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Kinetic Analysis of Protein Kinase C Inhibition by Staurosporine: Evidence That Inhibition Entails Inhibitor Binding at a Conserved Region of the Catalytic Domain But Not Competition with Substrates

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SUMMARY

The indole carbazole staurosporine is an extraordinarily potent antiproliferative agent that inhibits the growth of cultured mammalian cells at concentrations of <1 nM. The antiproliferative activity of staurosporine is attributed to its potent inhibition of diverse protein kinases, but the mechanism of staurosporine inhibition has not been elucidated for any protein kinase. Protein kinase C (PKC) is a family of Ca^{2+}- and phosphatidylserine-dependent protein kinases that are activated *in vivo* by the second messenger diacylglycerol. A fully active, Ca^{2+}- and phosphatidylserine-independent, catalytic fragment of PKC that contains only the catalytic domain of the enzyme can be produced by limited proteolysis. Previous studies indicated that staurosporine inhibits PKC by binding its catalytic domain. In this study, we define the kinetics of inhibition by staurosporine of a catalytic fragment of rat brain PKC-γ and of a catalytic fragment generated from a rat brain PKC-α/PKC-β mixture. Our kinetic results provide evidence that staurosporine inhibits PKC by binding to a site of the catalytic domain other than the ATP substrate and protein substrate binding sites. Staurosporine inhibition appears to entail binding at a conserved site in the catalytic domain of PKC, because staurosporine inhibited rat brain PKC-α, PKC-β, and PKC-γ, as well as the catalytic fragments of PKC-β and PKC-γ, with similar potencies. The kinetics of inhibition of the catalytic fragment of PKC-γ were uncompetitive with respect to histone III-S, providing evidence that the binding of histone III-S at the active site of the catalytic fragment precedes the binding of staurosporine to the enzyme. Taken in the context of previous mechanistic studies of PKC-catalyzed histone III-S phosphorylation, these results provide evidence that staurosporine binds to a complex of PKC, MgATP, and histone III-S, thereby forming a complex that cannot break down to products. In addition, the inhibitory kinetics observed when the ATP concentration was varied provided evidence that staurosporine reduces the affinity of MgATP for the catalytic fragment of PKC-γ. Thus, the kinetics of inhibition of the catalytic fragment of PKC-γ by staurosporine provide evidence that staurosporine inhibits PKC by a mixed mechanism.

The indole carbazole staurosporine is an extraordinarily potent antiproliferative agent that manifests cytotoxic activity against cultured mammalian cells at concentrations of <1 nM (1, 2). Staurosporine is also an effective antitumor agent *in vivo*, according to studies of human bladder carcinoma xenografts in athymic nude mice (3). The potential importance of staurosporine as a prototype of new anticancer agents is also suggested by its ability to antagonize tumor cell invasion *in vitro* (4) and by its antagonism of the phosphorylation (5) and drug efflux activity (6) of P-glycoprotein, the transport protein that plays a central role in multidrug resistance phenotypes of tumor cells.

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The antiproliferative activity of staurosporine is thought to result from the inhibition of protein serine/threonine kinases and PTKs by nanomolar concentrations of the indole carbazole (2, 7). Staurosporine appears to inhibit numerous protein kinases, based on its reported IC_{50} values against several distinct protein kinases, including the protein serine/threonine kinases PKC (IC_{50} = 6 nM) and cAMP-dependent protein kinase (IC_{50} = 15 nM) (3) and the PTK $pp60^{v\text{-}src}$ (IC_{50} = 6 nM) (2). In an effort to understand the basis for the extraordinary potency of staurosporine in cellular systems, recent studies have measured the relative abilities of staurosporine to inhibit specific protein kinases that play key roles in signal-transduction pathways that regulate cell growth. These comparative studies have demonstrated that the potency of staurosporine against the PTK activity of the platelet-derived growth factor receptor is about

ABBREVIATIONS: PTK, protein tyrosine kinase; PKC, protein kinase C; PS, phosphatidylserine; EGTA, ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid.

