Photoinduced Inactivation of Protein Kinase C by Dequalinium Identifies the RACK-1-Binding Domain as a Recognition Site*

(Received for publication, September 15, 1997, and in revised form, October 21, 1997)

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1,1'-Decamethylenebis-4-aminouqualdininium diiodide (DECA; dequalinium) is an anti-tumor agent and protein kinase C (PKC) inhibitor whose mechanism of action with PKC is unknown. This study reports that with human PKCα, DECA exhibited competitive inhibition (Kᵢ = 11.5 ± 5 μM) with respect to RACK-1 (receptor for activated C kinase-1), an adaptor protein that has been proposed to bind activated PKC following translocation (Ron, D., Luo, J., and Mochly-Rosen, D. (1995) J. Biol. Chem. 270, 24180–24187). When exposed to UV light, DECA covalently modified and irreversibly inhibited PKC (α or β), with IC₅₀ = 7–18 μM. UV/DECA treatment of synthetic peptides modeled after the RACK-1-binding site in the C2 region of PKCβ induced modification of Ser¹¹⁵-Leu-Asn-Pro-Glu-Trp-Asn-Glu-Thr²²⁶, but not of a control peptide. This modification occurred at a tryptophan residue (Trp²²⁶) that is conserved in all conventional PKC isoforms. In overlay assays with native RACK-1 that had been immobilized on nitrocellulose, UV-treated control PKCα bound well to RACK-1, whereas UV/DECA-inactivated PKCα had reduced binding activity. The significance of these findings is shown with adenocarcinoma cells, which, when pretreated with 10 μM DECA and UV light, exhibited diminished 12-O-tetradecanoylphorbol-13-acetate-activated PKCα translocation. Overall, this work identifies DECA as a tool that prevents PKC translocation by inhibiting formation of the PKC-RACK-1 complex.

Protein kinase C (PKC),¹ a monomeric Ca²⁺- and phospholipid-dependent serine/threonine protein kinase, plays a critical role in growth factor-activated signaling and malignant transformation (1, 2). A current focus of investigation is to identify a specific inhibitor of PKC that is targeted to a structural element of the protein that influences PKC behavior in cells. Because the regulatory domain of PKC distinguishes it from other protein kinases, it is this segment of PKC, contained in the N-terminal portion of the protein, that attracts the greatest interest. This domain houses the binding regions for PKC-activating ligands such as phosphatidylinerine (PS), diacylglycerol (DAG), and phorbol esters (C1 region) as well as for Ca²⁺ (C2 region).

¹ The abbreviations used are: PKC, protein kinase C; PS, phosphatidylinerine; DAG, diacylglycerol; DECA, 1,1'-decamethylenebis-4-aminouqualdininium diiodide; TPA, 12-O-tetradecanoylphorbol-13-acetate; Me₂SO, dimethyl sulfoxide; HPLC, high pressure liquid chromatography.

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This work was supported by National Institutes of Health Grant CA60618 and by the Gustavus and Louise Pfeiffer Research Foundation.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 4, Issue of January 23, pp. 2390–2395, 1998
Printed in U.S.A.

This paper is available on line at http://www.jbc.org
peptide adduct was carried out by Dr. J. Farmar (New York Blood Center, New York).

Synthesis of DECA—Dequalinium diiodide was synthesized by an established method (10, 13). Elemental analysis was as follows. Calculated: 50.74% C, 5.63% H, 7.89% N; found: 50.63% C, 5.57% H, 7.62% N (Desert Analytics, Tucson, AZ). It should be noted that commercially available dequalinium salts contain a major impurity.

Assay of PKC—PKC activity was taken as the difference in \( ^{32}P \)-Pi transferred from \( [\gamma-^{32}P]ATP \) to the modified pseudosubstrate peptide RFARKGSLRQKNV in the presence and absence of lipid activators (20 \( \mu \)g of PS and 1 \( \mu \)g of DAG) as described previously (10). Assays were conducted in triplicate, and the results were averaged. Values were typically within 10% error. Curve fitting was carried out with Cricketgraph software.

UV-induced Inactivation of PKC by DECA—The UV light-induced photolysis of DECA was carried out with a Black-Ray 365-nm ultraviolet lamp (Model UVL-56) that was positioned 2.3 ± 0.1 cm above a porcelain well plate seated in an ice bath. From this distance, the lamp delivered an intensity of 2700 microwatts/cm\(^2\). Into each well was placed a welled plate seated in an ice bath. From this distance, the lamp was angled, and the immunoprecipitated protein was prepared for SDS-polyacrylamide gel electrophoresis.

