Inhibition of Rodent Protein Kinase C by the Anticarcinoma Agent Dequalinium


ABSTRACT

Dequalinium has previously been shown to be an anticarcinoma agent (M. J. Weiss et al., Proc. Natl. Acad. Sci. USA, 84: 5444–5448, 1987). The present study demonstrates that it can inhibit protein kinase C, isolated from an overproducing cell line with a 50% inhibitory concentration of 8–15 μM. Further examination of the inhibition by using structural analogues of dequalinium reveals that the length of the methylene bridge between the two quinaldinium moieties, the presence of the ring substituents, and the bipartite character of the compound each contributes to the inhibitory potency. Related studies show that the analogues display the same rank order of inhibitory potency when tested with the trypsin-generated catalytic fragment of the enzyme, indicating that dequalinium inhibits kinase activity through an interaction with the catalytic subunit. Further studies argue that the ability of a given analogue to inhibit phosphotransferase activity correlates with its ability to compete with [3H]phorbol-12,13-dibutyrate binding on the intact enzyme (50% inhibitory concentration of 2–5 μM). This suggests that the inhibitor is either binding directly to the regulatory subunit as well, or that due to its interaction with the catalytic subunit, dequalinium produces an indirect effect on sites defined by phorbol ester binding. Kinetic analysis revealed that inhibition is noncompetitive with respect to ATP or phosphatidyl-serine. Studies conducted with types I, II, and III rat brain isozymes, resolved by hydroxylapatite chromatography, demonstrate that dequalinium inhibits each of them with similar potency (50% inhibitory concentration of 11 μM) and imply that the site of contact on the enzyme is a highly conserved region. Morphology studies with dequalinium in intact cells demonstrate that the inhibitor can protect control cells against phorbol ester-induced morphology changes but cannot protect protein kinase C-overproducing cells, suggesting that an elevation in protein kinase C levels alone is sufficient to overturn the protection conferred by dequalinium. On the basis of these results, we propose that protein kinase C could be a critical in vivo target of dequalinium.

INTRODUCTION

Over the last five years the fields of biochemistry and molecular biology have been harnessed to study the structure and function of PKC, a calcium- and phospholipid-dependent protein kinase that participates in many cell regulatory processes. This enzyme serves as both the receptor for phorbol ester tumor promoters (1) and as a key enzyme in growth factor-mediated signal transduction (2). It is activated by diacylglycerol, a product of phosphatidylinositol turnover, which, in turn, is stimulated by a variety of hormones and agonists (2). The enzymatic activity termed "PKC" is expressed by a family of at least seven known structurally and functionally related isozymes (3–9) whose distribution varies widely among somatic and neural tissues (10, 11). Rat brain expresses at least four isozymes, α, β1, β2, and γ, each of which is distinct immunogenically and is found in characteristic anatomical locations of this tissue (11, 12). Whether the diversity of enzyme structure and tissue distribution reflect differences in the response to stimuli or in the selection of substrates is not yet known, although recent studies provide evidence for subtle functional differences in cofactor requirements between individual PKC isozymes (11, 13).

One approach to studying the PKC isozymes individually has been to utilize the cDNA clone for a given isozyme in order to overproduce the protein product in a suitable cell type (14, 15). This laboratory recently cloned a cDNA encoding the rat brain β1 isozyme. The cDNA was inserted into the retroviral vector pMV7 (16) which was then packaged into defective retrovirus particles by ψ 2 cells (17). Rat fibroblasts were virally infected with the vector such that the β1 enzyme was constitutively expressed at high levels. The overexpression of PKC caused abnormalities in morphology and growth control (14). More recently, we used the same strategy to develop derivatives of the murine embryo fibroblast cell line C3H10T½ that stably express high levels of rat PKC-β1 (18). This approach provided a novel cell culture model for functional studies of PKC and also permitted the convenient isolation of a single isozyme in large quantity. Biochemical characterization of this PKC activity with regard to its chromatographic behavior on hydroxylapatite, response to tumor promoters, reactivity with PKC-specific antibodies, and sensitivity to known PKC inhibitors, indicated that the overproduced PKC-β1 enzyme exhibits properties consistent with those of native brain PKC (19).

Because PKC is thought to have a key role in regulating growth in many cell types, it may be a candidate target enzyme for the design of novel antineoplastic agents (20). The present work describes the investigation of dequalinium as a potential inhibitor of PKC, a study prompted by the published report (21) that this lipophilic, antimicrobial agent exhibits anticarcinoma activity. In that study, dequalinium was shown to be 125-fold more toxic to human carcinoma cells such as MCF-7 than to normal epithelial cells such as CV-1. Furthermore, dequalinium potently inhibited the growth of human colon carcinoma (MB49 or CX1), or rat colon carcinoma (W163) that had been s.c. implanted in nude mice. Dequalinium also inhibited growth and led to the regression of mammary tumors chemically induced in situ by the carcinogen 7,12-dimethylbenz(a)anthracene (22). The selective toxicity of this drug apparently arises from the higher membrane potential maintained across membranes of mitochondria in carcinoma cells (23). Thus, mitochondria concentrate lipophilic compounds such as dequalinium to a much higher degree than normal cells leading, in response to the large concentration gradient, to a slow release of the drug into the cytoplasm. A recent study has shown that one of the cyto-
plasmic effects of dequalinium is to act as an antagonist of calmodulin (24).

