Resveratrol Preferentially Inhibits Protein Kinase C-Catalyzed Phosphorylation of a Cofactor-Independent, Arginine-Rich Protein Substrate by a Novel Mechanism†

Jubilee R. Stewart,‡ Nancy E. Ward,‡ Constantin G. Ioannides,§ and Catherine A. O'Brian*,‡

Departments of Cancer Biology and Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 173, Houston, Texas 77030

Received April 15, 1999; Revised Manuscript Received July 26, 1999

ABSTRACT: Resveratrol, a polyphenolic natural product abundantly present in grape skins, is a candidate cancer chemopreventive agent that antagonizes each stage of carcinogenesis and inhibits protein kinase C (PKC), a key mediator of tumor promotion. While resveratrol has been shown to antagonize both isolated and cellular forms of PKC, the weak inhibitory potency observed against isolated PKC cannot account for the reported efficacy of the polyphenol against PKC in cells. In this report, we analyze the mechanism of PKC inhibition by resveratrol. Our results indicate that resveratrol has a broad range of inhibitory potencies against purified PKC that depend on the nature of the substrate and the cofactor dependence of the phosphotransferase reaction. Resveratrol weakly inhibited the Ca^{2+}/phosphatidylserine-stimulated activity of a purified rat brain PKC isozyme mixture ($IC_{50} = 90\ \mu M$) by competition with ATP ($K_i = 55\ \mu M$). Consistent with the kinetic evidence for a catalytic domain-directed mechanism, resveratrol inhibited the lipid-dependent activity of PKC isozymes with divergent regulatory domains similarly, and it was even more effective in inhibiting a cofactor-independent catalytic domain fragment (CDF) of PKC generated by limited proteolysis. This suggested that regulatory features of PKC might impede resveratrol inhibition of the enzyme. To explore this, we examined the effects of resveratrol on PKC-catalyzed phosphorylation of the cofactor-independent substrate protamine sulfate, which is a polybasic protein that activates PKC by a novel mechanism. Resveratrol potently inhibited protamine sulfate phosphorylation ($IC_{50} = 10\ \mu M$) by a mechanism that entailed antagonism of the activation of PKC by protamine sulfate and did not involve competition with either substrate. On the basis of the presence of PKC isozymes at subcellular sites rich in polybasic proteins, it has been proposed that certain endogenous polybasic PKC substrates may activate PKC in cells by the same mechanism as protamine sulfate. Our results suggest that antagonism by resveratrol of the phosphorylation of cellular PKC substrates that resemble protamine sulfate in their interactions with PKC may contribute to the efficacy of resveratrol against PKC in cells.

Resveratrol is a polyphenolic antifungal natural product that is present in high abundance in grape skins (*1*, *2*). Resveratrol exhibits potent anti-cancer activity in experimental model systems (*2*). Carcinogenesis models divide the development of cancer into the stages of initiation, promotion, and progression (*3*). Resveratrol has been shown to possess potent chemopreventive activity against each one of these stages, and direct antagonism of phorbol ester-mediated tumor promotion by resveratrol has been demonstrated in the two-stage mouse skin carcinogenesis model (*2*).

The phorbol ester tumor-promoter receptor protein kinase C (PKC)[1] is an isozyme family comprising at least 11 members (*4*, *5*). PKC isozymes are divided into three subfamilies based on differences in their regulatory domains and allosteric cofactor requirements (*4*, *5*). Common (cPKC) isozymes (α, β_1, β_2, and γ) are Ca^{2+}-dependent and phorbol ester-responsive; novel (nPKC) isozymes (δ, ϵ, θ, η, and μ) are Ca^{2+}-independent and phorbol ester-responsive; and atypical (aPKC) isozymes [ι (λ) and ζ] are Ca^{2+}- and phorbol ester-independent (*4*, *5*). The potent and selective activation of cPKC and nPKC isozymes by phorbol ester tumor promoters such as 12-(*O*-tetradecanoyl)phorbol 13-acetate (TPA) implicates PKC as a key mediator of tumor promotion (*3*, *4*, *6*). In support of this, it has been demonstrated that enforced expression of the PKC isozymes cPKC-β_1 and nPKC-ϵ, respectively, induces partial and full transformation of cultured fibroblasts (*7*, *8*). In addition, PKC isozymes are targets of the cancer chemopreventive agents piroxicam (*9*), ursodeoxycholate (*10*), curcumin (*11*), and tamoxifen (*12*, *13*).

Phorbol ester-mediated activation of PKC results in the translocation of PKC isozymes from the cytosol to the cell membrane and cytoskeleton (*4*, *5*). In a recent report, 15 μM resveratrol was shown to inhibit TPA-mediated PKC translocation and TPA-induced cyclooxygenase 2 (COX-2) transcription in human epithelial cells (*14*). In addition,

† This work was supported by grants from The Robert A. Welch Foundation (G-1141) and the National Institutes of Health (CA 74831).

* To whom correspondence should be addressed: Telephone 713-792-7969; Telefax 713-792-8747; Email obrian@mdacc.tmc.edu.

‡ Department of Cancer Biology.

§ Department of Gynecologic Oncology.

[1] Abbreviations: COX-2, cyclooxygenase 2; IC_{50}, inhibitor concentration that achieves 50% inhibition; PKC, protein kinase C; aPKC, atypical protein kinase C; cPKC, common protein kinase C; nPKC, novel protein kinase C; PS, phosphatidylserine; [Ser25]PKC(19−31), Arg-Phe-Ala-Arg-Lys-Gly-Ser^{25}-Leu-Arg-Gln-Lys-Asn-Val; TPA, 12-(*O*-tetradecanoyl)phorbol 13-acetate.

10.1021/bi990875u CCC: $18.00
Published on Web 09/15/1999

enforced expression of cPKC-α induced COX-2 transcription in the cells, and this was also blocked by 15 μM resveratrol (*14*). These observations suggest that tumor promotion antagonism by resveratrol (*2*) may involve inhibitory effects against PKC. In fact, resveratrol has been reported to inhibit partially purified PKC activity, but with an inhibitory potency (IC_{50} = 175 μM) (*1*) that cannot account for its above-described effects (*14*) against the action of phorbol esters and PKC-α in epithelial cells.