Overlay Assay—Immunoprecipitated native human RACK-1 (5 \( \mu \)g) or whole cell lysates (60 \( \mu \)g) were subjected to 9% SDS-polyacrylamide gel electrophoresis, followed by electrophoretic transfer to a nitrocellulose filter. The nitrocellulose filter was blocked overnight at 4°C in a 5% powdered milk suspension containing 0.1% Tween 20 and 0.01% sodium azide. The overlay assay utilized individual lanes, either by excising them as nitrocellulose strips or by sequestering the lanes of an intact filter in a Deca-Probe (Pharmacia Biotech Inc.). Purified recombinant human PKCa (0.27 \( \mu \)g) was UV-irradiated for 5 min in the presence of 1 mM DECA or 10% (v/v) Me\( _2 \)SO as a vehicle control. Following irradiation, each PKCa sample was tested for enzymatic activity in triplicate and for high affinity binding to immobilized native RACK-1. For the binding assay, each lane was treated with the PKC sample (3.5 \( \mu \)l) in Ca\(^{2+}\), DAG, and PS and incubated with gentle rocking for 30 min at room temperature as described previously (6). Lanes that were used in the overlay step were washed and detected with PKCa antisera. Additional lanes that excluded the overlay step were analyzed immunochromographically with either anti-RACK-1 antisera to demonstrate the RACK-1 signal at 33 kDa or with PKCa antisera to demonstrate the position of native PKCa (77 kDa) in cell lysates.

Cell Culture and Translocation Assay—MDA-MB-231 cells were cultured to 60–70% confluence on 150-mm plates in complete medium (Isove’s modified Dulbecco’s medium with l-glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin). In serum-free medium, plated cells were treated with 10 \( \mu \)M DECA (or Me\( _2 \)SO) for 30 min at room temperature and then, as indicated, subjected to 5 min of irradiation using a 365-nm UV lamp (American Ultraviolet Co., Murray Hill, NJ) at an intensity that delivered 2000 microwatts/cm\(^2\). Cells were washed with phosphate-buffered saline and replaced with complete medium containing TPA (1 \( \mu \)M) or 0.1% (v/v) Me\( _2 \)SO for 1 h at room temperature. Cells were washed with phosphate-buffered saline and harvested, and cell lysates were prepared by sonication (3 x 3 s). The homogenate was centrifuged at 200 \( \times \) g for 10 min to remove unlysed cells, and the resulting supernatant was centrifuged at 100,000 \( \times \) g for 1 h. The supernatant (soluble fraction) was analyzed for protein content and prepared for electrophoresis. The pellet (particulate fraction) was resuspended in buffer (20 mM Tris, pH 7.4, 250 mM sucrose, 2 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 \( \mu \)g/liter leupeptin, 10 \( \mu \)g/liter soybean trypsin inhibitor, and 10 \( \mu \)g/ml calpain inhibitor II) by Dounce homogenization (40 strokes), followed by sonication (3 x 3 s). The homogenate was centrifuged at 200 \( \times \) g for 10 min to remove unlysed cells, and the resulting supernatant was centrifuged at 100,000 \( \times \) g for 1 h. The supernatant (soluble fraction) was analyzed for protein content and prepared for electrophoresis. The pellet (particulate fraction) was resuspended in buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 5 mM EGTA, and 5 mM 2-mercaptoethanol plus protease inhibitors) containing 0.1% Triton X-100, placed on ice for 30 min with periodic Vortex mixing, and prepared for electrophoresis. Soluble (10 \( \mu \)g/lane) and particulate (5 \( \mu \)g/lane) fractions were analyzed by Western blotting and immunochromatically stained for PKCa (Santa Cruz Biotechnology).

RESULTS

Mode of Dequalinium-mediated Inhibition of PKCa with Respect to RACK-1—The binding interactions of recombinant RACK-1 and DECA. Lanes purified human PKCa were first explored by classical kinetics. For these studies, conditions of reversible binding of DECA were employed. Pilot experiments established that measurements of PKCa catalytic activity lay on the initial velocity component of the reaction curve. The Dixon plot (17) shown in Fig. 2 depicts concentration-dependent inhibition by DECA conducted in the presence of fixed nanomolar concentrations of recombinant RACK-1. The results reveal that DECA-mediated inhibition is competitive with re-
Dequalinium Interacts with the RACK-1-binding Domain of PKC

Dequalinium (DECA) was treated with DECA in the presence (●) and absence (□) of UV light. The data points reflect the means of triplicate measurements that were within 10% error. The plot is representative of four independent experiments.