Using PKC-β₁ isolated from the C3H10T½/2 cell system described above, we show that dequalinium is also an inhibitor of protein kinase C-β₁ activity in vitro and present structure-activity studies that examine the structural determinants of the inhibition. We determine the sites on the enzyme with which the inhibitor interacts by testing for its interference with phosphotransferase activity of the isolated catalytic fragment, and for competition with phorbol ester binding on the regulatory subunit of the holoenzyme and in intact cells. We also test whether the individual rat brain isoforms types I, II, and III resolved by hydroxylapatite chromatography exhibit a difference in sensitivity to dequalinium. Preliminary studies with intact C3H10T½/Cells which overproduce PKC-β₁, and the vector control cell line, provide evidence that dequalinium can interfere directly with a PKC-mediated event in vivo.

MATERIALS AND METHODS

Materials

Tissue culture medium and calf serum were purchased from Flow Laboratories, Inc., 150-mm plates were from Nunc, and G418 was from Gibco. Proteamine sulfate, histone III-S, DEAE-Sepharose, bovine serum albumin, bovine γ-globulin, polyethylene glycol (M, 6000-8000), TPCK-trypsin-agarose, and phosphatidylserine were from Sigma. Phenyl-Sepharose was purchased from Pharmacia, hydroxylapatite (Bio-Gel HT) was a product of Bio-Rad, and [7-32P]ATP (0.5-3 Ci/mmol) was purchased from Amersham. The EGF receptor synthetic peptide substrate (R-K-R-T-L-R-R-L) was synthesized by J. Wideman (Columbia University), and leupeptin was a gift from W. Troll (New York University). [3H]PDBu was purchased from Du Pont New England Nuclear, TPA and nonradioactive PDBu were products of LC Laboratories, Inc., 150-mm plates were from Nunc, and G418 was from Gibco. Protamine sulfate, histone III-S, DEAE-Sephacel, bovine serum albumin, bovine γ-globulin, polyethylene glycol (M, 6000-8000), TPCK-trypsin-agarose, and phosphatidylserine were from Sigma.

Methods

Cell Culture. C3H10T½-PKC-4 cells (18) were cultured in Dulbecco's modified Eagle's medium containing 10% calf serum for a maximum of 2 months. With the exception of the final passage prior to harvesting, the cells were maintained under selection with 50 µg/ml G418 (14). Cells were grown in 150-mm plates for 4 days or until confluent. These cells have an 11-fold increase in PKC activity when compared to control C3H10T½ (18).

Isolation of PKC. PKC-β₁, derived from C3H10T½-PKC-4 cells was isolated by using previously described methods (14). Briefly, C3H10T½-PKC-4 cells were harvested at confluence, typically from twenty-five 150-mm plates (2.7 x 10⁶ cells/plate), and lysed in 10 mM Tris, pH 7.5, 5 mM EGTA, 2 mM EDTA, 0.25 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml soybean trypsin inhibitor, 15 mM 2-mercaptoethanol, and 0.1% Triton X-100 (v/v) (extraction buffer).

Following 25 strokes with a Dounce homogenizer and centrifugation at 9000 rpm for 20 min at 4°C, the extract was applied to a disposable Bio-Rad column containing 1 ml DEAE-Sepharose that had been equilibrated with extraction buffer containing no Triton X-100. After the flowthrough activity (typically <1 unit/ml) was collected, the column was washed with 10 column volumes of Buffer A (20 mM Tris, pH 7.5, 2 mM EGTA, 2 mM EDTA, 1 mM diithiothreitol, 1 mM PMSF, 10 µg/ml soybean trypsin inhibitor), and PKC was then eluted with 3-5 column volumes of Buffer A plus 100 mM NaCl. When stored at -70°C in 10% glycerol and 0.05% Triton X-100, this crude preparation of PKC-β₁ showed significant loss of activity, sometimes as high as 50%, probably due to the freeze-thaw process. The length of storage at -70°C, however, did not further affect the recovered activity. The specific activity of the enzyme used for the experiments described was typically 6-17 units/mg, where 1 unit is 1 nmol 32P transferred to substrate per min (see below). Partially purified PKC-β₁ preparations were judged adequate for these studies since, with respect to total PKC activity, such preparations consisted of approximately 93% β₁ isoform. This homogeneity was shown by hydroxylapatite chromatography (19), and represents the highest enrichment in PKC-β₁ reported for this isoform.

Isolation of the mixture of isoforms (3.4 units/mg) from rat brain was carried out similarly, except that the extraction buffer contained 20 mM Tris, pH 7.5, 10 mM EGTA, 2 mM EDTA, 5 mM dithiothreitol, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor. Further resolution of the rat brain enzyme preparation into individual isoforms was accomplished by using a step elution procedure with phenyl-Sepharose, in order to exclude a largely hydrophilic population of proteins, followed by elution from hydroxylapatite with a potassium phosphate gradient (20-200 mM). The hydroxylapatite chromatography is a method previously shown to resolve the individual isoforms of the brain holoenzyme (25, 26). For following storage at -70°C, the specific activities of types I, II, and III isoforms on the day of the experiment were 71, 29, and 40 units/mg, respectively. Purification of the β₁ isoform from C3H10T½-PKC-4 cells was carried out in an identical manner.

Protein content of each enzyme sample was determined by the Bio-Rad protein assay with lysozyme as standard.