In this report, we first confirmed that resveratrol is a weak inhibitor of purified Ca^{2+}- and PS-dependent PKC activity (IC_{50} = 90 μM). The inhibitory mechanism entailed competition with the substrate ATP, based on kinetic analysis and inhibition of a catalytic-domain fragment of PKC. The apparent inhibitory potency of resveratrol was considerably greater against the catalytic-domain fragment compared with intact PKC. This suggested that regulatory features of PKC might impede resveratrol inhibition of the enzyme. To explore this, we examined the effects of resveratrol on PKC-catalyzed phosphorylation of a cofactor-independent substrate. The arginine-rich protein protamine sulfate is a cofactor-independent PKC substrate by virtue of its ability to activate the enzyme by a novel mechanism (*15*, *16*). Resveratrol potently antagonized the protamine sulfate phosphorylation reaction of PKC (IC_{50} = 10 μM) by a mechanism that was not competitive with respect to either the nucleotide or protein substrate but did entail antagonism of the activation of PKC by protamine sulfate. Our results suggest that the reported efficacy of resveratrol against cellular PKC (*14*) may reflect antagonism of the phosphorylation of arginine-rich PKC substrates that bear resemblance to protamine sulfate in their interactions with PKC.

MATERIALS AND METHODS

Enzymes, Substrates, and Other Reagents. Rat brain PKC was purified to near-homogeneity as previously described (*17*). The purified PKC preparation is a mixture of the isozymes α, β, γ, ε, and ζ (*18*). The histone kinase activity of the PKC preparation was stimulated more than 10-fold by 0.2 mM Ca^{2+} and 30 μg/mL PS but was unaffected by either cofactor alone. A fully active catalytic-domain fragment preparation was produced from rat brain PKC by limited trypsinolysis, as previously described (*19*). The histone kinase activity of the catalytic-domain fragment was stimulated less than 1.5-fold by 0.2 mM Ca^{2+} and 30 μg/mL PS. Baculovirus-produced, purified human PKC isozymes were purchased from Pan Vera Corp. (Madison, WI). Resveratrol (*trans*-3,4′,5-trihydroxystilbene) was purchased from Calbiochem (San Diego, CA). The protein substrates protamine sulfate and histone III-S were purchased from Sigma Chemical Co., the synthetic peptide substrate [Ser25]-PKC(19−31) was obtained from BACHEM (King of Prussia, PA), and [γ-^{32}P]ATP was from Amersham Corp. (Arlington Heights, IL). Bovine brain L-α-phosphatidylserine (PS) (≅98% purity) and all other reagents, including poly(Arg,-Ser) (3:1), ATP, L-1-tosylamido-2-phenyl chloromethyl ketone-treated trypsin, buffers, chelators, and protease inhibitors, were purchased from Sigma.

Kinase Assays. The protein and peptide kinase activities of purified rat brain PKC were assayed as previously described (*18*). Peptide kinase reaction mixtures contained

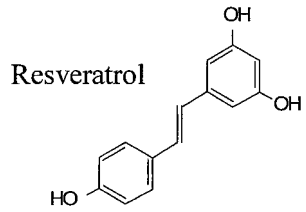

FIGURE 1: Structure of resveratrol.

20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$ (or 0.2 mM EGTA), 30 μg/mL PS (or none), 6 μM [γ-^{32}P]ATP (5000−8000 cpm/pmol), 10 μM [Ser25]PKC-(19−31), and 1−10 ng of PKC. PS was added to the reaction mixtures in the form of a sonicated dispersion. In assays of histone or protamine sulfate phosphorylation, [Ser25]PKC-(19−31) was replaced with either 0.67 mg/mL histone III-S or 0.20 mg/mL protamine sulfate. Where indicated, resveratrol was included in the reaction mixtures. Resveratrol was prepared as a 100 mM stock in DMSO; the final DMSO concentration in the reaction mixtures (<0.25%) was without effect on PKC activity. A 10-min reaction period at 30 °C was initiated by the addition of [γ-^{32}P]ATP, and substrate phosphorylation was quantitated on the basis of substrate binding to phosphocellulose paper, as previously described (*18*, *19*).

In assays of baculovirus-produced, purified human PKC isozymes, the above procedures were employed with the following modifications. Because each of the isozymes subjected to analysis phosphorylates 50 μM [Ser25]PKC-(19−31) efficiently (*20*), reaction mixtures contained 50 μM [Ser25]PKC(19−31) and 100 ng of the PKC isozyme under investigation. Ca^{2+} was omitted from assays of nPKC and aPKC isozymes.

All kinase assays were performed in triplicate and expressed as the mean value ± SD. To analyze inhibitory kinetics, Lineweaver−Burk, Dixon, and Hill plots, and nonlinear curves (*V* versus *S* graphs) (*21*) were generated by regression analysis with Microsoft software. All experimental results shown were determined to be reproducible in separate experiments.

RESULTS

Inhibition of cPKC, nPKC, and aPKC Isozymes by Resveratrol. [Ser25]PKC(19−31) is a defined peptide substrate that is based on the pseudosubstrate sequence of cPKC-α, and it is universally recognized as an excellent substrate by PKC isozymes (*20*). Consistent with the recognition of [Ser25]PKC(19−31) by both Ca^{2+}-dependent and Ca^{2+}-independent PKC isozymes, we determined that the Ca^{2+} and PS dependence of [Ser25]PKC(19−31) phosphorylation by a purified rat brain PKC isozyme mixture consisting of cPKC-α, -β, and -γ, nPKC-ε, and aPKC-ζ (*18*) was 3.0−3.5-fold. We first investigated the effect of the cancer chemopreventive agent resveratrol (Figure 1) on the [Ser25]-PKC(19−31) kinase activity of the purified PKC isozyme mixture in the presence of Ca^{2+} and PS. Representative results are shown in Figure 2. Resveratrol (5−200 μM) inhibited the PKC isozyme mixture in a concentration-dependent manner (Figure 2, ∇), with an IC_{50} of 90 ± 7 μM and a maximal inhibition of 65% ± 6% (the IC_{50} and maximal inhibition are average values obtained from three independent experiments).

