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Deletion Analysis of Protein Kinase Cα Reveals a Novel Regulatory Segment[1]

Susan A. Rotenberg,*,[2] Jianwei Zhu,‡ Hans Hansen,‡ Xiao-dong Li,* Xiao-guang Sun,* Corinne A. Michels,† and Heimo Riedel‡,[2,3]

*Departments of *Chemistry & Biochemistry and †Biology, Queens College of the City University of New York, Flushing, NY 11367, USA; and ‡Section on Molecular Biology, Joslin Diabetes Center and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02215, USA*

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Using a combined pharmacological and genetic approach, we have identified aa 260-280 in the C2 region as a critical factor in the catalytic function of protein kinase Cα (PKCα). Progressive truncations from the N-terminus as well as selected internal deletion mutants were expressed in *Saccharomyces cerevisiae* and tested for altered sensitivity to dequalinium, a PKC inhibitor whose target site was previously mapped to the catalytic domain. PKC mutants representing truncations of up to 158 amino acid residues (aa) from the N-terminus (ND84 and ND158) displayed 60-63% inhibition of kinase activity by 50 μM dequalinium, somewhat more sensitive than the wild-type PKCα enzyme (45% inhibition). Mutant ND262, lacking N-terminal aa 1-262, was inhibited by almost 72% with 50 μM dequalinium, but mutant ND278, which lacked an additional 16 aa, was inhibited by only 9% of total activity. This result suggests that a C-terminal segment of the C2 region (aa 263-278) influences inhibition by dequalinium at low micromolar concentrations. An internal deletion mutant (D260-280) which retains the entire primary structure of PKCα except for aa 260-280, was similarly inhibited by only 4% with 50 μM dequalinium. In the absence of dequalinium and despite the presence of a nearly complete regulatory domain, this mutant exhibited constitutive activity (both *in vitro* and in a phenotypic assay with *S. cerevisiae*) that could not be further stimulated even by the potent activator TPA. Taken together, our findings suggest that, in the native structure of PKCα, the segment described by aa 260-280 regulates PKCα activity and influences the sensitivity of PKCα to dequalinium.

Key words: cDNA expression, deletion mutagenesis, dequalinium, drug interaction, *Saccharomyces cerevisiae*.

Protein kinase C (PKC) is involved in many signal transduction pathways that govern cellular growth and differentiation (reviewed in Ref. *1*). The general structure of PKC is characterized as a monomer with an amino terminal regulatory domain connected *via* a flexible hinge region to a catalytic domain (*2*). Activators of PKC such as phosphatidylserine (PS), diacylglycerol (DAG) or phorbol ester, and Ca^{2+} bind to the enzyme at discrete sites in the regulatory domain, while ATP and substrates bind to specific sites in the catalytic domain (*3*). Comparison of the known primary structures of conventional Ca^{2+}-dependent PKC isoforms (α, β, and γ) and of the "novel" (δ, ε, η, μ, θ) and "atypical" Ca^{2+}-independent isoforms (ζ,ι), has revealed highly conserved (C) and variable (V) regions (Fig. 1; Ref. *4*). The structure of a typical Ca^{2+}-dependent PKC isoform is depicted as NH_2-V1-C1-V2-C2-V3-C3-V4-C4-V5-COOH whereby amino terminal sequences V1 through C2 comprise the regulatory domain, V3 defines the hinge region, and regions C3, V4, and C4 represent the catalytic domain (*4*).

A significant effort has been focused on identifying functional segments of the PKC protein that are critical to PKC activation. The pseudosubstrate sequence, a highly cationic region which is near the amino terminus (aa 19-31), functions as an auto-inhibitory peptide that binds to the substrate binding site and thus maintains the enzyme in an inactive conformation (*5*). In the presence of activating ligands, the flexible hinge region (roughly defined at aa 292-317) allows for changes in the physical structure of the enzyme (*6, 7*) such that the relative positions of the regulatory and catalytic domains describe an open, active conformation (*8*). During this process, the pseudosubstrate domain is displaced and entry of exogenous substrates occurs at the substrate binding domain. This idea was

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[2] To whom correspondence should be addressed.

[3] Present address: Department of Biological Sciences, Wayne State University, 2171 BIO SCI, 5047 Gullen Mall, Detroit, MI 48202-3917, USA; Member, Barbara Ann Karmanos Cancer Institute.

Abbreviations: cDNA, complementary DNA; DMSO, dimethyl sulfoxide; IC_{50}, 50% inhibitory concentration; PKC, protein kinase C; PS, phosphatidylserine; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; wt, wild-type.

formulated on the basis of a synthetic peptide modeled after the pseudosubstrate sequence that is a potent inhibitor of the enzyme (*5*). Furthermore, a modified pseudosubstrate peptide (^{25}SER peptide) bearing a serine in place of alanine at position 25 represents a high-affinity phosphorylation substrate (*5*). Antisera raised against the pseudosubstrate peptide were found to activate the enzyme in the absence of phospholipid and diacylglycerol (*9*). Anionic synthetic peptides modeled after a putative substrate binding region in the catalytic domain, led to activation of the enzyme (*10*), presumably due to an electrostatic interaction that displaced the cationic pseudosubstrate sequence from the anionic substrate binding site. Although these studies have identified functional sites, the three-dimensional structure of PKC remains unknown.