Photoinduced inactivation of human PKCα by DECA. PKCα was treated with DECA in the presence (●) and absence (□) of UV light. The data points reflect the means of triplicate measurements that were within 10% error. The plot is representative of four independent experiments.

Photoinduced Inactivation of PKC—The following experiment demonstrates that irradiation with 365-nm light causes irreversible inhibition of PKCα activity by DECA, as revealed by dilution of the enzyme-DECA complex into the assay medium. Dilution would be expected to dissociate a reversibly bound complex such that full enzymatic activity is recovered. However, if PKC is irreversibly inhibited, the dilution step should be ineffective.

Our findings indicate that a 5-min UV irradiation (365 nm) of mixtures of PKCα and DECA produced an inactive PKC-DECA covalent complex that persisted despite the dilution step. As shown in Fig. 3, UV irradiation of PKCα and DECA alone produced a dose-dependent decrease in the recoverable kinase activity as the DECA concentration was increased; with 20 μM DECA, only 5% of the total PKCα activity remained. The IC₅₀ for UV-induced inactivation by DECA is 12 ± 5 μM (average of four independent experiments) and was consistent with Kᵢ = 11.5 ± 5 μM for human PKCα (Fig. 2) and with IC₅₀ = 11 μM previously obtained for recombinant rat PKCβ1 (10), the latter two values having been measured under conditions of reversible binding (i.e. no UV light). With recombinant human PKCβ1, UV-induced inactivation by DECA was again observed in the same concentration range, exhibiting IC₅₀ = 14 μM ± 4, the average of four independent measurements (data not shown).

In control experiments that omitted UV irradiation, reversible binding of DECA to PKCα was verified by assay of PKCα activity incubated with increasing concentrations of DECA. It can be seen in Fig. 3 that, in the absence of UV irradiation, incubation of PKC with up to 20 μM DECA produced 80–100% of the total activity following a 24-fold dilution into the assay medium. Losses of enzymatic activity attributable to UV light alone were usually within 5–20% of the control PKC activity. Western blot analysis of the irradiated enzyme showed no degradation of pure PKCα protein following UV irradiation for 5 min (data not shown).

Photolabeling of Synthetic C2 Domain Peptides—In view of results that indicate that DECA competes with RACK-1 for the RACK-1-binding domain, it was next considered that synthetic peptides previously shown to define the RACK-1-binding domain of PKC (6) may be covalently modified by DECA when irradiated with UV light. For these experiments, highly purified peptides were employed whose sequences had been modeled after the corresponding amino acid sequences of the C2 domain of PKCβ (Fig. 4A). The PKCβ peptide sequences are closely aligned with the corresponding sequences of PKCα, although minor deviations are evident, as shown by the underlined residues. The segment of the C2 domain described by these sequences lies C-terminal to the phorbol ester-binding domain (amino acids 102–144) (18) and within the general region that has been ascribed to Ca²⁺ binding (amino acids 187–249) (19, 20).

Irradiation of 100 nmol of each peptide for 45 min in the presence of 12 nmol of DECA was carried out. Separation of the unreacted material from the product(s) was performed by HPLC with a gradient of water and acetonitrile, with detection at 333 nm (λₘₐₓ of free DECA). Of the three peptides tested, only βC2-4 (amino acids 218–226) yielded a detectable signal at 333 nm, indicative of a DECA-peptide adduct (Fig. 4B, lower trace). While DECA itself typically eluted at 45% acetonitrile, this new peak eluted at 17% acetonitrile. This peak was not detected in the untreated peptide, peptide irradiated alone, or peptide mixed with DECA but not irradiated (data not shown). When a scrambled control peptide of βC2-4 (Fig. 4A) was UV-irradiated with DECA under identical conditions, no major peaks at 17% acetonitrile were detected (Fig. 4B, upper trace). This finding implied a sequence-specific interaction of DECA with βC2-4.