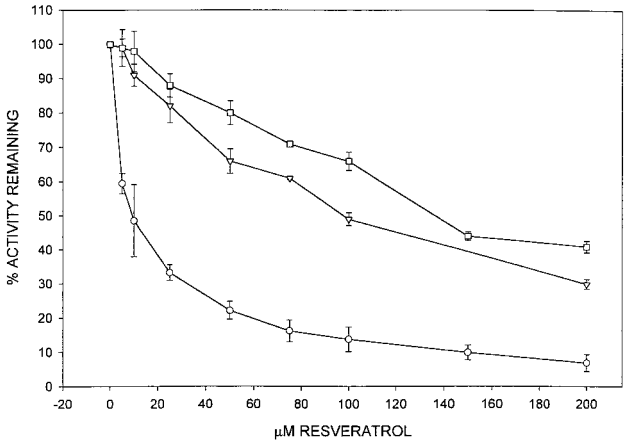

FIGURE 2: Inhibition of a purified PKC isozyme mixture and purified recombinant cPKC-α by resveratrol. The concentration-dependent inhibition achieved by resveratrol against the protamine-sulfate kinase activity (○) and [Ser25]PKC(19−31) kinase activity (▽) of a purified PKC isozyme mixture and against the [Ser25]-PKC(19−31) kinase activity of purified recombinant cPKC-α (□) is shown. Assays contained 10 μM [Ser25]PKC(19−31) or 0.20 mg/mL protamine sulfate, and resveratrol was present in the assay mixtures at the concentrations shown. [Ser25]PKC(19−31) phosphorylation was measured in the presence of the cofactors 0.2 mM $CaCl_2$ and 30 μg/mL PS, and protamine sulfate phosphorylation was measured in their absence. For other experimental details, see Materials and Methods. Full activity values (100%) were 8 ± 1 pmol of ^{32}P transferred/min (○), 17 ± 2 pmol of ^{32}P transferred/min (▽), and 23 ± 2 pmol of ^{32}P transferred/min (□). Each point represents the average ± SD of triplicate determinations, and the inhibition curves shown were reproduced in separate experiments.

Ca^{2+} selectively activates cPKC isozymes in the presence of PS through interactions with the C2 domain of the isozymes (*4*). Phorbol esters and diacylglycerol activate cPKC and nPKC but not aPKC isozymes in the presence of PS by binding interactions with the C1 domain of PKC (*4*). When the phorbol ester TPA (100 nM) was substituted for Ca^{2+} in the [Ser25]PKC(19−31) kinase assay mixtures, the inhibitory potency of resveratrol was virtually unchanged. Resveratrol (5−200 μM) inhibited PKC in the presence of TPA and PS in a concentration-dependent manner, with an IC_{50} of 101 ± 8 μM and a maximal inhibition of 70% ± 3% at 200 μM resveratrol. (The IC_{50} and maximal inhibition values are both averages obtained from three independent experiments.)

To determine whether the inability of resveratrol (5−200 μM) to fully inhibit the purified PKC isozyme mixture was due to the presence of resveratrol-insensitive isozymes in the PKC preparation, we next surveyed the effects of 100 μM resveratrol on the [Ser25]PKC(19−31) kinase activity of seven PKC isozymes, including representatives of each PKC isozyme subfamily and all of the isozymes present in the purified PKC preparation. cPKC, nPKC, and aPKC subfamilies are distinguished primarily on the basis of their modes of regulation and the structures of their regulatory domains (*4*). The isozymes examined were baculovirus-produced, purified human cPKC-α, -β_1, -β_2, and -γ, nPKC-δ and -ϵ, and aPKC-ζ. Because each of the isozymes exhibited robust activity in the absence of C1 domain-interacting activators, the isozymes were assayed under conditions equivalent to those employed in Figure 2, i.e., in the presence of the allosteric cofactors Ca^{2+} and PS (cPKCs) or PS alone (nPKCs and aPKC-ζ). Table 1 shows that each of the isozymes was sensitive to the inhibitory effects of resveratrol. The degrees of inhibition achieved by 100 μM resveratrol against the isozymes ranged from 31% to 63%. Consistent with the above evidence for little isozyme selectivity in resveratrol inhibition of PKC, we established that resveratrol generated similar inhibitory curves against recombinant human cPKC-α (Figure 2, □) and the purified rat brain PKC isozyme mixture (Figure 2, ▽). The cPKC-α results shown in Figure 2 are representative of three experiments. Resveratrol inhibited cPKC-α catalysis in a concentration-dependent manner (Figure 2, □), with an IC_{50} of 140 ± 5 μM and maximal inhibition was 63% ± 4%. The results show that resveratrol inhibits PKC isozymes with divergent regulatory domains with comparable efficacy.

Table 1: Inhibition of Purified PKC Isozymes by 100 μM Resveratrol[a]

isozyme	% inhibition	isozyme	% inhibition
cPKC-α	39 ± 7	nPKC-δ	63 ± 1
cPKC-β_1	56 ± 1	nPKC-ϵ	49 ± 3
cPKC-β_2	59 ± 3	aPKC-ζ	31 ± 5
cPKC-γ	55 ± 1		

[a] Each assay mixture contained 100 ng of the indicated purified recombinant human PKC isozyme, 50 μM [Ser25]PKC(19−31), and 30 μg/mL PS. cPKC assay mixtures also contained 0.2 mM $CaCl_2$. For other assay conditions and assay procedures, please see Materials and Methods. Results shown are an average ± SE of three experiments done in triplicate.

Resveratrol Is a Catalytic Domain-Directed PKC Inhibitor. Having established that resveratrol inhibits PKC isozymes with divergent regulatory domains comparably, we next investigated whether the inhibitory effects of resveratrol against PKC were catalytic domain-directed. The hydrophobic and aromatic properties of resveratrol (Figure 1) suggested that resveratrol might inhibit PKC by competing with the substrate ATP. To investigate the kinetics of inhibition, it was first necessary to determine whether the observed inhibition was reversible. To accomplish this, PKC was preincubated alone (positive control representing 100% activity) or in the presence of 100 μM resveratrol for 5 min at 25 °C and then placed on ice. Preincubation mixtures were then diluted 20-fold into PKC assay mixtures with [Ser25]-PKC(19−31) as the substrate. The final resveratrol concentration in the assay mixture (5 μM) was noninhibitory (see Figure 2). Following preincubation of PKC with 100 μM resveratrol and dilution into standard assay mixtures, 92% ± 7% of the [Ser25]PKC(19−31) activity of the enzyme was recovered. In contrast, when PKC was preincubated alone and then assayed in reaction mixtures containing 100 μM resveratrol, the enzyme was inhibited 60% ± 3%. These results were reproduced in a second experiment. Thus, the inhibitory action of resveratrol against PKC is reversed upon dilution, indicating a reversible inhibitory mechanism.