The tools of molecular genetics have mapped the sites on PKC that are recognized by activating ligands. Site-directed mutagenesis has established the location of the pseudosubstrate sequence (*11*), the phorbol ester binding site(s) to the two cysteine-rich tandem repeats in the C1 region (*12-15*), and a possible Ca^{2+}-binding domain in the C1 region (*16*).

The recognition sites of ligands that stimulate enzyme function have been critical in defining important structure-function relationships in PKC. The study of ligands that impede enzyme function, *i.e.*, inhibitors, should be similarly informative (*17*). The PKC inhibitor dequalinium (*18*) is a lipophilic, dicationic PKC inhibitor designated as "mixed-type" since it affects activities of both regulatory and catalytic domains (as shown with the catalytic fragment) (*19*). Structure-activity relationships of dequalinium and related analogues revealed that the length and flexibility of the 10-carbon methylene bridge (Fig. 1B) and the bipartite nature of the molecule were critical determinants of enzyme inhibition (*18*). The mechanism of dequalinium-mediated inhibition of PKC eluded traditional kinetic analysis since all PKC ligands studied to date showed non-competitive behavior with dequalinium (*18*). Our recent findings showed that the RACK-1 binding region (located in the C2 segment of the regulatory domain) contains a target site for dequalinium specifically at aa 218-226 (*20*). It is not known however, whether binding of dequalinium to this site is obligatory for inhibition of catalytic activity to occur.

To understand the role of the regulatory domain in the inhibitory action of dequalinium, this study employs deletion mutants of bovine PKCα and enzymatic analysis to identify whether there are sites in the regulatory domain that influence dequalinium-mediated inhibition of catalytic activity. Because stable expression of PKC mutants in mammalian cells was not successful for many years, we selected a strain of *Saccharomyces cerevisiae* (strain 334) to express deletion mutants of bovine PKCα (*17, 21, 22*) which, unlike mammalian systems, carries genetic alterations which reduce protease activity (*26*). Following the characterization of the expressed mutants, we demonstrate *in vitro* and *in vivo* that a sequence in the C2 region lying immediately N-terminal to the flexible hinge region, mediates the basal activity of PKC and influences the sensitivity of PKC to inhibition by dequalinium.

MATERIALS AND METHODS

Materials—Dequalinium diiodide was obtained from Aldrich (Milwaukee, WI); DEAE-Sephacel, PMSF, leupeptin, and glass beads (0.45 μm diameter) from Sigma (St. Louis, MO); growth media, uracil- and leucine-free amino acid supplements for yeast cell culture from Bio-101 (La Jolla, CA); PKCα-specific and secondary antisera from Santa Cruz Biotechnology (Santa Cruz, CA); Purified PKCα standard from PanVera Corporation (Madison, WI); nitrocellulose membranes from Pharmacia Biotech (Piscataway, NJ); [γ-^{32}P]ATP (3,000 Ci/mmol) from NEN-Dupont (Wilmington, DE); protein dye reagent from Bio-Rad (Hercules, CA); synthetic di-oleyl phosphatidylserine from Avanti Polar Lipids (Alabaster, AL); Ecoscint scintillation solution from National Diagnostics (Atlanta, GA). The ^{25}SER peptide (RFARKGSLRQKNV) was synthesized by N. Pileggi of the Protein Core Facility, Columbia University (New York, NY). All curve-fitting was carried out with Cricketgraph™ software.

PKC Mutagenesis and cDNA Construction—The complete protein-coding region of bovine PKCα (*2*) was linked at the *Nco*I site at the translation initiation codon with a synthetic A-rich *Hin*dIII-*Nco*I adapter (5′-AGCTTAAAA-AA-3′ and 3′-ATTTTTTGTAC-5′) upstream of the ATG codon for improved translation efficiency (*23*). The cDNA was truncated from the 3′ end by use of exonuclease *Bal*31 and then joined with a synthetic blunt-end *Xba*I adapter (5′-TAACTAACTAAT-3′ and 3′-ATTGATTGATTAGA-TC-5′) that introduced stop codons in all three reading frames. A complete protein-coding cDNA including 10 bp of the 3′ untranslated sequence was used for complete PKCα expression.

Amino terminal deletion mutations in the PKCα cDNA were created in which 84-294 amino terminal amino acids were truncated from the 5′ end by *Bal*31 digestion. For improved translation efficiency, a synthetic *Sal*I blunt-end adapter (5′-TCGACAAAAAAAAAAATGGCT-3′ and 3′-GTTTTTTTTTTTACCGA-5′) was joined to restore the methionine initiation codon and A-rich 5′ untranslated sequences. The cDNA was inserted into the *Sal*I and *Xba*I sites under control of galactose-inducible *GAL10* transcriptional elements of the high-copy-number yeast episomal expression plasmid YEp51 containing the *LEU2* gene for selection (*24*). With this strategy, we created deletion mutants lacking 84 (ND84), 158 (ND158), 262 (ND262), 278 (ND278), 294 (ND294), or 322 (ND322) amino-terminal amino acids. The methionine initiation codon had been restored in all mutants. It was followed by a new alanine codon in ND84, ND158, ND262, and ND294. After ligation, all plasmids were amplified in *Escherichia coli* DH5α and were identified and confirmed by restriction and DNA sequence analysis.