100 times greater than its potency against the PTK activity of the epidermal growth factor receptor (8) and also that staurosporine inhibits the PTK activity of the insulin receptor approximately 100 times more effectively than the PTK activity of insulin-like growth factor I receptor (9).

Although the ability of staurosporine to inhibit widely divergent members of the protein kinase family suggests that staurosporine functions by binding the enzymes at a region that is conserved throughout the protein kinase family (7), the mechanism of staurosporine inhibition has not been elucidated for any protein kinase (2, 7, 8). PKC represents a family of Ca^{2+}- and PS-dependent protein kinases that play a central role in the regulation of cell growth (10, 11). PKC is activated *in vivo* by the second messenger diacylglycerol (10, 11), and a fully active, Ca^{2+}- and PS-independent, catalytic fragment of PKC that contains only the catalytic domain can be produced by limited proteolysis (12). Studies with the catalytic fragment of PKC provide evidence that staurosporine inhibits the enzyme by binding to its catalytic domain. Staurosporine inhibits PKC and its catalytic fragment with indistinguishable potencies (13), and a radiolabeled analog of staurosporine binds to the catalytic fragment of PKC with a K_d (2.4 nM) (14) that is similar to the IC_{50} of staurosporine against PKC activity (13). Furthermore, staurosporine does not inhibit binding of the PKC activator phorbol dibutyrate to the regulatory domain of PKC (13). Although the evidence that staurosporine inhibits PKC by binding to its catalytic domain is strong, the mechanism of PKC inhibition by staurosporine remains unclear (2, 7).

The kinetics of both PKC and PKA inhibition by the staurosporine analog K252a (2) are competitive with respect to ATP (15). However, in a preliminary examination of the kinetics of PKC inhibition by staurosporine, the inhibition was described as "not competitive" with respect to ATP (1). A straightforward interpretation of these preliminary kinetic results is precluded. The kinetics of staurosporine inhibition were obtained using an assay of PKC activity that included a DEAE-extracted mixture of rat brain PKC isozymes, the substrate histone, and the allosteric cofactors Ca^{2+}, PS, and diacylglycerol (1), and it has been pointed out that the presence of multiple cofactors and their interactions with the catalytic domain of PKC and other reaction mixture components in assays of the Ca^{2+}- and PS-dependent histone kinase activity of PKC preclude simple kinetic analysis of the enzyme activity (12). Because staurosporine inhibits PKC by binding its catalytic domain (13, 14), the kinetics of PKC inhibition by staurosporine can be studied in a simplified assay system that uses the catalytic fragment of PKC in order to circumvent the complicated interactions introduced by allosteric cofactors (12). In this report, we define the kinetics of staurosporine inhibition of a catalytic fragment of rat brain PKC-γ and a catalytic fragment generated from a mixture of rat brain PKC-α and PKC-β.

Materials and Methods

[γ-^{32}P]ATP was purchased from Amersham Corp (Arlington Heights, IL), and frozen rat brains were from Pel-Freez (Rogers, AR). The synthetic peptide [Ser-25]PKC(19–31) was purchased from Peninsula Laboratories (Belmont, CA), hydroxylapatite resin from Bio-Rad Corp (Richmond, CA), and phosphocellulose paper from Fisher Scientific (Houston, TX). Staurosporine was purchased from Boehringer Mannheim (Indianapolis, IN) and stored at a concentration of 1 mg/ml, in dimethyl sulfoxide, at −20°. Histone III-S and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Purification of rat brain PKC and its isozymic forms. Rat brain PKC was purified to near-homogeneity, according to silver-stained polyacrylamide gels, as previously described, in a procedure that entailed elution of PKC from melittin-agarose with MgATP (16, 17). The purified enzyme was fully autophosphorylated, and its histone kinase activity was stimulated approximately 10-fold by 1 mM Ca^{2+} and 30 μg/ml PS but was not stimulated by either Ca^{2+} or PS alone (17). According to hydroxylapatite chromatography (18), the isozyme composition of the purified enzyme was approximately 60% PKC-β and 40% PKC-α. The rat brain isozymes PKC-α, PKC-β, and PKC-γ were prepared by hydroxylapatite chromatography, as described by Huang *et al.* (18).