Protein Kinase C Assay. PKC activity was taken as the difference in the amount of 32Pi, transferred from [γ-32P]ATP to a synthetic peptide sequence of the EGF receptor (R-K-R-T-L-T-L-R-R-L) in the presence and absence of PS. The reaction medium (0.12 ml), placed in 13-x 100-mm disposable glass test tubes, consisted of 20 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM CaCl₂, and the following components were added in order: 4.0 µg synthetic peptide, 10 µg PS or 10 µl H₂O, 10 µl enzyme (3.8 µg), and 10 µl of test compound. All dequalinium solutions were made up fresh on the day of the experiment. They were solubilized in methanol and serially diluted into water for preparation of working solutions. At no time did the final concentration of methanol exceed 8%; control experiments demonstrated that the presence of methanol up to 10% had no effect on PKC phosphotransferase activity. For each inhibitor concentration, duplicate sets of tubes were prepared with and without phospholipid. PS working solutions were prepared by drying the stock PS (dissolved in chloroform), using N₂, adding 1 ml sterilized, deionized H₂O, and sonicating with three 10-s bursts with a sonifier cell disrupter, Model W185 (Heat Systems, Ultrasonors, Inc.). Following the addition of test compound, the tubes were incubated on ice for 10 min. The kinase reaction was initiated by the addition of 73 µM [γ-32P]ATP (approximately 200 cpm/pmol) to each tube, which was immediately transferred to a 30°C waterbath for 10 min. The reaction was quenched by transferring 40 µl of the reaction medium to a 3-x 3-cm square of phosphocellulose paper and immersing it in a 1.0-liter beaker of tap water. After terminating all reactions, the squares were washed 5 times with 1 liter of water. Finally, each square was placed in a test tube containing 7 ml hydroxylapatite and was measured for 32P content. The results for each duplicate set of tubes were averaged. The difference between the (+) PS and the (-) PS tubes was judged to be Ca²⁺/PS-dependent activity. The presence of PS typically stimulated the kinase reaction 6- to 10-fold with the synthetic EGFR peptide as substrate.

For assays measuring phosphorylation of histone III-S (average M, 16,000), the conditions were identical to those described above except that histone III-S (15, 21, or 42 µM) replaced the EGF receptor peptide. For assays in which proteamine sulfate phosphorylation was measured, the reaction medium (0.12 ml) consisted of 20 mM Tris, pH 7.5, 10 mM MgCl₂, proteamine sulfate (0.083 - 0.67 mg/ml), 10 µM enzyme (0.3 mg/ml), and test compound which was added last; no phospholipid or Ca²⁺ was present. The kinase reaction was carried out in triplicate under the conditions described above. The reported results were given as the means of these triplicates which differed by less than 15%. All experiments were repeated at least twice.

Isolation of Catalytic Fragment. Using PKC prepared by DEAE-Sepharose chromatography, as described above, the catalytic fragment of the enzyme was released by limited proteolysis with trypsin. For the purpose of controlling the time of exposure to protease, TPCK-trypsin immobilized on agarose was used since centrifugation rapidly removes it from the reaction medium. The reaction was initiated by adding the
TPCK-trypsin-agarose to the enzyme suspension (1 mg/ml) in an Eppendorf tube such that there was 100 μl TPCK-trypsin-agarose/500 μl enzyme. The tube was rotated for 2 min at 8°C, and immediately centrifuged for 1 min in a Fisher microcentrifuge (Model 235C). The supernatant, containing the catalytic fragment, was quickly transferred to a fresh tube to which PMSF (1 μM) and soybean trypsin inhibitor (10 μg/ml) were further added. The extent of proteolysis was confirmed by demonstrating the loss of PS and Ca²⁺ dependence of the kinase reaction and the 6- to 8-fold higher level of activity in the absence of PS. In order to resolve the catalytic fragment from any remaining intact enzyme, the product mixture was first diluted 3-fold with Buffer A, followed by chromatography on 0.5 ml DEAE-Sephacel. Typically, an unproteolyzed preparation of the PKC-β1 enzyme yields a single peak when chromatographed on DEAE-Sephacel and eluted with 80 mM NaCl in Buffer A.¹ Using a 10-ml gradient of 0–4 M NaCl in Buffer A, the trypsin-proteolyzed PKC preparation eluted at two distinct activity peaks (27). The first peak, representing residual intact PKC, displayed a 6-fold enhancement when PS was added. The second peak, representing the catalytic fragment, exhibited less than 2-fold stimulation by PS. Fractions (0.7 ml) representing the second peak were pooled to a total volume of 2.1 ml. The PKC catalytic fragment in this pool (77 units/mg) was used for triplicate assays with the dequalinium analogues, as described, but without added phospholipid. The results are given as the means of these triplicates which differed by less than 10%.

¹³H]Phorbol Ester Binding. [³H]PDBu binding was assayed in a reaction (0.25 ml) consisting of 80 mM Tris-Cl, pH 7.4, 0.1 mM calcium chloride, 7.5 mM magnesium acetate, 1 mM dithiothreitol, phosphatidylyserine (25 μg/ml), bovine serum albumin (5 mg/ml), enzyme preparation (3.8 μg), 10 nM [³H]PDBu, and test compound added to the indicated concentration. In order to assess nonspecific binding of [³H]PDBu, a duplicate set of tubes was prepared for each concentration of test compound that included 30 μM nonradioactive PDBu. The reaction was conducted in 1.5-ml Eppendorf tubes which were incubated for 5 min at 30°C and then cooled in an ice water bath. Estimation of bound [³H]PDBu was by the polyethylene glycol precipitation method (28), which entails the addition of bovine γ-globulin (0.5 mg) and polyethylene glycol (12% final concentration) to each tube. After at least 30 min on ice, the tubes were microfuged for 15 min at 8°C. Following the removal of the supernatant, the ³H content of the pellet was measured by severing the tip of the Eppendorf tube and placing it in 7 ml Hydrofluor. In order to allow solubilization of the pellet material, scintillation cocktail was added and the tubes were incubated 1–2 h later. Specific binding was taken as the difference between the ³H content in the pellets resulting from binding reactions conducted with and without nonradioactive PDBu. Typically, the nonspecific binding represented 30% of the total ³H in the pellet. The results are reported as the means of triplicate values which differed by less than 15%.