Sequence analysis (Table I) of the DECA-βC2-4 adduct demonstrated that the modification had occurred at the tryptophan residue of βC2-4, which corresponds to Trp223 of PKC. These results suggest that Trp223 is a site in the intact enzyme that can be covalently modified by DECA during PKC inactivation. It is of interest to note that this amino acid is strictly conserved in the C2 domains of the conventional PKC isoforms (α, β1/β2, and γ) (21).

Overlay Experiments—The ability of the DECA-inactivated enzyme to bind RACK-1 in an overlay assay was addressed. Under conditions that are optimal for RACK-1 binding (3, 7), samples of recombinant human PKCα (in the presence of PS/Ca²⁺/DAG) were incubated with strips of nitrocellulose representing individual lanes of a membrane onto which native human RACK-1 (immunoprecipitated from MDA-MB-231 cells) had been immobilized by electrophoretic transfer, as described under “Experimental Procedures.” Purified recombinant human PKCα was subjected to UV treatment with or without DECA, prior to the overlay step. For these experiments, DECA-inactivated PKCα was irreversibly inhibited by 70–90% as compared with the UV-treated control enzyme. As shown in Fig. 5A, UV-treated control PKCα was observed to bind well to endogenous RACK-1 (lane 2), whereas UV/DECA-inactivated PKCα displayed significantly reduced binding to RACK-1 (lane 3). These results demonstrate that, coincident with its inactivation by DECA, PKCα is rendered unable to bind RACK-1. This finding is consistent with the idea that DECA and RACK-1 recognize the same binding site in PKCα.

Additional high affinity interactions with human PKCα were observed with immobilized whole lysates of MDA-MB-231 cells (Fig. 5B, lane 2) that were also eliminated when the enzyme was inactivated by UV/DECA treatment (lane 3). This result suggests that, in addition to RACK-1, UV/DECA treatment blocks the ability of PKCα to bind other PKC-binding proteins as well, possibly localized in different subcellular compartments (reviewed in Ref. 22).

DECA Reduces Translocation of PKC in Human Breast Adenocarcinoma Cells—Because activated PKC is thought to bind RACK-1 as a consequence of translocation (6), the ability of DECA to intervene in phorbol ester-induced translocation of

![Fig. 3. Photoinduced inactivation of human PKCα by DECA.](image-url)
PKC was tested in human breast adenocarcinoma cells (MDA-MB-231). In these cells, PKCa is the most abundant isoform as well as the only isoform with a C2 domain. (The other isoforms found by Western blotting in these cells were PKCh, PKCi, and PKCm (data not shown).) Following pretreatment with DECA (10 μM) for 30 min and subsequent UV irradiation for 5 min, the cells were stimulated with the tumor-promoting phorbol ester TPA to induce translocation of cytosolic PKC into cellular membranes. The Western blot shown in Fig. 6 indicates that preincubation with UV/DECA followed by TPA treatment produced a marked retention of PKCa in the soluble fraction (lane 10) as compared with no retention when UV treatment alone preceded TPA addition (lane 8). Similarly, a decrease in TPA-induced PKCa recruitment to the particulate fraction was

**TABLE I**

Sequence analysis of unmodified and DECA-modified βC2-4

| Cycle | Amino acid | Unmodified βC2-4 | DECA-modified βC2-4 |
|-------|------------|-----------------|---------------------|
|       |            | pmol*           |                     |
| 5     | Glu        | 81.73           | 35.75               |
| 6     | Trp        | 54.32           | 2.93                |
| 7     | Asn        | 87.69           | 30.07               |

* Picomoles recovered.
Dequalinium Interacts with the RACK-1-Binding Domain of PKC

Evidence of covalent modification by DECA of the RACK-1-binding domain in PKC was presented with the synthetic peptide βC2-4 (amino acids 218–226). This peptide is one of three peptides known to define the RACK-1-binding site (Fig. 4A) and has been shown to interfere with PKC/RACK-1 binding interactions in cardiac myocytes and Xenopus (6). The modification of a tryptophan residue in βC2-4 by DECA is consistent with an earlier study with mitochondrial F1-ATPase in which a phenylalanine residue was derivatized by this compound (23). The preferential modification of βC2-4 as compared with the scrambled control peptide underscores the sequence specificity of the modification event and argues against random reactivity with tryptophan residues in the holoenzyme.