Generation of a catalytic fragment of PKC. Fully active catalytic fragments of PKC were generated from PKC-γ, PKC-β, and the PKC-α/PKC-β mixture by trypsinolysis, under previously described conditions (16). In each case, the PKC preparation was incubated with trypsin (650–1300 units/ml) for 30 min in 20 mM Tris·HCl at pH 7.5 and 4°. Proteolysis was terminated with 1 mM phenylmethylsulfonyl fluoride. The histone kinase activity of each proteolyzed PKC preparation was stimulated <2-fold by 1 mM Ca^{2+} and 30 μg/ml PS. In agreement with a previous report (19), we found that PKC-α was completely resistant to trypsinolysis under these conditions, providing evidence that the catalytic fragment produced from the mixture of PKC-α and PKC-β is mainly derived from PKC-β.

Assay of PKC activity. The histone kinase activity of PKC was assayed as previously described (20). Reaction mixtures (120 μl) contained 20 mM Tris·HCl pH 7.5, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 30 μg/ml PS, 6 μM [γ-^{32}P]ATP (3500–6000 cpm/pmol), 0.67 mg/ml histone III-S, and purified PKC. Where indicated, staurosporine was included in the assay mixtures. Dimethyl sulfoxide was present in the reaction mixtures at a concentration of <0.5% and had no effect on the enzyme activity. In assays of the catalytic fragment of PKC and in assays of the basal histone kinase activity of PKC, $CaCl_2$ was replaced by 1 mM EGTA and PS was omitted. Alternative histone III-S and [γ-^{32}P]ATP concentrations were used as specified, and in indicated assays histone was replaced by 5 μM [Ser-25]PKC(19–31). Reactions were initiated by the addition of [γ-^{32}P]ATP and proceeded from 5 to 10 min at 30° with linear kinetics. Reactions were terminated by pipetting an aliquot (40 μl) of the reaction mixture onto phosphocellulose paper. The radioactivity incorporated into histone III-S or [Ser-25]PKC(19–31) was then measured as previously described (20). All assays were performed in triplicate.

Results

To define the kinetic mechanism of PKC inhibition by staurosporine, we analyzed the kinetics of staurosporine inhibition of a catalytic fragment of PKC produced by limited proteolysis of a purified rat brain PKC preparation (16, 17) that contained PKC-α and PKC-β. A simplified assay system that included Mg, ATP, histone-III-S, and the catalytic fragment and that lacked Ca^{2+}, PS, and diacylglycerol was used in these studies. In parallel experiments, we determined the kinetics of inhibition of a catalytic fragment of rat brain PKC-γ.

Mixed competitive/noncompetitive inhibition describes Lineweaver-Burk inhibitory plots in which the lines cross to the left of the y-axis but above the x-axis, whereas the lines cross on the x-axis in fully noncompetitive inhibition (21). Fig. 1A shows that staurosporine inhibited histone III-S phosphorylation, catalyzed by a catalytic fragment of PKC generated from a mixture of PKC-α and PKC-β, by a mechanism that was mixed competitive/noncompetitive (21) with respect to ATP. Because the highly charged polypeptide histone III-S could have confounding effects on the kinetic analysis of PKC