Studies with Intact Cells. Cultures of 10T½-MV7-5 and 10T½-PKC-4 cells grown as described previously (18), were seeded at one-third to one-half saturation density on 60-mm plates. Forty-eight hours later the cultures were treated with either 10 μM DECA in DMSO [0.1% DMSO final concentration (v/v)] or DMSO alone. Fifteen minutes after the cells were treated with 100 ng/ml TPA in DMSO [0.01% solvent final concentration (v/v)] or DMSO alone. The cultures were viewed microscopically every 15–30 min for 6 h and were photographed 90 min following TPA treatment.

Synthetic Methods

General. All chemicals and reagents used for synthesis of the dequalinium series of compounds were purchased from the Aldrich Chemical Co. (Milwaukee, WI). HPTLC-HLF chromatography plates were purchased from Analtech (Newark, DE). The synthetic methods described below are well established and have appeared in earlier studies (29–31). With the exception of HAQ, they included elemental analysis and melting point data of the products. Characterization of the purity of the products used for the present studies was by melting point determination with a Meltemp and by TLC. The most discriminating solvent system found for compounds of this type was chloroform/methanol/acetic acid/water (50/20/10/5). All compounds were stored protected from light at —20°C.

HAQ. Prepared in the same manner as MAQ except that iodomethane was replaced by 1-iodoethane. Purity was assessed at greater than 99% by TLC. The melting point range was 232–233°C.

HEXA. Three equivalents of 4-aminooquinidine were refluxed with 1 equivalent of 1,6-diiodohexane for 72 h in 2-butanol. The precipitate was collected, washed with acetone and diethyl ether, and recrystallized 1 time from ethanol and methanol. TLC showed a major product (>95%) and a secondary contaminant which turned brown upon prolonged exposure to air. This contaminant is likely to be 1-(4-iodoethane-methylen)-4-aminooquinuidinium iodide, a result of incomplete reaction. A trace amount of other impurities was present which is likely to represent isomeric products in which the alkylation occurred at the amino nitrogen rather than the heterocyclic nitrogen. The melting range of the product was 294–300°C; reported melting, 295–296°C (29, 30).

OCTA. Prepared in the same manner as HEXA except that 1,6-diiodohexane was replaced by 1,8-diiodooctane. TLC showed a single major product (>98%). The melting range was 288–291°C; reported melting range, 294–295°C (29, 30).

DECA. Prepared in the same manner as HEXA except that 1,6-diiodohexane was replaced by 1,10-diiododecane. TLC showed a major product (>95%). The impurities are likely to be similar to the isomers discussed by HEXA. The melting range was 302–304°C; reported melting range, 308–309°C (29, 30). The dichloride salt of DECA (DECA-CI) is available commercially from Sigma. The major component of DECA-CI migrated at nearly the same rate as the iodide salt, but the number and quantities of impurities were noticeably greater. The melting range for DECA-CI from Sigma was 303–306°C; reported melting range, 317–318°C (29).

BQ-10. Prepared in the same manner as DECA except that 4-aminooquinidine was replaced by quinoline. TLC showed a major product (>90%) and a single other component. The melting range was 230–232°C; reported melting range, 213–214°C (31).

RESULTS

Our initial studies focused on the DECA analogue of the dequalinium series (Fig. 1A) since it is this compound that was used in the anticarcinoma studies (21), and thus serves as the parent compound of the series. As shown in Fig. 2, we found...
that the DECA analogue exerts a strong inhibitory effect on the kinase activity of PKC-β₁, exhibiting a IC₅₀ in the range of 8–14 μM. This discovery prompted us to explore the structure-activity relationships of a series of analogues in which the length of the methylene bridge was varied. The HEXA and OCTA analogues were synthesized, representing lengths of the methylene bridge as 6 and 8 carbons, respectively (Fig. 1A). Fig. 3 presents the results of testing each compound on the β₁ isozyme in the manner described for the DECA compound in Fig. 2. The results indicate that inhibitory potency increases as the methylene bridge is lengthened. In this series, the HEXA compound was found to be the least effective PKC-β₁ inhibitor. For four determinations, the IC₅₀ value for this compound was 71 ± 11 μM (SD), which is about 6 times higher than that of DECA, signifying a diminished inhibitory effectiveness by the presence of 6 methylene carbons instead of 10. Increasing the number of carbon units in the methylene bridge to 8 and 10 enhanced the inhibitory potency as shown by the progressively lower IC₅₀ values obtained from three determinations for the OCTA (IC₅₀ = 25 ± 3 μM), and DECA (IC₅₀ = 11 ± 3 μM) structures. We tested a related structure, BQ-10 (Fig. 1B), which retains the 10 carbon methylene bridge but lacks the amino and methyl substituents on both ring systems. Relative to the parent compound, BQ-10 was >40 times less potent, exhibiting a IC₅₀ >400 μM. Thus, inhibition of PKC depends on the presence of the exocyclic ring substituents. The absence of effect on kinase activity by the monoquinaldinium analogues (Fig. 1C) HAQ and MAQ (no inhibition up to 0.83 mM), demonstrates that the bis structure of an analogue is also a critical determinant of its potency.