Internal deletion mutant D157-247, in which amino acids 157-247 were specifically deleted, was created by cleavage of the PKCα plasmid at a unique *Bam*HI site in the PKC coding region, *Bal*31 digestion, and religation. Mutant D260-280 was created by using the Bio-Rad Mutagenesis Kit with oligonucleotide 5′-GACTTCATGGGGTCCCTT-GGTACCGAGGAGGGCGAGTACTAC-3′. The deleted codons were replaced by a *Kpn*I site (sequence underlined) encoding Gly-Thr. Plasmids were amplified in *E. coli*

DH5α and were characterized by restriction and DNA sequence analysis.

Yeast Strains and Culture Conditions—PKC expression plasmids and control plasmids were introduced by lithium acetate transformation (*25*) into *S. cerevisiae* strain 334 (*MATα pep4-3 prb1-1122 ura3-52 leu2-3,112 reg1-501 gal1*) (*26*). Dequalinium inhibition studies were performed with mutants inserted into vector YEp51 which carries an integrated GAL10-GAL4 fusion cassette. This provides for galactose-inducible expression of Gal4p (*27*) and, in turn, high levels of expression of the PKCα alleles fused to the GAL10 promoter of YEp51. Phenotypic assays were conducted with cells transformed with the pVT100-U plasmid carrying a constitutive ADH1 gene transcriptional promoter (*28*) and either the cDNA insert for wild-type bovine PKCα or PKCα lacking aa 260-280. Each mutant was grown in the absence of the appropriate nutrient for selection of transformants.

Cells were grown at 30°C in suspension with vigorous shaking. The synthetic medium contained 2% glucose and was uracil- or leucine-free to select for stable propagation of the expression plasmids, as previously described (*21*). Cultures of 180 ml were inoculated from freshly saturated cultures and grown overnight or until an optical density of 0.5 O.D.$_{600}$ was reached. PKC cDNA expression was induced by addition of galactose to 2% (w/v) and subsequent incubation at 30°C with vigorous shaking for 3-6 h.

Isolation of PKC Mutants from Yeast—Each yeast cell suspension was distributed among five 50-ml centrifuge tubes, sedimented at 2,500$\times g$ and 4°C for 15 min, and the supernatants were discarded. To each pellet was added approximately 1 ml glass beads (0.45 μm diameter) and 1 ml buffer A (20 mM Tris, pH 7.5, 2 mM EGTA, 2 mM EDTA, 1 mM 2-mercaptoethanol) with protease inhibitors 0.25 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor. Each pellet was vortexed six times for 30 s with intermittent cooling in ice. By use of a pipet tip diameter which excluded the glass beads, the homogenates were removed, pooled and centrifuged for 20 min at 9,750$\times g$. The supernatant was applied to a disposable 15-ml Econocolumn (Bio-Rad) containing 0.5 ml DEAE-Sephacel pre-equilibrated in buffer A. The column was washed with 5.0 ml buffer A and eluted with 1.5 ml buffer A containing 150 mM NaCl. Total protein content of eluates obtained by DEAE-Sephacel chromatography was quantitated by use of the Bio-Rad protein dye reagent.

Assay of PKC Catalytic Activity—Wild-type PKCα or mutant activity was assayed in with enzyme preparations that had been isolated by DEAE-Sephacel chromatography, as previously described (*18*), with the modified pseudosubstrate peptide (^{25}SER peptide) as substrate (*5*). The assay medium (0.12 ml) consisted of 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 26.6 μM ^{25}SER peptide, PS (83 μg/ml) and 1 μM TPA, mutant PKC or control plasmid preparation (typically 15 μg total protein), 66 μM [γ-^{32}P]ATP (2.5 μCi/assay), and dequalinium (or DMSO) added to the indicated concentration, such that the DMSO concentration in each assay was 4.2% (v/v). Because many mutants were not responsive to PS/Ca^{2+} or PS/TPA, the background activity for these assays was defined as the kinase activity present in the control plasmid preparation, unless otherwise indicated. The reaction was initiated by the addition of [γ-^{32}P]ATP and carried out for 30 min in a 30°C water bath. A 100-μl aliquot of each reaction was applied to a phosphocellulose square (2$\times$2 cm) and immersed immediately in 1 liter H_2O. The squares were washed five more times, placed in a vial containing 10 ml Ecoscint solution, and quantified for ^{32}P in an Isocap/300 liquid scintillation counter (Searle Analytic).