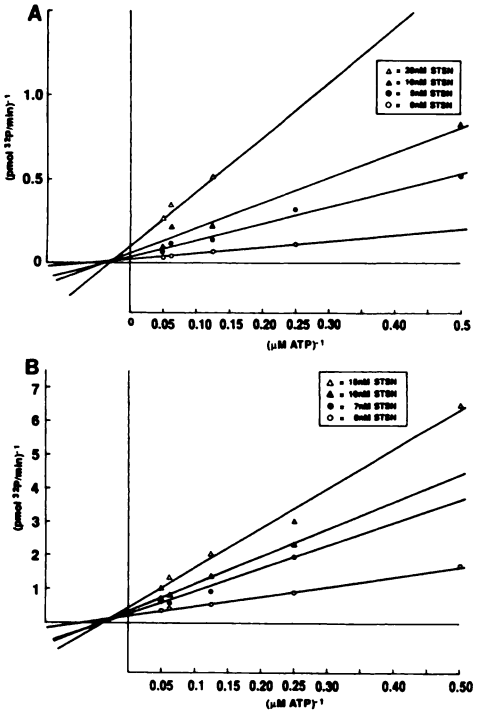

Fig. 1. Kinetics of inhibition of the catalytic fragment of PKC by staurosporine (*STSN*) as a function of ATP. Lineweaver-Burk analyses of the inhibition by staurosporine of a catalytic fragment of PKC generated from a mixture of PKC-α and PKC-β (A) and of the catalytic fragment of PKC-γ (B) are shown. Because PKC-α is resistant to proteolysis, the catalytic fragment generated from the isozyme mixture (approximately 60% PKC-β) (A) was mainly a product of PKC-β. Assays were conducted in the absence of allosteric cofactors (Ca^{2+} and PS). The [γ-^{32}P]ATP concentration was varied as indicated, and the specific activity of the nucleotide was held constant. For other assay conditions, see Materials and Methods. *pmol ^{32}P/min*, rate of transfer of ^{32}P from [γ-^{32}P]ATP to histone III-S. Each *point* represents a mean of triplicate assays. *Lines* were determined by least-squares regression analysis. In A, linear correlation coefficients were 0.999 (0 nM staurosporine), 0.992 (5 nM staurosporine), 0.991 (10 nM staurosporine), and 0.987 (20 nM staurosporine). In B, linear correlation coefficients were 0.998 (0 nM staurosporine), 0.986 (7 nM staurosporine), 0.998 (10 nM staurosporine), and 0.995 (15 nM staurosporine). These experiments were reproducible in their entirety.

TABLE 1

Comparison of the kinetics of inhibition of a catalytic fragment of PKC by staurosporine when ATP is varied and the phosphoacceptor substrate is either histone III-S or [Ser-25]PKC(19–31)

$K_{m_{app}}$(ATP) values were obtained from Lineweaver-Burk plots, and K_i(staurosporine) values were determined using Dixon plots. Patterns of inhibition were interpreted as previously described (21). The catalytic fragment of PKC was prepared by limited proteolysis of a purified rat brain PKC-α/PKC-β mixture (see Materials and Methods).

Phosphoacceptor substrate	$K_{m_{app}}$(ATP)	K_i(staurosporine)	Inhibitory pattern
	μM	nM	
[Ser-25]PKC(19–31)	24	2.6	Mixed competitive/noncompetitive
Histone III-S	20	3.3	Mixed competitive/noncompetitive

catalysis (12), we also determined the kinetics of inhibition of the catalytic fragment by staurosporine when the phosphoacceptor substrate was the oligopeptide [Ser-25]PKC(19–31) (22) instead of histone III-S. Table 1 shows that similar $K_{m_{app}}$(ATP) values were obtained in assays of histone III-S and [Ser-25]PKC(19–31) phosphorylation and that staurosporine inhibited both histone III-S and [Ser-25]PKC(19–31) phosphorylation with mixed competitive/noncompetitive kinetics (21) and indistinguishable potencies. The $K_{m_{app}}$(ATP) values in Table 1 primarily reflect the Ca^{2+}- and PS-independent protein kinase activity of the catalytic fragment of rat brain PKC-β (see Materials and Methods), and they are similar to the $K_{m_{app}}$(ATP) value (18 μM) reported for the Ca^{2+}- and PS-independent protamine sulfate phosphorylation reaction catalyzed by rat brain PKC-β (23).