Under our standard assay conditions (see “Materials and Methods”) the IC₅₀ values described above were highly reproducible. Inhibition by DECA was found to be equally effective with PKC-β₁ preparations of different levels of purity. Thus, enzyme that had been isolated by DEAE-Sepharose, phenyl-Sepharose, and hydroxylapatite chromatographies, was as sensitive to DECA as enzyme prepared only by step elution from DEAE-Sepharose, i.e., despite an 8-fold difference in protein content. Systematic variations in certain components of the reaction system, however, yielded pronounced alterations in the apparent IC₅₀, indicating that the IC₅₀ values reported here are not absolute. One component found to be critical to inhibitor sensitivity was the PKC substrate chosen for the assay. Our standard assay contained as substrate a 30 μM concentration of a synthetic octapeptide fragment of the EGFR, which contains the threonine site phosphorylated by PKC in vitro (33). When histone III-S was examined as a substrate of PKC-β₁, the enzyme was about 5-fold less sensitive to inhibition by the DECA analogue (IC₅₀ = 50 μM). In addition, we addressed the inhibitory effectiveness of the DECA analogue by using protamine sulfate as substrate. This artificial substrate of PKC can be phosphorylated in the absence of phospholipid and calcium (34) and thereby reports solely on PKC catalytic function. We found that over a wide range of protamine sulfate concentration (0.083–0.67 mg/ml), PKC-β₁ activity was consistently refractory to inhibition by DECA, even when tested at 100 μM (data not shown here). These data indicate that the choice of substrate can dramatically determine the occurrence and extent of inhibition by dequalinium of PKC activity. In studies with staurosporine, a potent inhibitor of PKC (35), we have observed a similar dependence of IC₅₀ on the substrate used in the assay.4

In addition to choice of substrate, variations in the concentrations of two other assay components, ATP or PS, affected the extent of inhibition by DECA. Shown in Fig. 4 are Lines-waver-Burk analyses of the dependence of inhibition on the concentration of ATP and PS, respectively. For ATP concentrations in the range of 3–50 μM, noncompetitive action by DECA was observed (Fig. 4A) such that Kᵢ = 50 μM (i.e., the inhibitor concentration which doubled the slope of the reciprocal plot). At ATP concentrations higher than 50 μM (data not shown), no ATP-dependent effect of DECA was observed. Since the standard assay medium contained 73 μM ATP, the observation of 50% inhibition by 8–14 μM DECA under standard conditions (Fig. 2) cannot be explained by interference with ATP binding to the enzyme. Noncompetitive inhibition by DECA was also observed with respect to activation of PKC by the phospholipid PS (Fig. 4B). Such findings indicated that over the range of 60–240 μM PS, inhibition by DECA was decreased as the PS concentration was progressively elevated. The noncompetitive character of the plots for ATP and PS dependence implies that DECA is not interacting directly with the sites occupied by these cofactors.

To gain further insight into the mechanism of dequalinium-mediated inhibition, we sought to identify the site or sites on the enzyme with which DECA directly interacts to produce inhibition. We first considered that dequalinium might interfere directly with the function of the catalytic subunit. To test this, we prepared the catalytic fragment by subjecting PKC-β₁ to limited proteolysis by trypsin, followed by chromatography on
DEAE-Sephacel to resolve the catalytic fragment from residual intact enzyme ("Materials and Methods"). The results, shown in Fig. 5, demonstrate the effects of the DECA, HEXA, and BQ-10 compounds on the activity of the catalytic fragment. It was found that the DECA analogue inhibited phosphotransferase activity with an IC_{50} of 10 μM; the HEXA analogue was significantly less inhibitory (IC_{50} = 120 μM), and the BQ-10 compound was ineffective. Significantly, the rank order of inhibitory potencies of these analogues obtained with the catalytic fragment was consistent with the rank order observed with the intact enzyme (Fig. 3). Thus, the catalytic domain of PKC-β, is a target for inhibition by DECA. When protamine sulfate was used as a substrate of the catalytic fragment, there was again no interference by the DECA compound even when tested at 100 μM (data not shown).

It is noteworthy that at DECA concentrations sufficient to inhibit >95% of the total activity of the holoenzyme (Fig. 1), there was only 75% inhibition of the activity obtained with the isolated catalytic fragment activity (Fig. 5). Unlike the experiments with the intact enzyme which measured the difference in phosphotransferase activity with and without phospholipid, the activities reported for the catalytic fragment represented total kinase activity in the absence of phospholipid. Thus, the residual activity evident in Fig. 5 may reflect either the presence of other kinases which are able to phosphorylate the EGF receptor peptide substrate, and/or a subpopulation of the PKC catalytic fragment molecules that is resistant to inhibition by DECA.

In light of the dual character of some other PKC inhibitors which can interact with both the catalytic and regulatory subunits (36), the inhibitory potency of the dequalinium analogues was assessed in terms of another function residing in the regulatory subunit, the region on PKC recognized by diacylglycerol and by phorbol esters such as PDBu. By using [3H]PDBu as a probe, we examined the ability of dequalinium to displace this radioactive marker from the intact enzyme. As shown in Fig. 6, the specific binding of [3H]PDBu to PKC-β, was dramatically reduced by the DECA analogue, with an IC_{50} of 2–5 μM, in the presence of 25 μg PS/ml assay medium. HEXA was consistently less potent than DECA, while BQ-10 and HAQ had no inhibitory effect (data not shown). Thus, these studies demonstrate that the structural features which determine the potency for inhibiting the phosphotransferase activity of either the intact enzyme or the catalytic fragment are also reflected in the ability to inhibit phorbol ester binding.

The identification of the regulatory subunit as another possible locus of action of dequalinium led us to consider that the inhibitor may discriminate among the α, β, and γ isoforms from rat brain since each isoform has a characteristic amino acid sequence at its NH₂ terminus (11). PKC activity assays were performed with DECA on a mixture of rat brain isoforms and also on the PKC isoforms resolved by hydroxylapatite, types I, II, and III. The results of these assays are shown in Table 1. In agreement with the potency of the DECA compound

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![Fig. 4. Kinetic analysis of inhibition of PKC-β, by dequalinium. A, dependence on ATP concentration. Lineweaver-Burk plot of PKC-β, activity (7 μg enzyme/assay) was measured in duplicate at various concentrations of [γ-32P]ATP having a constant specific radioactivity of 613 cpm/pmol. The Kₐ for ATP was determined to be 15 μM. The non-competitive behavior of inhibitor was measured at DECA concentrations of 50 μM (●) and 100 μM (▲). B, dependence on PS concentration. Lineweaver-Burk plot of PKC-β, activity (3.5 μg enzyme/assay) was measured in duplicate at various concentrations of PS (Avanti Polar Lipids) in the presence of DECA concentrations of 26 μM (●), 52 μM (▲), and 75 μM (▲). In the absence of inhibitor, the enzyme activity was a constant value over the range of PS concentrations presented (60–240 μM).