We analyzed the kinetics of inhibition of purified rat brain PKC by resveratrol, by varying the concentration of ATP above and below the K_m (3.3−40 μM ATP; K_m = 15 ± 4 μM). Figure 3 shows that the mechanism of PKC inhibition by resveratrol was competitive with ATP. The results shown were reproduced in a separate analysis, and the K_i was calculated from the data by Dixon analysis. The average K_i obtained from the two analyses was 55 ± 4 μM.

As a test of the kinetic evidence that resveratrol is a catalytic domain-directed PKC inhibitor, we next investigated

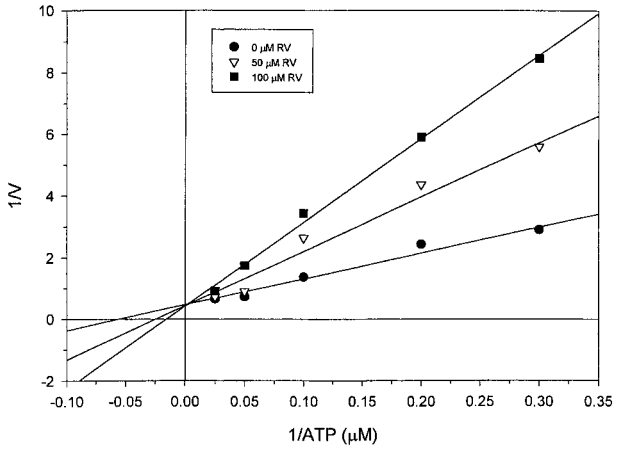

FIGURE 3: Inhibitory kinetics of resveratrol against PKC-catalyzed [Ser25]PKC(19−31) phosphorylation are competitive with the nucleotide substrate. The [Ser25]PKC(19−31) kinase activity of purified rat brain PKC was assayed in the presence of 0.2 mM Ca^{2+} and 30 μg/mL PS. Concentrations of resveratrol and the substrate [γ-^{32}P]ATP were varied, as indicated in the double-reciprocal analysis shown. $1/V$ = (pmol of ^{32}P/min)$^{-1}$. The competitive kinetics shown were reproduced in a separate experiment.

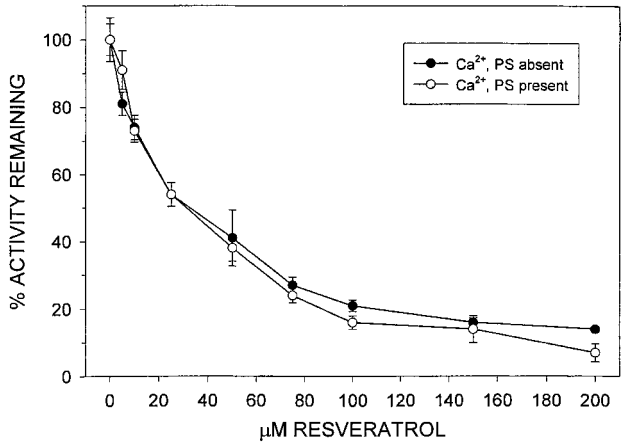

FIGURE 4: Inhibition of the catalytic-domain fragment of PKC by resveratrol. The concentration-dependent inhibition of the catalytic-domain fragment of PKC by resveratrol in assays containing (○) or lacking (●) 0.2 mM Ca^{2+} and 30 μg/mL PS is shown. [Ser25]-PKC(19−31) (10 μM) was employed as the phosphoacceptor substrate. The catalytic-domain fragment was produced from purified rat brain PKC by limited trypsinolysis, and its activity was stimulated less than 1.5-fold by Ca^{2+} and PS. Full activity (100%) was 8.1 ± 0.6 pmol of ^{32}P transferred/min in the absence of Ca^{2+} and PS. Resveratrol was present in the assay mixtures at the indicated concentrations. For other experimental details, see Materials and Methods. Each point represents the average ± SD of triplicate determinations, and the results shown were reproduced in separate experiments.

the ability of resveratrol to inhibit a Ca^{2+}- and PS-independent catalytic-domain fragment derived from the purified rat brain PKC isozyme mixture by limited trypsinolysis (see Materials and Methods). Resveratrol exhibited enhanced potency against the catalytic-domain fragment compared with intact PKC. Resveratrol (5−200 μM) inhibited [Ser25]PKC(19−31) phosphorylation catalyzed by the catalytic-domain fragment in the absence of Ca^{2+} and PS in a concentration-dependent manner, with an IC_{50} of 30 ± 5 μM and a maximal inhibition of 87% ± 2% (average values from three independent experiments). Representative results are shown in Figure 4 (●).

The enhanced inhibitory efficacy of resveratrol observed in assays of the catalytic-domain fragment could not be ascribed to the absence of the cofactors Ca^{2+} and PS from the reaction mixtures, because a virtually identical inhibitory curve against the catalytic-domain fragment was generated by resveratrol when assays were done in the presence of 0.2 mM Ca^{2+} and 30 μg/mL PS (Figure 4, ○). The similarity of the inhibitory curves generated by resveratrol against the catalytic-domain fragment in the presence (○) and absence (●) of Ca^{2+} and PS also indicates that the inhibitory potency of resveratrol is not compromised by phospholipid sequestration of the inhibitor. The IC_{50} and maximal inhibition achieved by resveratrol against the catalytic-domain fragment in the presence of Ca^{2+} and PS were 37 ± 3 μM and 92% ± 1%, respectively (average values from three independent experiments). The ability of resveratrol to inhibit the catalytic-domain fragment more potently and to a greater extent than intact PKC [compare Figure 2 (▽) and Figure 4] demonstrates that the mechanism of PKC inhibition by resveratrol includes a major catalytic domain-directed component and suggests that features of the regulatory domain of PKC may offer protection against the inhibitory action of resveratrol.

Potent Inhibition of PKC-Catalyzed Protamine Sulfate Phosphorylation by Resveratrol through a Distinct Kinetic Mechanism. On the basis of our evidence of greater efficacy of resveratrol against the Ca^{2+}/PS-independent catalytic-domain fragment of PKC compared with intact Ca^{2+}/PS-dependent PKC, we investigated whether resveratrol would show superior efficacy against PKC-catalyzed phosphorylation of a Ca^{2+}/PS-independent substrate compared with conventional PKC substrates that are phosphorylated in a Ca^{2+}/PS-dependent manner. Protamine sulfate is a polybasic, Arg-rich PKC substrate that activates PKC by a novel mechanism involving binding of protamine sulfate at an allosteric site (*15*, *16*). As a result, PKC-catalyzed protamine sulfate phosphorylation is Ca^{2+}/PS-independent (*15*, *16*). The polybasic, Arg-rich nature of protamine sulfate is implicated in its activation of PKC, because protamine free base and an Arg-rich synthetic peptide substrate corresponding to a sequence in protamine likewise activate PKC, but the Lys-rich substrate histone H1 does not (*15*, *22*).