In general, a straightforward interpretation of inhibitory kinetics with respect to ATP against a catalytic fragment generated from PKC-α and PKC-β may be precluded by both the presence of a mixture of isozymes in the reaction mixtures and the potential existence of two ATP binding sites in the catalytic domain of each isozyme (24, 25). Rat brain PKC-α and PKC-β contain homologous consensus sequences for ATP binding sites outside of their active sites (25), as do bovine brain PKC-α and PKC-β (24). It is not yet known whether these non-active site consensus sequences actually encode functional ATP binding sites (24–26). Consensus sequences for ATP binding sites in protein kinase active sites include an essential lysine residue, which is implicated in both ATP binding and protein kinase catalysis (27–29). Although rat brain PKC-γ bears homology with PKC-α and PKC-β at the putative second ATP binding site, PKC-γ contains an arginine residue in place of the essential lysine residue (25). In addition, bovine brain PKC-γ contains an arginine residue at this site (24). Previous studies with several protein kinases indicate that a lysine to arginine mutation in the ATP-binding consensus sequence at the active site abolishes enzyme activity (27). Fig. 1B shows that, as in the case of the catalytic fragment generated from a mixture of PKC-α and PKC-β, staurosporine inhibited histone III-S phosphorylation catalyzed by a catalytic fragment of PKC-γ with mixed competitive/noncompetitive kinetics with respect to ATP. Secondary plots (data not shown) (21) indicated that the potencies of staurosporine against the catalytic fragment of PKC-γ (K_i = 6.0 nM) and against the catalytic fragment generated from PKC-α and PKC-β (K_i = 3.3 nM) were comparable. The finding that both the potencies and the inhibitory patterns of staurosporine against catalytic fragments generated from a PKC-α/PKC-β mixture (Fig. 1A) and from PKC-γ (Fig. 1B) were similar, despite the critical difference between the sequences encoding the putative second ATP binding sites in the PKC-α/PKC-β and PKC-γ preparations, provides evidence that the mechanism of staurosporine inhibition of PKC does not involve the putative second ATP binding site. The result with the catalytic fragment of PKC-γ also shows that the complicated inhibitory kinetics observed in the inhibition of the catalytic fragment of PKC when ATP is varied cannot be ascribed simply to the presence of multiple isozymes.

Because the kinetics of staurosporine inhibition of the catalytic fragment of PKC with respect to ATP (Fig. 1; Table 1) argued against an inhibitory mechanism involving binding of staurosporine at the ATP substrate binding site, we next examined whether the kinetics of staurosporine inhibition would support a mechanism that entailed binding at the phosphoacceptor substrate binding site. Mixed noncompetitive/uncompetitive inhibition describes Lineweaver-Burk inhibitory plots that have lines crossing to the left of the *y*-axis and below the

x-axis, whereas the lines are parallel in fully uncompetitive inhibition (21). Fig. 2A shows that staurosporine inhibited the catalytic fragment generated from the PKC-α/PKC-β mixture with mixed noncompetitive/uncompetitive kinetics (21) with respect to histone III-S. At the highest staurosporine concentration tested (20 nM), the kinetics approached a fully uncompetitive pattern. When the kinetics of staurosporine inhibition with respect to histone III-S were studied with the catalytic fragment of PKC-γ, the pattern of inhibition was fully uncompetitive (Fig. 2B) and the K_i of staurosporine was 13 nM. The complicated kinetics shown in Fig. 2A may be due to the use of a catalytic fragment produced from a mixture of PKC isozymes. In any case, it is clear that the inhibitory kinetics shown in Fig. 2 do not support a mechanism involving staurosporine binding at the phosphoacceptor substrate binding site.