![Fig. 5. Effect of DECA analogues on kinase activity of the catalytic fragment of PKC-β,]. Assays of the phosphotransferase activity of the catalytic fragment in the presence of increasing concentrations of the indicated analogue were carried out under the conditions described for the intact enzyme (see "Materials and Methods"), but without the addition of phosphatidylserine. All assays were conducted in triplicate for each concentration of drug and data are expressed as the average values. DECA (●), HEXA (▲), and BQ-10 (■).

![Fig. 6. Effect of DECA analogues on [3H]PDBu binding. Assays for specific binding of [3H]PDBu to PKC-β, were carried out at the indicated concentrations of the dequalinium analogues, as described in "Materials and Methods." PKC-β, was prepared by DEAE-Sephacel chromatography. DECA (●), HEXA (▲), and BQ-10 (■).

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measured with PKC-\(\beta_1\) isolated from the C3H10T\(\frac{1}{2}\)-PKC-4 cells, the mixture of rat brain isozymes exhibited a \(IC_{50}\) of 11 \(\mu\)M. When tested with the DECA analogue, the rat brain peak activities I, II, and III, which correspond to isozymes \(\gamma, \beta,\) and \(\alpha\), respectively (37), displayed a similar \(IC_{50}\) value. Thus, dequalinium effectively inhibits the native PKC activities from brain but apparently does not recognize differences among the individual isozymes. Because there may be other isozymes in rat brain that were not resolved by hydroxylapatite, we should stress that the \(\alpha, \beta,\) and \(\gamma\) preparations from rat brain tested here are probably not homogeneous.

Inhibition of PKC by DECA in Intact Cells. We next determined whether DECA could inhibit PKC in intact cells. To accomplish this, we used two derivations of the murine embryo fibroblast line C3H10T\(\frac{1}{2}\) developed in our laboratory (18). Cells designated 10T\(\frac{1}{2}\)-PKC-4 contain a retroviral expression vector into which has been inserted a rat brain PKC-\(\beta_1\) cDNA. In these cells there is an 11-fold greater PKC enzyme activity than in a control cell line, 10T\(\frac{1}{2}\)-MV7-5, which harbors the retrovector without a cDNA insert (18). 10T\(\frac{1}{2}\)-MV7-5 cells, like parental 10T\(\frac{1}{2}\) cells, exhibit a characteristic morphological response when exposed to TPA; 10T\(\frac{1}{2}\)-PKC-4 cells show an exaggerated response (18). We therefore tested whether DECA could inhibit the morphological response of these cell lines to the phorbol ester TPA. Cultures of 10T\(\frac{1}{2}\)-MV7-5 and 10T\(\frac{1}{2}\)-PKC-4 were pretreated with DMSO (i.e., solvent control) or 10 \(\mu\)M DECA for 15 min and then challenged with 100 ng/ml TPA. Ninety min after TPA treatment, 10T\(\frac{1}{2}\)-MV7-5 cells pretreated with DMSO showed a typical morphological response relative to non-TPA-treated control cultures (compare Fig. 7, A and B). In contrast, cultures of 10T\(\frac{1}{2}\)-MV7-5 cells pretreated with DECA show a much less profound alteration in morphology following TPA treatment (Fig. 7C). Treatment with DECA alone had no effect on the morphology of these cells (data not shown). Thus, DECA can inhibit a primary response of intact cells to TPA. 10T\(\frac{1}{2}\)-PKC-4 cells, which are somewhat morphologically altered without TPA treatment (18), showed an exaggerated morphological response to TPA, relative to 10T\(\frac{1}{2}\)-MV7-5 cells (in Fig. 7, compare A and B with D and E). However, DECA was unable to inhibit the response to TPA in these cells (Fig. 7F). DECA had a slight inhibitory effect in 10T\(\frac{1}{2}\)-PKC-4 at very early time points (\(\approx 15\) min), before the full morphological response was achieved (data not shown). Thus, overproduction of PKC can overcome the inhibition by DECA of the effects of TPA. These data strongly suggest that the ability of DECA to inhibit the morphological response to TPA in 10T\(\frac{1}{2}\)-MV7-5 cells occurs by its ability to inhibit PKC directly.

It should be noted that the inhibitory effect of DECA in 10T\(\frac{1}{2}\)-MV7-5 cells was transient. Five h after TPA treatment, the cultures receiving DECA and TPA showed a strong morphological response and the cultures were indistinguishable from cultures that received only TPA. Whether this is due to metabolism of DECA to an inactive form, its uptake by mitochondria, or the gradual accumulation of TPA-induced, PKC-mediated phosphorylation events, is unclear.

