible site for general anesthetics on PKC might be at the lipid-protein interface. Our work suggests this is unlikely because the residues where mutations affect lipid insertion following phorbol binding, Leu-20, Trp-22, and Leu-24 (24), are found on the opposite face of PKCδ C1B from Tyr-6 (see Fig. 11B for location of Trp-22). However, we cannot rule out that lipid association of phorbol-bound PKCδ C1B may possibly cause indirect effects on the structure of C1B, or its force field, that might be transmitted to the general anesthetic site.

The most detailed functional studies of alcohol and anesthetic action on PKC have been carried out on human PKCa. How similar is it to the mouse δ isoform that we have studied here? Fig. 11B shows a surface representation of the structure of PKCδ C1B, in which those residues that are not identical in human PKCa are colored green (see legend for details). In addition Tyr-6 of PKCδ C1B, which is conservatively replaced by a histidine in PKCa C1B, is colored red. This diagram reveals that Tyr-6 is on a boundary dividing two surface regions. One of these is highly conserved and the other quite variable. Overall, with the current evidence there is no reason to suppose that alcohol binding should not occur in the α-isoform, although minor differences in the alcohol structure-activity relationships might be expected.

In conclusion, we have demonstrated an allosteric interaction between the anesthetic and phorbol sites on PKCδ C1B, and have located the allosteric alcohol site as being in the proximity of Tyr-6. This new data supports the hypothesis that discrete alcohol-binding sites exist in the phorbol ester-binding regulatory domain, C1, of PKC as proposed by Slater et al. (20). Because the structure of PKCδ C1B has been reported, our study also suggests the possibility that high-resolution structural studies will be able to explore the binding site and the mechanism with atomic resolution in this isoform.

Acknowledgments—We are grateful to Dr. P. M. Blumberg of the National Cancer Institute, Bethesda, MD, for providing us with the DNA construct of PKCδ C1B. We also thank Dr. Jonathan Lee for providing the CD spectrometer at Boston University.
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