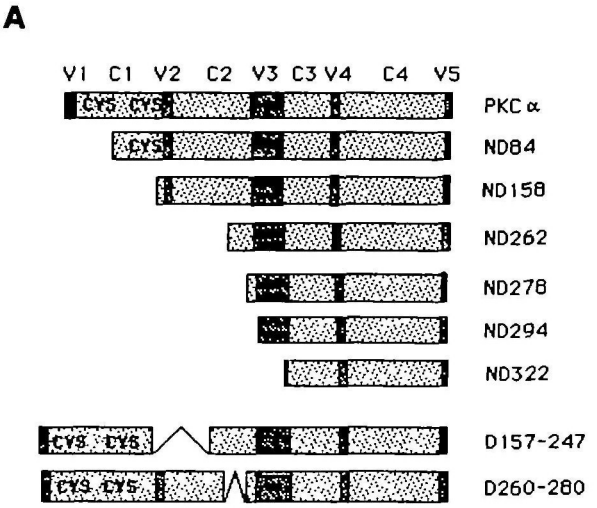
Immunoblot Assay—Following partial purification of cell extracts by DEAE-Sephacel chromatography (*18*), the samples were characterized by Western blot. To quantitate the amount of PKC protein in each sample (ng PKC/20 μg protein), additional lanes were compared in the same gel to which known amounts of highly pure PKCα protein (1-25 ng) had been applied. Samples were resolved on a 9% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Pharmacia Biotech) (*29*). Blots were incubated with PKCα-specific antisera recognizing an epitope in the catalytic domain, followed by incubation with anti-IgG-horseradish peroxidase conjugate. Signals were detected using chemiluminescence (Amersham Life Science, Arlington Heights, IL), and quantitated by two-dimensional scanning densitometry (Molecular Dynamics).

RESULTS

Characterization of PKC Mutant Proteins—We employed a series of deletion mutants of PKCα, isolated from a *Saccharomyces cerevisiae* expression system, which represents progressive truncation of the regulatory domain from the N-terminus, as shown in Fig. 1A. Each PKCα mutant was isolated by chromatography on DEAE-Sephacel, as described in "MATERIALS AND METHODS," and the catalytic activity for each mutant is reported in Table I. It was observed that most mutants had lost the ability to be stimulated significantly by PS, TPA, and Ca^{2+}, as previously observed (*17*). This characteristic is consistent with the deletion of the pseudosubstrate segment (aa 19-36) in those mutants that had been truncated from the N-terminus.

While many mutant proteins exhibited absolute catalytic activity in a similar range as wild-type PKCα, both ND262 and D157-247 consistently exhibited very low absolute catalytic activities (units/mg). Because the expression levels of the mutant proteins were roughly equivalent (Table I), the low catalytic activities of ND262 and D157-247 could be related to the intrinsic nature of each mutant protein. In any given experiment however, catalytic activities (in total units) for D157-247 and ND262 exceeded the phosphotransferase activity present in the plasmid control.

Western blot analysis of mutant proteins revealed their faithful expression into protein products. As shown in Fig. 2, mutant proteins that had been partially purified by DEAE-Sephacel chromatography, displayed a progressive reduction in the size of immunochemically reactive bands, consistent with their expected molecular weights. Listed in Table I are the relative abundances of the partially purified mutant proteins which were in the range of 3-6 ng PKC protein/20 μg total protein (0.015-0.03% purity). It was noted that the apparent M.W. of ND262 was slightly lower than expected, and may be related to the low activity measured for this mutant (Table I). This higher mobility for ND262 was observed consistently in several preparations, and found to be equivalent with regard to its abundance in both eluates of DEAE-Sephacel chromatography



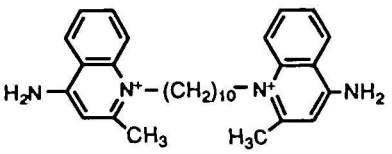


Fig. 1. **A: Primary structure of deletion mutants in the regulatory domain of PKCα.** The deduced amino acid (aa) sequences of wild-type PKCα and deletion mutants ND84, ND158, ND262, ND278, ND294, ND322, D157-247, and D260-280 are aligned. These mutants are lacking 84, 158, 262, 278, 294, 322 N-terminal aa, and aa 157-247, or 260-280, respectively. Regions of conserved (C1-C4) and variable (V1-V5) aa sequences are indicated by light and dark boxes, respectively. Each zinc finger-like repeat of six cysteine residues in C1 is represented as CYS. Deleted sequences have been omitted or indicated by ∧. **B: Chemical structure of dequalinium.**

TABLE I. **Catalytic activities of PKCα mutants.**

PKC mutant	Ca^{2+}/PS/TPA-stimulated catalytic activity [(units/mg)+SD]	*n*	-Fold stimulation by Ca^{2+}/PS/TPA	Relative phorbol ester binding[a]	Relative abundance[c] (ng PKC/20 μg protein)
PKCα (wt)	245±53	10	4	++	19.0
ND84	251±11	2	1-1.5	+	5.0
ND158	223±15	4	1-1.5	−	5.7
ND262	65± 2	4	1-1.5	−	4.6
ND278	281±59	8	1-1.5	−	3.4
ND294	222±11	8	1-1.5	−	6.0
ND322	135±12	5	1	−	6.4
D157-247	12± 1	6	1-3	++	5.4
D260-280[b]	387±14	6	1-1.5	n.d.	3.2
YEp51	29± 5	3	1	−	−