### DISCUSSION

The results of these studies indicate that micromolar concentrations of dequalinium markedly inhibit PKC activity. A series of analogues was synthesized to assess the contribution of the methylene bridge, the ring substituents, and the \(\text{bis}\) structure to the inhibition. The inhibitory potencies of these analogues were determined by using the partially purified \(\beta_1\) isofrom of PKC derived from C3H10T\(\frac{1}{2}\) cells genetically engineered to overproduce this isofrom (14, 18). Of those compounds tested, we found that a \(\text{bis}\) structure containing a methylene bridge of 10 carbons and that retains the ring substituents is the most potent inhibitor of the \(\beta_1\) isofrom. Analogues having a shorter methylene bridge (6 or 8 carbons) attenuated the inhibitory potency and analogues lacking either the ring substituents or the \(\text{bis}\) structure were noninhibitory, implying that these features are critical to inhibition. A future direction of the present work should consider analogues having a methylene bridge of 12 or more carbons in order to establish an optimal bridge length.

Our studies also revealed that the sensitivity of PKC to inhibition by dequalinium is dependent on the substrate used in the assay. Inhibition was highest with the EGFR synthetic peptide (R–K–R–T–L–R–L–R) which contains the threonine site phosphorylated by PKC \textit{in vivo} (33). A 5-fold higher concentration of the drug was required to produce comparable inhibition when the substrate was histone, and no inhibition was seen when protamine was the substrate, even when the DECA concentration was 100 \(\mu\)M. Given that in all cases both substrate and inhibitor were cationic, it is unlikely that this substrate-related effect is due to an interaction of substrate and inhibitor alone, although more extensive kinetic analysis would be required to exclude this possibility. An alternative explanation of this phenomenon is suggested by previous studies of PKC with synthetic peptides and histone substrates (38, 39). Such systematic surveys have indicated that the substrate affinity is enhanced significantly by increasing the number of cationic amino acids adjacent to the phosphorylation site. Moreover, relative to the peptide substrates tested, histone proteins, in general, were observed to have much lower \(K_a\) values, suggesting that tertiary structure also has an important role in determining this kinetic parameter. It is possible, therefore, that protein substrates like histone and protamine, which have considerably more cationic character and tertiary structure than the EGFR peptide, can compete more effectively with DECA for critical anionic residues in the substrate binding site of PKC. Further studies are required, however, to determine the exact mechanism. Nevertheless, our studies indicate that the choice of substrate should be considered carefully when screening potential PKC inhibitors.

Another consideration emerging from these studies is our finding that increasing concentrations of PS decreased the inhibitory effect of DECA on PKC activity (Fig. 4B). In kinetic studies, phospholipid and DECA exhibited noncompetitive behavior implying that DECA does not bind directly to the PS-binding site. In view of the lipophilic nature of dequalinium, the drug may simply partition into the phospholipid micellar phase such that its availability to the enzyme is diminished. Consistent with this possibility, we found that high PS concentrations can attenuate the inhibitory potency of DECA even with the catalytic fragment of PKC (data not shown), \textit{i.e.}, under

#### Table 1 Effect of DECA on PKC isozymes from rat brain

The mixture of PKC isozymes that eluted from DEAE-Sephacel (see "Materials and Methods") and the three individual peaks resolved by hydroxylapatite chromatography were assayed for inhibition of kinase activity by the DECA analogue. For additional details see "Materials and Methods." Earlier studies (37) have established that types I, II, and III correspond genetically and immunochemically to \(\gamma, \beta,\) and \(\alpha\) isofroms, respectively.

<table>
<thead>
<tr>
<th>Rat brain isozyme</th>
<th>(IC_{50}) ((\mu)M)</th>
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<tbody>
<tr>
<td>Mixture</td>
<td>11</td>
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<td>Type I</td>
<td>11</td>
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<td>Type II</td>
<td>9</td>
</tr>
<tr>
<td>Type III</td>
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In these cells there is an 11-fold greater PKC enzyme activity than in a control cell line, 10T\(\frac{1}{2}\)-MV7-5, which harbors the retrovector without a cDNA insert (18). 10T\(\frac{1}{2}\)-MV7-5 cells, like parental 10T\(\frac{1}{2}\) cells, exhibit a characteristic morphological response when exposed to TPA; 10T\(\frac{1}{2}\)-PKC-4 cells show an exaggerated response (18). We therefore tested whether DECA could inhibit the morphological response of these cell lines to the phorbol ester TPA. Cultures of 10T\(\frac{1}{2}\)-MV7-5 and 10T\(\frac{1}{2}\)-PKC-4 were pretreated with DMSO (i.e., solvent control) or 10 \(\mu\)M DECA for 15 min and then challenged with 100 ng/ml TPA. Ninety min after TPA treatment, 10T\(\frac{1}{2}\)-MV7-5 cells pretreated with DMSO showed a typical morphological response relative to non-TPA-treated control cultures (compare Fig. 7, A and B). In contrast, cultures of 10T\(\frac{1}{2}\)-MV7-5 cells pretreated with DECA show a much less profound alteration in morphology following TPA treatment (Fig. 7C). Treatment with DECA alone had no effect on the morphology of these cells (data not shown). Thus, DECA can inhibit a primary response of intact cells to TPA. 10T\(\frac{1}{2}\)-PKC-4 cells, which are somewhat morphologically altered without TPA treatment (18), showed an exaggerated morphological response to TPA, relative to 10T\(\frac{1}{2}\)-MV7-5 cells (in Fig. 7, compare A and B with D and E). However, DECA was unable to inhibit the response to TPA in these cells (Fig. 7F). DECA had a slight inhibitory effect in 10T\(\frac{1}{2}\)-PKC-4 at very early time points (\(\approx 15\) min), before the full morphological response was achieved (data not shown). Thus, overproduction of PKC can overcome the inhibition by DECA of the effects of TPA. These data strongly suggest that the ability of DECA to inhibit the morphological response to TPA in 10T\(\frac{1}{2}\)-MV7-5 cells occurs by its ability to inhibit PKC directly.