We examined the ability of resveratrol to inhibit protamine sulfate phosphorylation by purified rat brain PKC in the absence of Ca^{2+} and PS. Figure 2 (○) shows that resveratrol was much more potent against protamine sulfate phosphorylation compared with [Ser25]PKC(19−31) phosphorylation (▽) by PKC. The concentration-dependent inhibitory curve against protamine sulfate phosphorylation in Figure 2 is representative of curves generated in three independent experiments, which yielded average IC_{50} and maximal inhibition values of 10 ± 2 μM and 91% ± 2%, respectively. For comparison, we examined the inhibitory potency of resveratrol against PKC phosphorylation of histone H1, which is a conventional polybasic and Lys-rich PKC substrate (*15*). Resveratrol was considerably less effective against the Ca^{2+}/PS-dependent phosphorylation of histone, exhibiting an IC_{50} of 91 ± 8 μM and a maximal inhibition of 72% ± 12% (average values from three experiments). To determine whether the Arg-rich nature of protamine sulfate was sufficient to account for the superior potency of resveratrol against this substrate, we also examined the

inhibitory potency of resveratrol against PKC phosphorylation of an Arg-rich polypeptide that serves as a conventional PKC substrate. The copolymer poly(Arg,Ser) is phosphorylated by PKC in a phospholipid-dependent manner, and, despite its structural resemblance to protamine, it does not activate the enzyme (*23*). PKC-catalyzed phosphorylation of poly(Arg,Ser) (40 μg/mL) was stimulated 6-fold in the presence of Ca^{2+}/PS, and the cofactor-stimulated reaction was inhibited by resveratrol with an IC_{50} of 120 $\pm$ 18 μM and to a maximal extent of 67% $\pm$ 4% (average values from three experiments). Thus, the superior inhibitory potency exhibited by resveratrol against PKC phosphorylation of protamine sulfate was not observed with either Lys-rich or Arg-rich polypeptide substrates that lack the ability to activate PKC.

We conducted a kinetic analysis to investigate the basis for the preferential inhibition of PKC-catalyzed protamine sulfate phosphorylation by resveratrol. To determine whether resveratrol reversibly inhibited PKC-catalyzed protamine sulfate phosphorylation, we employed the experimental approach that was taken to show that resveratrol inhibited the [Ser25]PKC(19−31) phosphorylation reaction reversibly. In these experiments, PKC was preincubated for 5 min at 25 °C with/without resveratrol and then diluted 20-fold into assay mixtures. Preincubation of PKC with resveratrol (10−15 μM) resulted in <10% inhibition of the protamine sulfate phosphorylation reaction of the enzyme, while the reaction was inhibited 45−50% when PKC was preincubated alone and 10−15 μM resveratrol was present in the assay mixtures. These results indicate that resveratrol reversibly inhibits PKC-catalyzed protamine sulfate phosphorylation.

To compare the inhibitory kinetics of resveratrol against PKC-catalyzed [Ser25]PKC(19−31) phosphorylation (Figure 3) and protamine sulfate phosphorylation, we first analyzed the inhibitory kinetics for protamine sulfate phosphorylation at variable ATP concentrations. In plots of initial velocity versus initial substrate concentration (*V* versus *S*), the data points obtained in this analysis in the presence of 0, 12.5, 25, and 50 μM resveratrol (Figure 5) were better fit by sigmoid curves (solid lines) than hyperbolic curves (not shown) generated by nonlinear regression analysis, and the data did not conform to Michaelis−Menten double-reciprocal plot analysis. V_{max} values were calculated as the asymptotic values corresponding to the sigmoidal *V* versus *S* curves. Resveratrol had a pronounced and concentration-dependent effect on the V_{max} of the protamine sulfate phosphorylation reaction. The average V_{max} values obtained from Figure 5 and a second analysis (not shown) were 18.5 $\pm$ 2.1 (0 μM resveratrol), 11.8 $\pm$ 0.8 (12.5 μM resveratrol), 8.1 $\pm$ 1.2 (25 μM resveratrol), and 5.0 $\pm$ 1.5 (50 μM resveratrol) pmol of ^{32}P transferred min^{-1} (10 ng of PKC)$^{-1}$. Thus, the highest resveratrol concentration included in the analysis (50 μM) caused a 3.7-fold reduction in the V_{max} value when ATP was the varied substrate. Figure 3 shows that the kinetics of resveratrol inhibition of PKC-catalyzed [Ser25]PKC(19−31) phosphorylation are competitive with respect to ATP, i.e., the V_{max} value does not change when [ATP] is varied. In contrast, the kinetics of inhibition of protamine sulfate phosphorylation (Figure 5) rule out simple competition with ATP.

When protamine sulfate was the varied substrate, nonlinear regression analysis of the data generated a *V* versus *S* curve

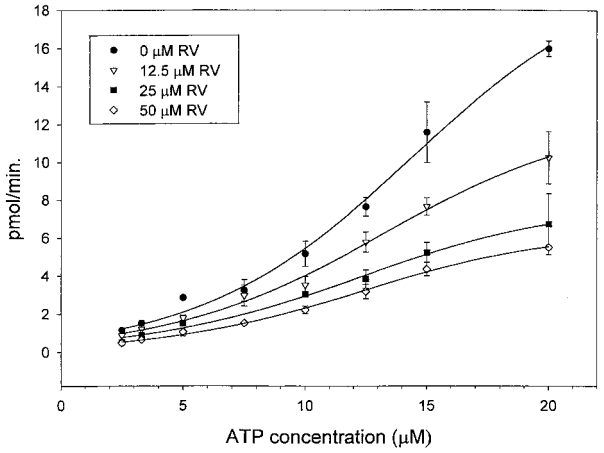

FIGURE 5: Inhibitory kinetics of resveratrol against PKC-catalyzed protamine sulfate phosphorylation, with [γ-^{32}P]ATP as the varied substrate. The protamine sulfate kinase activity of purified rat brain PKC was assayed in the absence of Ca^{2+} and PS, as described in Materials and Methods. Concentrations of resveratrol and the substrate [γ-^{32}P]ATP were varied, as indicated in the *V* versus *S* analysis shown. Sigmoidal curves (solid lines) were generated by nonlinear regression analysis of the data. Pmol/min = picomoles of ^{32}P transferred to protamine sulfate per minute per 10 ng of PKC. Data points represent average values obtained from triplicate assays. The results shown were reproduced in a separate experiment.