PKCα wild-type (wt) and mutant proteins were expressed in *S. cerevisiae* and partially purified by DEAE-Sephacel chromatography, followed by assay of phosphotransferase activity and purity, as described in "MATERIALS AND METHODS." All activity measurements were based on 15 μg protein per assay except for mutant D157-247 (0.18 mg protein). YEp51 is the control plasmid strain. One unit = 1 pmol ^{32}P transferred/min. (*n*), number of independent measurements; wt, wild-type; n.d., not determined. [a][^{3}H]Phorbol dibutyrate binding as reported in Refs. *15*, *21*, and unpublished results. [b]Background activity for D260-280 = 166±5 units/mg. [c]Quantitation of Western blot signals presented in Fig. 2.

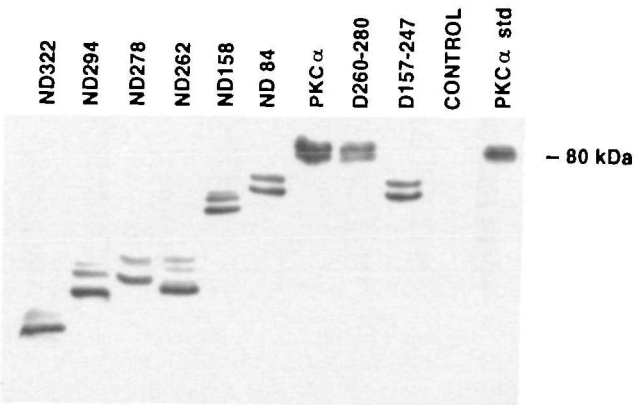


Fig. 2. **Western blot analysis of PKCα mutant proteins.** Samples isolated by DEAE-Sephacel chromatography from cells transformed with control plasmids (control) or plasmids expressing wild-type PKCα or mutants ND84, ND158, ND262, ND278, ND294, ND322, D157-247, and D260-280 were analyzed as described in "MATERIALS AND METHODS." Each lane was based on 20 μg sample protein. Highly pure recombinant PKCα (5 ng) was used as a positive control (PanVera).

and detergent extracts. This phenomenon and the appearance of doublets in most cases may indicate incomplete processing of specific PKC mutant proteins by phosphorylation (*30*), a possible outcome of the specific yeast strain used in this study (strain 334), as well as the mutant protein itself. For the objectives of the present study, we did not pursue the significance of this anomaly.

Inhibition Studies with PKC Mutants—Experiments were conducted to compare the activity of a given mutant in the presence and absence of 50 μM dequalinium, and to calculate the percentage of inhibition of protein kinase activity. Assays were carried out in the presence of PS/Ca^{2+} and TPA for all mutants in order to compare dequalinium potency with the wildtype PKCα under uniform conditions. A dequalinium concentration of 50 μM was selected because it caused substantial inhibition (45%) of wild-type PKCα activity and was therefore used to compare the sensitivities of various PKC mutants, as shown in Fig. 3. Mutant ND84, which represents truncation of the first 84 amino acids, was inhibited by 60%, somewhat higher than that obtained for the wild-type enzyme. The N-terminal segment missing in ND84 contains the pseudosubstrate sequence and most of the first cysteine-rich repeat (Fig. 1, CYS). Mutants ND158 and ND262 were inhibited by 63 and 72%, respectively (Fig. 3). As depicted in Fig. 1A, ND158 represents truncation of the V1 region and virtually all of the C1 region, which includes the site of phorbol ester binding (*12, 13*). The internal deletion mutant D157-247, which lacks aa 157-247 and most of the Ca^{2+}-binding domain (aa 182-257) but retains an intact phorbol ester binding site, was inhibited 75% by 50 μM dequalinium (Fig. 3). Mutant ND262, representing an additional deletion of V2 plus three-fourths of the C2 region, also displayed a strong degree of inhibition (72%) by 50 μM dequalinium (Figs. 1A and 3). Progressive deletion of PKCα up to aa 262 therefore resulted in a progressive increase in dequalinium sensitivity at 50 μM concentra-

tion, including internal deletion mutant D157-247 (Fig. 3).

Further truncation of ND262 by an additional 16 amino acids however, led to mutant ND278 whose activity was inhibited only 9% with 50 μM dequalinium (Fig. 3). Mutant ND278, retains the C-terminal border of the C2 segment, the complete hinge region, roughly defined by residues 292-317 [Ref. 2], and the complete catalytic domain (Fig. 1A). This result suggests that aa 263-278 of PKCα harbors a site that directly or indirectly plays a role in determining the sensitivity of the enzyme to dequalinium, or alternatively, that the ND278 mutation itself results in a structural defect that renders it unresponsive to 50 μM dequalinium.