It should be noted that the inhibitory effect of DECA in 10T\(\frac{1}{2}\)-MV7-5 cells was transient. Five h after TPA treatment, the cultures receiving DECA and TPA showed a strong morphological response and the cultures were indistinguishable from cultures that received only TPA. Whether this is due to
conditions in which the kinase activity is independent of phospholipid. This result implies that PS can physically sequester the inhibitor. Thus, the \( IC_{50} \) values we obtained for DECA with the holoenzyme, and that have been determined for certain other lipophilic cations whose inhibition of PKC is a function of the phospholipid concentration (40), are not absolute values. Consequently, it is difficult to predict from these subcellular studies the inhibitory potency of this compound for PKC in vivo.

The present studies suggest that DECA inhibits PKC by causing perturbations in the function of both the catalytic and regulatory domains of this enzyme. Since we found that DECA and related analogues inhibited the phosphotransferase activity of the catalytic fragment with potencies similar to those seen with the holoenzyme, it would appear that the primary target of the drug is the catalytic domain. The fact that the inhibitory efficacy of DECA is a function of the phosphotransferase substrate and the ATP concentration is also consistent with this interpretation. At the same time, where previous studies have established that the PDBu-binding site resides exclusively on the regulatory domain (41), we found that DECA inhibited \(^{3}H\)PDBu binding to the PKC holoenzyme. Competition of DECA with phorbol esters is compatible with the mechanism observed for other PKC inhibitors that are lipophilic amines, i.e., the antineoplastic lipidal amine CP-46,665-1 (42), and sphingosine (43).

It is noteworthy that the potency with which DECA displaced phorbol ester (\( IC_{50} = 2-5 \mu M \)) was similar to the potency with which it inhibited phosphotransferase activity (\( IC_{50} = 8-14 \mu M \)). In addition, the DECA, HEXA, and BQ-10 analogues had the same rank order of potency in inhibiting \(^{3}H\)PDBu binding as they did in inhibiting the kinase activity of the intact enzyme. These binding studies were carried out in the absence of the peptide substrate, demonstrating that DECA and its analogues could bind in a substrate-independent manner consistent with their inhibitory potencies. Although the \( IC_{50} \) values observed in the binding studies were consistently lower than those determined for kinase inhibition, we attribute this higher sensitivity to the fact that the PS concentration in the binding studies (25 \( \mu g/ml \)) was lower than the PS concentration in the kinase inhibition assay (83 \( \mu g/ml \)).

Thus, it is possible that DECA interacts with specific binding sites on both the catalytic and regulatory domains of PKC. Alternatively, the inhibitor might occupy a single major binding site (on the catalytic domain) which blocks or induces a conformational change such that phorbol ester-binding sites on the regulatory domain become inaccessible. This would be consistent with current models of PKC activation (20, 44) which invoke an unfolding of the protein in the presence of activating cofactors. A complex or mixed mode of inhibition has also been proposed for several other lipophilic cationic PKC inhibitors, including the aminoacridines (45), tamoxifen (36, 46), trifluoperazine (36), Adriamycin (36), and gossypol (36). Gossypol, in particular, has been shown to inhibit both \(^{3}H\)PDBu binding to the holoenzyme and phosphotransferase activity of the catalytic fragment (with histone III-S as substrate) with similar potency, a phenomenon observed with DECA in the present study. The site(s) of action of DECA on PKC is (are) apparently highly conserved, since our studies with the overexpressed rat \( \beta \), form and the individual rat brain isozymes revealed no significant differences in sensitivity to inhibition by this drug.

Using cell morphology as an indicator of phorbol ester action, we have shown that dequalinium does indeed interfere with the response of intact cells to phorbol ester. The transient protection from a saturating dose of phorbol ester was conferred on vector control cells by an external concentration of 10 \( \mu M \), consistent with its \( IC_{50} \) in vitro. This experiment indicated that, relative to TPA, when DECA is present at 60-fold excess, it
can cause effective inhibition of phorbol ester binding in vivo and that an elevation of PKC-β1 levels alone is sufficient to overturn that inhibition. It is also noteworthy that despite the ability of DECA to inhibit PKC with only certain substrates in vitro, our studies with intact cells demonstrate the inhibition of PKC action with native substrates.

Although this experiment showed that PKC can serve as an in vivo target of dequalinium, it did not examine other cellular components that could serve as targets in the drug concentration range studied. Dequalinium has been shown previously to act as an antagonist of calmodulin (IC₅₀ = 1 μM) in the in vitro activation of a cyclic AMP phosphodiesterase (24, 47), apparently by binding directly to calmodulin. This effect of DECA correlated with its potency as an antiproliferative agent in a Ca²⁺-astrocytoma cell line (47). That tamoxifen, another antitumor lipophilic cation, is both a PKC inhibitor and a calmodulin antagonist in the activation of a cyclic AMP phosphodiesterase (48) suggests that the antitumor effects of these and other lipophilic cationic drugs are due to interactions with more than one cellular target.

In the present study, the analysis of DECA-mediated inhibition of PKC was greatly facilitated by the use of preparations of the overproduced PKC-β₁ isoform which were virtually homogenous (93%) with respect to total PKC activity (19). Consequently, the possible ambiguity imposed by the presence of other PKC isoforms was avoided. We suggest that the use of cell systems that have been genetically engineered to overproduce a specific PKC isoform should provide valuable tools for examining the potency and specificity of any potential PKC inhibitor in both enzyme preparations and intact cells. Further studies are in progress regarding the possible in vivo role of PKC as a critical cellular target for the anticarcinoma activity of DECA and other potential PKC inhibitors, using these genetically engineered cell systems.

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REFERENCES

INHIBITION OF PROTEIN KINASE C BY DEQUALINIUM


Inhibition of Rodent Protein Kinase C by the Anticarcinoma Agent Dequalinium

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