In order to assess whether staurosporine inhibited PKC isozymes by binding to a conserved region of their catalytic domains, we compared the inhibitory potencies of staurosporine against hydroxylapatite-purified PKC-α, PKC-β, and PKC-γ (18) and against catalytic fragments of hydroxylapatite-purified PKC-β and PKC-γ (18). Because PKC-α is highly resistant to trypsinolysis in the absence of Ca^{2+} and PS (19) (see Materials and Methods), the potency of staurosporine against the catalytic fragment of PKC-α was not examined. Under standard assay conditions, (for details, see Materials and Methods), staurosporine inhibited the Ca^{2+}- and PS-dependent activities of PKC-α, PKC-β, and PKC-γ, as well as the Ca^{2+}- and PS-independent activities of the catalytic fragments of PKC-β and PKC-γ, with similar potencies; the IC_{50} values for the isozymes and their catalytic fragments ranged from 9 to 14 nM (Table 2). These results support an inhibitory mechanism that entails binding of staurosporine at a conserved region in the catalytic domain of PKC isozymes.

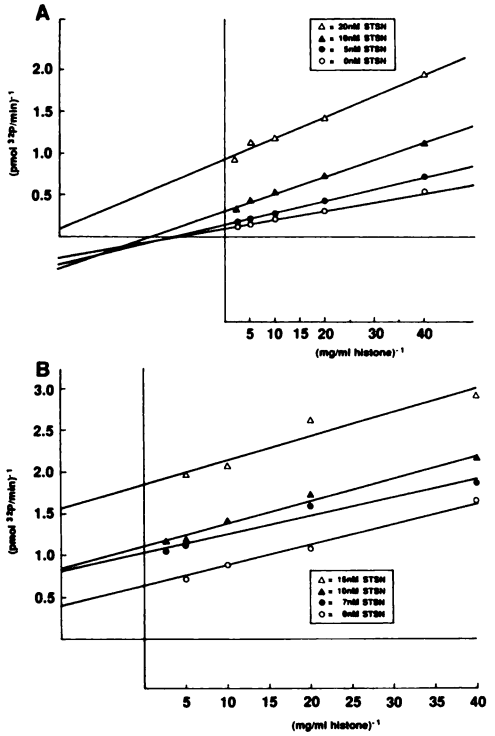

Fig. 2. Kinetics of inhibition of the catalytic fragment of PKC by staurosporine (*STSN*) as a function of histone III-S. Lineweaver-Burk analyses of the inhibition by staurosporine of a catalytic fragment of PKC generated from a mixture of PKC-α and PKC-β (A) and of the catalytic fragment of PKC-γ (B) are shown. The catalytic fragment generated from the isozyme mixture was mainly a product of PKC-β (for details, see legend to Fig. 1). For assay conditions, see Materials and Methods and the legend to Fig. 1. *pmol ^{32}P/min*, transfer of ^{32}P from [γ-^{32}P]ATP to histone III-S per minute. Each *point* represents a mean of triplicate assays. *Lines* were determined by least-squares regression analysis. In A, linear correlation coefficients were 0.999 (0 nM staurosporine), 0.999 (5 nM staurosporine), 0.995 (10 nM staurosporine), and 0.995 (20 nM staurosporine). In B, linear correlation coefficients were 0.997 (0 nM staurosporine), 0.980 (7 nM staurosporine), 0.993 (10 nM staurosporine), and 0.959 (15 nM staurosporine). These experiments were reproducible in their entirety.