Further truncation producing mutants ND294 and ND322, increasingly restored sensitivity to dequalinium at 50 μM concentration (Fig. 3). Both mutants possess the catalytic domain, and lack the entire regulatory domain. Mutant ND294 retains the hinge region (V3) at its amino terminus while mutant ND322 lacks this region (Fig. 1A). When tested with 50 μM dequalinium, mutant ND278 (9% inhibition) was surpassed in sensitivity by ND294 (22% inhibition), and by ND322 (97% inhibition) (Fig. 3).

ND278 and wild-type PKCα were compared in terms of their dose-dependent inhibition by dequalinium (Fig. 4). The diminished sensitivity of ND278 to 50 μM dequalinium (Fig. 3) was observed over a broad range of dequalinium concentrations. Overall, ND278 displayed an IC_{50} of 125 μM, which is twice that of the wild-type enzyme (IC_{50} = 62 μM).

In order to address the functional role of the segment between ND262 and ND278, which encompasses the C-terminal segment of the C2 region and part of the hinge

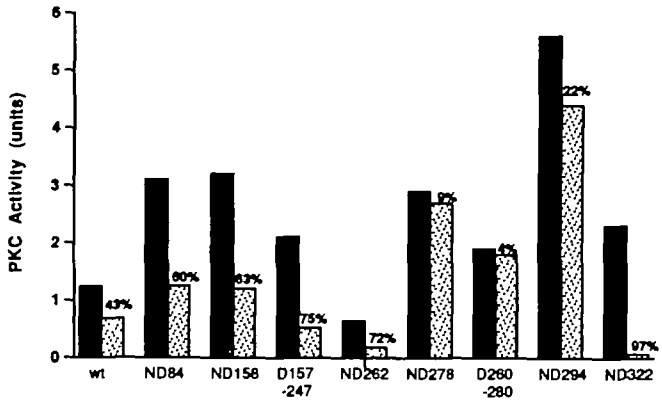


Fig. 3. **PKCα mutant inhibition profile for dequalinium.** Mutant protein activities (in total units) were measured in the absence (solid bars) and presence of 50 μM dequalinium (stipled bars), and the percentage of inhibition is indicated in the graph. Activity values of DEAE-Sephacel eluates were measured using 15 μg protein/assay, except for mutant D157-247 (180 μg/assay) and corrected for background activity as given by the control plasmid preparation (Table I). Each bar represents the average of 2 to 6 measurements at an error within 10%. The percentage of inhibition in each case is representative of three independent experiments, each consisting of duplicate measurements (1 unit = 1 pmol ^{32}P transferred/min).

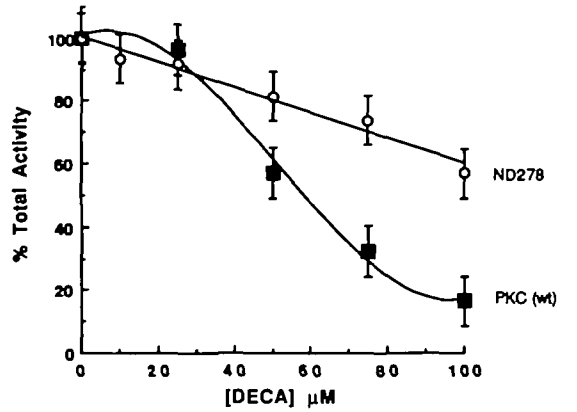
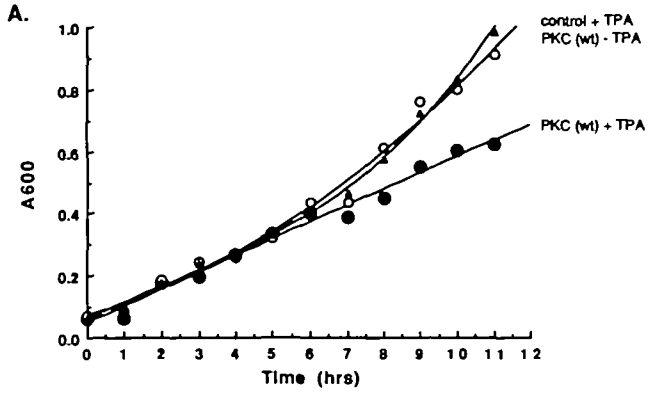


Fig. 4. **Dose-dependent inhibition of PKCα and ND278 by dequalinium.** Percentage of enzymatic activities (% total activity) for wild-type (wt) PKCα (■) or ND278 (○) were plotted against increasing concentrations of dequalinium (DECA). Mutant (2.9 units, 15 μg) or wild-type PKCα (1.5 units, 15 μg) activity values were measured with DEAE-Sephacel eluates and corrected for background activity (plasmid control). Data points are the average of triplicate measurements and variations are indicated by error bars. Results are representative of two independent experiments.