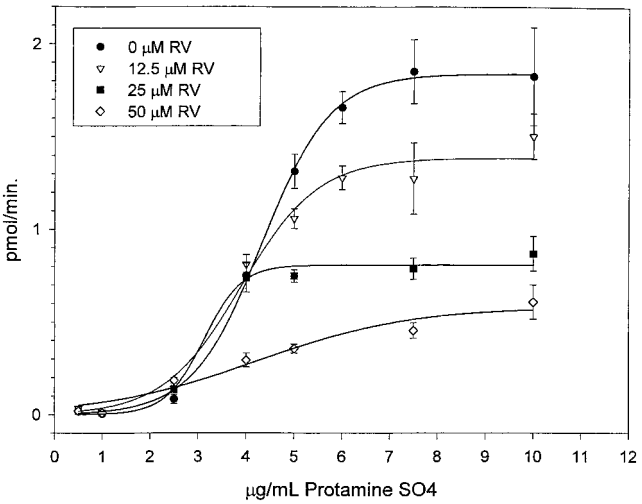

FIGURE 6: Inhibitory kinetics of resveratrol against PKC-catalyzed protamine sulfate phosphorylation, with protamine sulfate as the varied substrate. The kinetic analysis shown (*V* versus *S*) was done as described in the legend to Figure 5, except that the varied substrate was protamine sulfate. Pmol/min = picomoles of ^{32}P transferred to protamine sulfate per minute per nanogram of PKC. Data points represent average values obtained from triplicate assays. The results shown were reproduced in a separate experiment.

with pronounced sigmoidal character (Figure 6, ●). The average Hill coefficient obtained from Figure 6 (●) and a second analysis (not shown) was 6.0 $\pm$ 0.4 (Table 2). The observed positive cooperativity is in agreement with previous observations that protamine sulfate stimulates its own phosphorylation by PKC (*15*, *16*). In confirmation of results reported in ref *15*, the double-reciprocal plot corresponding to Figure 6 yielded parabolic curves (data not shown), which is the predicted outcome for a varied substrate that acts as an enzyme activator (*15*). Resveratrol caused a concentration-dependent drop in the V_{max} value obtained with protamine sulfate as the varied substrate (Table 2). The approximately 2-fold reduction in the V_{max} value achieved by resveratrol

Table 2: Kinetics of Resveratrol Inhibition of PKC with Protamine Sulfate as the Varied Substrate[a]

resveratrol concn (μM)	V_{max} [pmol of ^{32}P min^{-1} (ng of PKC)$^{-1}$]	Hill coefficient (n_H)
0	1.81 ± 0.03	6.0 ± 0.4
12.5	1.43 ± 0.04	3.6 ± 0.1
25	0.93 ± 0.11	3.9 ± 0.8
50	0.75 ± 0.16	2.8 ± 0.2

[a] The V_{max} and n_H values shown are averages from the analysis in Figure 6 and a second analysis (not shown). V_{max} values were calculated as described for Figure 5, i.e., by extrapolating the sigmoidal curves generated by nonlinear regression analysis of the *V* versus *S* data points to asymptotic values. Hill coefficients were calculated as the slopes of the corresponding Hill plots.

(Table 2) indicates that the inhibitory mechanism is not competitive with respect to protamine sulfate. Resveratrol also caused a 2-fold reduction in the Hill coefficient obtained with protamine sulfate as the varied substrate (Table 2), providing evidence that resveratrol interferes with the function of protamine sulfate as a PKC activator.

Bazzi and Nelsestuen (*23*) have shown that PKC phosphorylation of conventional lipid-dependent substrates occurs in association with substrate-induced aggregation of the lipid cofactor and the resultant formation of large complexes of substrate, lipid, and PKC. In addition, they have demonstrated that PKC-catalyzed protamine sulfate phosphorylation is associated with complex formation between PKC and self-aggregated protamine sulfate. We investigated whether resveratrol could interfere with the formation of large complexes of PKC and protamine sulfate, using an established method that measures sedimentation of PKC−protamine sulfate aggregates (*15*, *23*). In these studies, rat brain PKC (160 ng) was incubated with 250 μg/mL protamine sulfate ± 20 μM resveratrol in 20 mM Tris-HCl, pH 7.5, containing 1 mg/mL BSA for 5 min at 30 °C (total volume = 320 μL), and the samples were centrifuged at 14000*g* for 5 min at 4 °C to sediment PKC−protamine sulfate aggregates. The PKC activity remaining in the supernatant was measured in assay mixtures (120 μL) containing 0.6 mg/mL protamine sulfate as substrate and 10 μL of sample supernatant, in order that the contribution of protamine sulfate in the sample supernatant to the final substrate concentration in the assay mixture was negligible (<5%). Resveratrol had no protamine sulfate-independent effect on the recovery of PKC activity in the sample supernatant in this system. In samples containing 20 μM resveratrol and lacking protamine sulfate, 99% ± 4% of the activity was recovered in the supernatant (average result of two experiments). Consistent with previous observations (*15*, *23*), protamine sulfate caused substantial PKC activity loss from the supernatant. Only 57% ± 8% of the PKC activity was recovered in the supernatant of samples containing 250 μg/mL protamine sulfate, and inclusion of 20 μM resveratrol in protamine sulfate-containing samples resulted in the recovery of 53% ± 10% of the PKC activity in the supernatant (average results of two experiments). Thus, resveratrol was without measurable effect on either protamine sulfate self-aggregation or PKC binding to aggregated protamine sulfate, at a concentration that potently inhibited PKC-catalyzed protamine sulfate phosphorylation (Figure 2).

DISCUSSION

Resveratrol is a candidate cancer chemopreventive agent that antagonizes each stage of carcinogenesis (*2*) and inhibits PKC (*1*, *14*), a key mediator of the tumor promotion stage of carcinogenesis (*3*, *4*). At a concentration of 15 μM, resveratrol potently inhibits PKC in mammalian cells (*14*). Paradoxically, the reported IC_{50} of resveratrol against isolated PKC is 175 μM (*1*). To resolve this apparent contradiction, we analyzed the mechanism of PKC inhibition by resveratrol. We found that the potency of resveratrol against purified PKC depended on the nature of the substrate and the cofactor dependence of the phosphotransferase reaction. Furthermore, the range of inhibitory potencies observed against purified PKC overlapped with the reported potency of resveratrol against cellular PKC.