Previous work with the catalytic fragment of PKC showed that DECA causes inhibition of phosphotransferase activity with the same potency as with the intact enzyme (10), signifying that a critical target site is located in the catalytic domain. Neither MgATP nor peptide substrate is competitive with DECA, however (10).

Other work from this laboratory has shown that DECA is ineffective as an inhibitor up to 1 μm with other protein kinases such as the cAMP-dependent protein kinase catalytic subunit, the calmodulin-dependent myosin light chain kinase, and pp60src. The lack of effect with cAMP-dependent protein kinase is particularly compelling since the catalytic domains of PKC and cAMP-dependent protein kinase exhibit almost 50% sequence homology and are believed to have very similar three-dimensional structures (24).

The specificity of DECA for PKC may be explained in part by coincident binding interactions with both the catalytic domain (unidentified) and the regulatory domain (at the RACK-1-binding site). Inhibition of PKC by DECA involving the two domains could be due to either one or two molecules of DECA binding per molecule of PKC. A two-molecule binding model depicts the binding of one DECA molecule to the RACK-1-binding domain, which occurs in parallel with the binding of a second molecule of DECA to its target site in the catalytic domain. In a one-molecule binding model, the aminoquinoidalmin mieties of DECA bind simultaneously to the RACK-1-binding domain and catalytic domain of the intact enzyme. Thus, the bipartite structure of DECA (Fig. 1) participates in a unique two-point contact such that the regulatory and catalytic domains are tethered. This idea is lent support by the structure-activity relationships of DECA analogues and their inhibition of PKCβ1 (10), from which it is known that the 10-carbon distance that exists between the aminoquinoidalmin mieties is a critical determinant of the inhibitory potency of DECA, thus implicating both ends of the molecule in DECA-mediated inhibition. A one-molecule binding model is also strengthened by the present study, which showed that nanomolar concentrations of RACK-1 (C2 domain interaction) were sufficient to diminish the DECA-mediated inhibition of PKC activity (catalytic domain interaction) (Fig. 2).

An important finding of this work is that interaction of DECA with the RACK-1-binding domain of PKC produces significant inhibition of translocation. This result may explain the ability of DECA to delay the morphological response by fibroblasts treated with phorbol ester (10), to act as an anti-tumor agent (8, 25), and to inhibit cell motility and invasion (26). That DECA did not entirely abolish TPA-induced translocation but merely suppressed it may be explained by the artificially strong stimulus provided by TPA (27) that also produces direct interactions of PKC with membrane lipids (28). In view of the proposal that RACK-1 binds activated PKC (29), the present study suggests that DECA impedes this association (as well as

\[ \text{DISCUSSION} \]

In this work, we demonstrate that, under reversible binding conditions, DECA and RACK-1 display competitive binding kinetics. When irradiated with UV light (365 nm), DECA causes irreversible inhibition of recombinant human PKC activity (Fig. 3) with concomitant loss of high affinity binding by PKC to immobilized RACK-1 (Fig. 5A) and other unidentified proteins (Fig. 5B). The biological significance of PKC inactivation by DECA was observed in cells whose treatment with UV/DECA significantly diminished TPA-induced translocation of PKCα.

Evidence of covalent modification by DECA of the RACK-1-binding domain in PKC was presented with the synthetic peptide βC2-4 (amino acids 218–226). This peptide is one of three peptides known to define the RACK-1-binding site (Fig. 4A) and has been shown to interfere with PKC/RACK-1 binding interactions in cardiac myocytes and Xenopus (6). The modification of a tryptophan residue in βC2-4 by DECA is consistent with an earlier study with mitochondrial F1-ATPase in which a phenylalanine residue was derivatized by this compound (23). The preferential modification of βC2-4 as compared with the scrambled control peptide underscores the sequence specificity of the modification event and argues against random reactivity with tryptophan residues in the holoenzyme.

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\[ \text{3 A. Francis-Smith, S. A. Rotenberg, unpublished results.} \]
other unidentified high affinity protein/protein interactions), thereby blocking downstream PKC-directed signaling events.

Acknowledgments—We thank Dr. Daria Mochly-Rosen (Stanford University Medical Center) for generously providing the C2 domain peptides and the bacterial RACK-1 expression system used in this study. Stimulating discussions with Prof. Corinne A. Michels, Prof. William S. Allison, and Dr. Susan Jaken are gratefully acknowledged. Technical assistance was provided by Regina Sullivan.

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