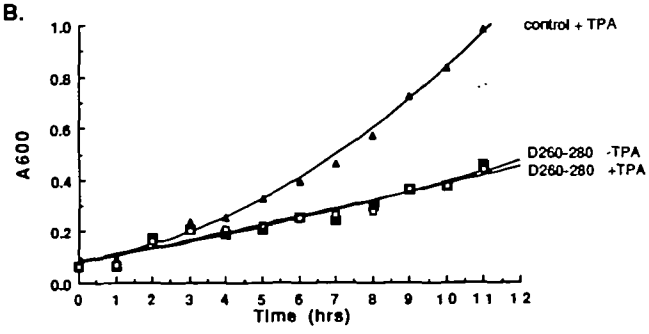


Fig. 5. **The impact of wild-type PKCα and mutant D260-280 on the growth rate of *S. cerevisiae*.** Results are representative of three independent experiments. A: Growth curves for cells constitutively expressing wild-type (wt) bovine PKCα. Cell density was measured by the O.D.$_{600}$ for cultures treated with (●) and without (○) 2 μM TPA. The growth curve for cells carrying the control plasmid grown with 2 μM TPA (▲) is shown for comparison. B: Growth curves for cells expressing PKC mutant D260-280 treated with (■) and without (□) 2 μM TPA. The growth curve for control plasmid cells grown with TPA (▲) is shown for comparison.

region, we tested an internal deletion mutant D260-280, depicted in Fig. 1A. Despite the absence of only 21 aa in the otherwise intact primary structure of PKC, mutant D260-280 exhibited low but detectable basal enzyme activity and very low levels of stimulation with the activators PS and TPA (Table I). This mutant displayed a very low level of sensitivity to 50 μM dequalinium (4% inhibition), similar to the weak response observed for ND278 (Fig. 3).

The Effect of Mutant D260-280 on the Growth Rate of S. cerevisiae—In earlier studies, yeast cells that overproduce wild-type bovine PKCα were found to exhibit a significant increase in doubling time in response to activation by TPA (*21, 31, 32*). This effect was marginal in PKCα-producing cells that had not been treated with phorbol ester, indicating that the activated form of the mammalian enzyme produces this altered phenotype in *S. cerevisiae* (*21, 31*) and *S. pombe* (*32*).

We employed this phenotypic assay to analyze the effect of D260-280 on cell growth with and without TPA. Figure 5, A and B, shows growth curves for wild-type PKCα, internal deletion mutant D260-280, and a control plasmid. Each strain was grown in the presence or absence of 2 μM TPA. TPA caused a 2-fold increase in the doubling time of cells overproducing wild-type PKCα, compared with untreated wild-type PKCα overproducing cells, as previously described (*21, 31*), but had no effect on the growth rate of TPA-treated cells transformed with the control plasmid (Fig. 5A). In the absence of TPA, cells overproducing mutant D260-280 (Fig. 5B), exhibited a 3-fold longer doubling time than the plasmid control or the unstimulated wild-type PKCα (Fig. 5A). The addition of TPA to mutant D260-280 cells did not further slow the growth rate (Fig. 5B). Thus, even in the absence of TPA, a PKC protein lacking aa 260-280 exhibits the phenotypic effects displayed by the TPA-activated wild-type PKCα. Taken together with Table I and Fig. 3, these findings imply that the mutant D260-280 protein represents a constitutively active enzyme with reduced sensitivity to inhibition by dequalinium.

DISCUSSION

We have presented evidence that the C-terminal portion of the C2 region (aa 260-280) modulates PKCα catalytic activity, and in parallel influences the inhibitory potency of dequalinium. The impact of progressive truncation of PKCα on the sensitivity to dequalinium revealed the importance of aa 263-278 as a determinant of dequalinium action, as well as in the regulation of catalytic activity. This specific segment of the C2 region has not previously been associated with any known PKC function.

Our findings indicate that in wild-type PKCα, aa 260-280 may function to maintain a lower basal activity. Using a phenotypic assay in which increased PKC activity correlates with reduced cell growth rate (*21, 31*), we found that cells expressing mutant D260-280 displayed a suppressed growth rate in the absence of TPA, similar to the TPA-activated form of wild-type PKCα (Fig. 5A). The high basal activity of this mutant was refractory to further stimulation by TPA both *in vitro* (Table I) and *in vivo* (Fig. 5B). One interpretation of these results is that in the wild-type enzyme, the sequence defined by aa 260-280 stabilizes an inactive conformation of PKC, while in the mutant protein D260-280 this stabilization is lost and the enzymatic activity is constitutively elevated.