We first established that resveratrol weakly inhibits the Ca^{2+}/PS-stimulated [Ser25]PKC(19−31) kinase activity of a purified rat brain PKC isozyme mixture (IC_{50} = 90 μM) by competition with ATP (K_i = 55 μM). The previous observation that resveratrol also weakly inhibits the protein tyrosine kinase Lck (IC_{50} = 260 μM) (*1*), which is distantly related to PKC in the protein kinase superfamily (*24*), together with our observation that resveratrol is similarly effective against members of each PKC isozyme subfamily (cPKC, nPKC, and aPKC) suggest that resveratrol may be weakly inhibitory against a broad spectrum of protein kinases. Our kinetic results further suggest that, at least in the case of protein kinases closely related to PKC, this may entail binding of resveratrol at the nucleotide-substrate binding site. However, because these weakly inhibitory effects require resveratrol concentrations that are nonspecifically toxic to mammalian cells (*25*; Stewart and O'Brian, unpublished observations) and therefore not relevant to the chemopreventive properties of resveratrol (*2*), we did not pursue this line of investigation. Instead, we focused on the question of whether resveratrol exhibits enhanced potency against other types of PKC-catalyzed reactions that could potentially contribute to its chemopreventive action.

A fully active, Ca^{2+}/PS-independent catalytic-domain fragment (CDF) is produced from PKC by limited proteolysis (*4*, *19*). In support of our kinetic evidence that resveratrol inhibition of Ca^{2+}/PS-stimulated PKC activity is active-site-directed, we found that resveratrol was an effective inhibitor of [Ser25]PKC(19−31) phosphorylation by the CDF produced from purified rat brain PKC. The apparent inhibitory potency of resveratrol was 3-fold greater against the CDF compared with PKC. The properties of resveratrol revealed in the CDF analysis along with its uncharged aromatic structure distinguish it from numerous PKC inhibitors that fall into the class of cationic amphiphiles, e.g., tamoxifen, melittin, sphingosine, and trifluoperazine (*19*, *26*−*28*). In contrast with resveratrol, the inhibitory potencies of cationic amphiphiles against PKC primarily reflect interactions with the phospholipid cofactor (*19*, *26*−*28*). As a result, cationic amphiphiles are typically severalfold more potent in the inhibition of PKC compared with the lipid-independent CDF, where their comparatively weak inhibitory interactions with the enzyme itself are revealed (*19*, *28*). Furthermore, the inhibitory potency of cationic amphiphilic PKC inhibitors falls sharply as the phospholipid cofactor concentration is increased, due to lipid sequestration of the inhibitor (*28*). In

contrast, the potency of resveratrol against the CDF was virtually unaffected by the cofactors 0.2 mM Ca^{2+} and 30 μg/mL PS. Thus, the potency of resveratrol against PKC is predicted to be relatively independent of the lipid microenvironment of the cell. In contrast, the lipid microenvironment exerts a major influence over the ability of cationic amphiphiles to inhibit PKC (*26*−*28*).

The enhanced potency of resveratrol against the CDF compared with either Ca^{2+}/PS- or TPA/PS-activated forms of PKC provided evidence that structural features of the regulatory domain of PKC impede inhibitory interactions of resveratrol with PKC, even when the enzyme is fully activated by lipid-dependent mechanisms. This left open the possibility that resveratrol might exhibit improved potency against PKC when the enzyme is activated by lipid-independent mechanisms. Protamine sulfate is a polybasic PKC substrate that activates the enzyme by a lipid-independent mechanism. Protamine sulfate binds an allosteric site of PKC with high affinity independently of activating cofactors and MgATP (*15*). Engagement of the allosteric site by protamine sulfate induces autophosphorylation of PKC as well as phosphorylation of active-site-bound protamine sulfate (*15*). Furthermore, structural studies provide evidence that an activated conformation of PKC can be induced by both protamine sulfate and ARP, which is a cofactor-independent, Arg-rich synthetic peptide substrate of PKC that corresponds to a phosphorylation site in protamine sulfate (*16*, *22*).

We found that resveratrol was in fact 3−9-fold more effective against PKC-catalyzed protamine sulfate phosphorylation (IC_{50} = 10 μM) compared with lipid-dependent PKC-catalyzed [Ser25]PKC(19−31) phosphorylation (IC_{50} = 90 μM) and histone phosphorylation (IC_{50} = 91 μM) and with lipid-independent CDF catalysis (IC_{50} = 30 μM). The kinetic mechanism of resveratrol inhibition of protamine sulfate phosphorylation by PKC was distinct from the purely competitive mechanism with respect to ATP that characterized resveratrol inhibition of Ca^{2+}/PS-stimulated PKC activity. This was evident from the substantial reduction of the V_{max} for protamine sulfate phosphorylation caused by resveratrol when ATP was the variable substrate. Likewise, kinetic analysis with protamine sulfate as the variable substrate ruled out competition with the phosphoacceptor substrate as the inhibitory mechanism. The positive cooperativity of protamine sulfate phosphorylation by PKC evidenced by sigmoidal *V* versus *S* curves and confirmatory of ref *15* precluded double-reciprocal kinetic analysis (*21*). Hill analysis (*21*) with protamine sulfate as the variable substrate revealed a Hill coefficient of 6.0, indicative of marked stimulation of protamine sulfate phosphorylation by protamine sulfate itself (*15*). This value was halved in the presence of resveratrol. From these kinetic results, we conclude that resveratrol inhibition of protamine sulfate phosphorylation entails antagonism of protamine sulfate-induced PKC activation and does not involve competition with either substrate. This mechanism accounts for our observation that resveratrol is uniquely effective against the protamine sulfate phosphorylation reaction catalyzed by purified PKC.

The fact that the hydrophobic and uncharged aromatic structure of resveratrol bears no resemblance to the polar and polybasic pharmacophore represented by protamine sulfate and the synthetic peptide ARP (RRRRYGSRRRRY) is consistent with our kinetic evidence that resveratrol inhibition of PKC-catalyzed protamine sulfate phosphorylation does not involve competition with protamine sulfate at the substrate binding site. The structural dissimilarity also suggests that resveratrol does not compete with protamine sulfate at the allosteric binding site that is responsible for protamine sulfate activation of PKC. It is more likely that resveratrol interacts with hydrophobic site(s) in PKC that are exposed upon PKC activation (*22*). These hydrophobic site(s) have been identified on the basis of the induction of nonpolar binding interactions between cPKC-α and fluorescent probes, concomitant with isozyme activation by ARP (*22*). The hydrophobic site(s) are hypothesized to mediate the binding of activated PKC to biological membranes (*22*). In fact, ARP does induce binding interactions between cPKC-α and lipid vesicles, in association with cPKC-α activation (*22*). However, our results indicate that resveratrol inhibition of PKC is lipid-independent, and it remains to be determined whether the hydrophobic site(s) detected by fluorescent probes (*22*) can mediate inhibitory effects against PKC in the absence of the lipid cofactor.

Based on the presence of PKC isozymes at the cytoskeleton, focal adhesion plaques, and nuclei of cells, and on the polybasic nature of protamine sulfate and certain cytoskeletal and nuclear proteins, Leventhal and Bertics (*15*) have proposed that some polybasic cytoskeletal and nuclear proteins may activate PKC in cells by the same mechanism as protamine sulfate, i.e., independently of receptor-mediated lipid hydrolysis. In fact, a recent report showed that the proteoglycan syndecan 4 colocalizes to focal adhesion plaques with cPKC-α and that syndecan 4 activates purified cPKCα independently of lipid and other activating cofactors by the binding of a Lys-rich sequence in the cytoplasmic domain of syndecan 4 to the catalytic domain of cPKCα (*29*). Thus, receptor-mediated, lipid-dependent cPKC-α activation and colocalization of the isozyme with syndecan 4 at focal adhesion plaques may be followed by syndecan 4-dependent activation of cPKCα-catalyzed phosphorylation of other focal adhesion components (*29*). Our results provide evidence that resveratrol inhibition of the latter type of PKC-catalyzed reaction may be responsible for the observed efficacy of the tumor-promotion antagonist against cellular PKC.

REFERENCES

1. Jayatilake, G. S, Jayasuriya, H., Lee, E.-S., Koonchanok, N. M., Geahlen, R. L., Ashendel, C. L., McLaughlin, J. L., and Chang, C.-J. (1993) *J. Nat. Prod. 56*, 1805−1810.
2. Jang, M., Cai. L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W. W., Fong, H. H. S., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., and Pezzuto, J. M. (1997) *Science 275*, 218−220.
3. Weinstein, I. B. (1990) in *The Biology and Medicine of Signal Transduction* (Nishizuka, Y., Ed.) pp 307−316, Raven Press, New York.
4. Blobe, G. C., Stribling, S., Obeid, L. M., and Hannun, Y. A. (1996) *Cancer Surveys 27*, 213−248.
5. Jaken, S. (1996) *Curr. Opin. Cell Biol. 8*, 168−173.
6. O'Brian, C. A., and Ward, N. E. (1989) *Cancer Metastasis Rev. 8*, 199−214.
7. Housey, G. M., Johnson, M. D., Hsiao, W. L. W., O'Brian, C. A., Murphy, J. P., Kirschmeier, P., and Weinstein, I. B. (1988) *Cell 52*, 343−354.

8. Cacace, A. M., Guadagno, S. N., Krauss, R. S., Fabbro, D., and Weinstein, I. B. (1993) *Oncogene 8*, 2095−2104.
9. Roy, H. K., Bissonnette, M., Frawley, B. P., Wali, R. K., Niedziela, S. M., Earnest, D., and Brasitus, T. A. (1995) *FEBS Lett. 366*, 143−145.
10. Wali, R. K., Frawley, B. P., Hartmann, S., Roy, H. K., Khare, S., Scaglione-Sewell, B. A., Earnest, D. L., Sitrin, M. D., Brasitus, T. A., and Bissonnette, M. (1995) *Cancer Res. 55*, 5257−5264.
11. Lin, J.-K., Chen, Y.-C., Huang, Y.-T., and Lin-Shiau, S.-Y. (1997) *J. Cell. Biochem.* (*Suppl*). *28*/*29*, 39−48.
12. O'Brian, C. A., Liskamp, R. M., Solomon, D. H., and Weinstein, I. B. (1985) *Cancer Res. 45*, 2462−2465.
13. Su, H. D., Mazzei, G. J., Vogler, W. R., and Kuo, J. F. (1985) *Biochem. Pharmacol. 34*, 3649−3653.
14. Subbaramaiah, K., Chung, W. J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J. M., and Dannenberg, A. J. (1998) *J. Biol. Chem. 273*, 21875−21882.
15. Leventhal, P. S., and Bertics, P. J. (1993) *J. Biol. Chem. 268*, 13906−13913.
16. Orr, J. W., and Newton, A. C. (1994) *J. Biol. Chem. 269*, 8383−8387.
17. O'Brian, C. A., and Ward, N. E. (1990) *Biochemistry 29*, 4278−4282.
18. Ward, N.E., Gravitt, K. R., and O'Brian, C. A. (1996) *J. Biol. Chem. 271*, 24193−24200.
19. Ward, N. E., and O'Brian, C. A. (1993) *Biochemistry 32*, 11903−11909.
20. Kazanietz, M. G., Areces, L. B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J. F., and Blumberg P. M. (1993) *Mol. Pharmacol. 44*, 298−307.
21. Palmer, T. (1985) *Understanding Enzymes*, John Wiley & Sons, New York.
22. Bruins, R. H., and Epand, R. M. (1995) *Arch. Biochem. Biophys. 324*, 216−222.
23. Bazzi, M. D., and Nelsestuen, G. L. (1987) *Biochemistry 26*, 1974−1982.
24. Hanks S. K., Quinn, A. M., and Hunter, T. (1988) *Science 241*, 42−52.
25. Mgbonyebi, O. P., Russo, J., and Russo, I. H. (1998) *Int. J. Oncol. 12*, 865−869.
26. Bottega, R., and Epand, R. M. (1992) *Biochemistry 31*, 9025−9030.
27. Epand, R. M., and Lester, D. S. (1990) *Trends Pharmacol. Sci. 11*, 317−320.
28. O'Brian, C. A., and Kuo, J. F. (1994) in *Protein Kinase C* (Kuo, J. F., Ed.) Oxford University Press, New York.
29. Oh, E.-S., Woods, A., and Couchman, J. R. (1997) *J. Biol. Chem. 272*, 8133−8136.

BI990875U