Our finding that the sequence defined by aa 260-280 plays an important role in the regulation of PKC activity adds new information to the model used to describe the conversion of inactive PKC to its active conformation (*8*). According to this model, the intramolecular dynamics are governed by the binding of activating cofactors and the flexibility of the hinge region (V3 in Fig. 1A). Activators such as PS/TPA or PS/Ca^{2+} were shown by circular dichroism analysis to induce dramatic secondary structural changes in the enzyme (*6, 7*). During PKC activation, it is thought that the flexible hinge region displaces the pseudosubstrate domain (V1 in Fig. 1A) from its position in the substrate binding site (C3-V4-C4 in Fig. 1A) which consequently becomes accessible to exogenous substrates (*5*). Consistent with this model, the absence of the pseudosubstrate sequence (as in ND84) in the present work resulted in a constitutively active mutant protein that could not be significantly activated by cofactors (Table I). However, internal deletion of aa 260-280 in the C2 region, also led to a constitutively active mutant protein (Table I, Fig. 5B). This finding implies that, in addition to the pseudosubstrate region, the segment defined by aa 260-280 also regulates catalytic activity. The notion that a segment in the C2 region can influence catalytic activity usually centers on Ca^{2+} binding which occurs at aa 182-257 (*33, 34*). Recent studies with PKCβII suggested that phosphorylation of ^{660}SER, located near the C-terminus (C4 in Fig. 1A), is involved in regulating the affinities for Ca^{2+}, ATP, and substrate, and in determining protein stability (*35*). Because of the effect on Ca^{2+} binding affinity, the authors suggested that phosphorylation of ^{660}SER in the catalytic domain could promote a direct interaction with the Ca^{2+}-binding domain (aa 182-257) in the C2 region (*35*). Our findings are also consistent with a model in which an interaction occurs in the wild-type enzyme between aa 260-280 in the C2 region and the catalytic domain.

The concept of an interaction between aa 260-280 and the catalytic domain is again supported by our finding of diminished dequalinium sensitivity by mutants lacking this sequence. A fundamental observation in an earlier study established that a potent dequalinium inhibitory effect occurs with the catalytic fragment, prepared by limited trypsinolysis of wild-type PKCβ1 (*18*). Similarly, in the present work, the mutant protein ND322, which approximates the catalytic fragment of PKCα, also showed high sensitivity to dequalinium that exceeded that of the activated wild-type enzyme (Fig. 3). It is clear from these studies that inhibition of PKC activity occurs through the catalytic domain. Although earlier studies eliminated the ATP binding site as a target (*18*), the precise target site of dequalinium remains unknown. Since the target site is apparently fully available in ND322 (as shown by its potent inhibition by 50 μM dequalinium), it seems likely that ND278, ND294, and D260-280 lack a critical segment (presumably contained in aa 263-278) that is functional in the wild-type PKCα, and mutants ND84, ND158, and ND262, and that facilitates access by dequalinium to its target in the catalytic domain. This is supported by our deletion mapping experiment (Fig. 3) where mutant ND262 was potently inhibited by 50 μM dequalinium but mutants ND278, ND294, and D260-280 all exhibited very

low sensitivity. While there are no known phosphorylation sites in aa 263-278 of PKCα (^{263}V-S-E-L-M-K-M-P-A-S-G-W-Y-K-L-L^{278}), it is possible that within the regulatory domain, this segment contributes an important physical or chemical element that modulates the structural relationship between the catalytic and regulatory domains, influencing both basal catalytic activity and physical access by dequalinium to its inhibitory target site. This idea is consistent with the general function of the regulatory domain in governing access to the catalytic domain. In this light, it is not surprising that full restoration of dequalinium sensitivity occurs with ND322 (which was inhibited by 97%; Fig. 3) since it represents the complete removal of the regulatory domain and hinge region.

A current model for the binding of dequalinium to PKCα depicts two distinct target sites (one in the regulatory and one in the catalytic domain) that interact independently with two molecules of dequalinium, or co-dependently with a single molecule of dequalinium, since it is bipartite (*18*; Fig. 1B). Our recent work reported that when photolyzed with 366 nm light, dequalinium covalently modifies the RACK-1 binding site (aa 218-226), specifically at tryptophan-223 in the regulatory domain of human PKCα, and consequently inhibits PKCα translocation in human breast cancer cells (*20*). RACK-1 is an adaptor protein that is localized in the detergent-insoluble membrane fraction and has been proposed to bind only the activated form of PKC, as shown for PKCβ in cardiac myocytes (*36*). The RACK-1 binding domain (defined by aa 186-198 and 209-226) lies in the C2 region and is distinct from the phorbol ester binding domain located at aa 102-144 in the C1 region (*14*). In the present work, mutants D157-247 and ND262, which lack the RACK-1 binding domain, as well as the Ca^{2+} binding region (aa 182-257), are still sensitive to dequalinium, suggesting (i) that inhibition by dequalinium is not dependent on Ca^{2+} binding, and (ii) that the interactions by dequalinium with the RACK-1 domain and the catalytic domain can occur as independent events. We conclude therefore that the binding of dequalinium to the RACK-1 binding domain is not obligatory for dequalinium-mediated inhibition of catalytic activity, and is not related to the role played by aa 263-278 in modulating dequalinium-mediated inhibition.

In summary, the present work shows that deletion of aa 260-280 is mirrored both in the constitutive activation of PKC, and in impaired inhibition by dequalinium. The simplest interpretation of these results is that within the intact structure of the wild-type protein, the segment described by aa 260-280 in the regulatory domain mediates certain functional and structural aspects of the PKC catalytic domain. We propose that this region participates in a critical interdomain interaction.

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