Discussion

Previous studies of staurosporine inhibition of PKC provided strong evidence that staurosporine inhibits the enzyme by binding to its catalytic domain with high affinity (2, 7, 13, 14). Because the catalytic fragment of PKC can be assayed in a simplified system that facilitates straightforward kinetic analysis (12), we defined the kinetics of staurosporine inhibition of PKC by characterizing the inhibition of catalytic fragments of rat brain PKC that were generated from defined PKC isozymes. We found that the kinetics of staurosporine inhibition for the catalytic fragment of PKC-γ, as well as for a catalytic fragment generated from a PKC-α/PKC-β mixture, were not competitive with respect to either ATP or histone III-S. Similar inhibitory kinetics were observed with respect to ATP whether the phosphoacceptor substrate was histone III-S or [Ser-25]PKC(19–31), indicating that the kinetics of staurosporine inhibition did not result from direct binding interactions between staurosporine and histone. The kinetic data provide evidence that staurosporine inhibits PKC by binding to a site of the catalytic domain other than the ATP substrate and protein substrate binding sites. The mechanism of staurosporine inhibition of PKC most likely involves the binding of staurosporine to a conserved site in the catalytic domain of PKC isozymes, because staurosporine inhibited the rat brain PKC isozymes PKC-α, PKC-β, and PKC-γ, as well as the catalytic fragments of PKC-β and PKC-γ, with similar efficacies (Table 2).

The fact that the staurosporine analog K252a inhibits PKC and protein kinase A by competition with ATP (2, 15) suggests that staurosporine itself may bind ATP binding sites (2, 7). The catalytic domain of PKC contains two potential ATP binding sites (24, 25), and it is possible that the complicated kinetics observed for the inhibition of the catalytic fragment of PKC by staurosporine when ATP was varied (Fig. 1) could be due to the binding of staurosporine at the putative non-active site ATP binding site of PKC. However, this appears unlikely,

TABLE 2

Comparison of the inhibitory potencies of staurosporine against PKC isozymes and their catalytic fragments

IC_{50} represents the concentration of staurosporine required to inhibit 50% of the histone kinase activity of the indicated PKC isozyme under standard assay conditions. The catalytic fragments of PKC isozymes were assayed in the absence of Ca^{2+} and PS. In the analyses of the intact isozymes, the inhibitory potency of staurosporine against the Ca^{2+}- and PS-dependent activity of each isozyme was measured. Rat brain PKC isozymes were prepared as described in Ref. 18. For experimental details, see Materials and Methods.

Isozyme	IC_{50} against intact isozyme	IC_{50} against catalytic fragment
	nM	*nM*
PKC-α	12	
PKC-β	11	9
PKC-γ	14	14

because staurosporine inhibited the catalytic fragment of PKC-γ and a catalytic fragment generated from a PKC-α/PKC-β mixture with similar kinetics, despite the critical difference between the non-active site ATP-binding consensus sequence of PKC-γ and the homologous consensus sequences of PKC-α and PKC-β (24, 25). Furthermore, MgATP inhibits the binding of [^{3}H]dimethylstaurosporine to PKC only at ATP concentrations that greatly exceed those present in the protein kinase assays of this study [K_i(ATP) = 2.5 mM] (14).

Based on kinetic studies of PKC-catalyzed histone III-S phosphorylation, a mechanism has been proposed for the histone kinase reaction of PKC. Initial velocity measurements of histone III-S phosphorylation by the catalytic fragment of rat brain PKC in the presence of varying concentrations of each substrate indicate that the kinetic mechanism is sequential, i.e., both substrates bind to PKC before product release (12). The kinetics of inhibition of Ca^{2+}- and PS-activated rat brain PKC by product and dead-end inhibitors provide evidence that supports a mechanism in which the substrates ATP and histone III-S bind to PKC in a preferred order, with ATP binding first (30). In this study, we show that the kinetics of inhibition of the catalytic fragment of PKC-γ by staurosporine are uncompetitive when the concentration of histone III-S is varied. The uncompetitive kinetics provide evidence that the binding of histone III-S at the active site of the catalytic fragment precedes the binding of staurosporine to the enzyme (21). Taken in the context of previous studies of the kinetics of PKC-catalyzed histone III-S phosphorylation (12, 30), our results provide evidence for the following mechanism of PKC inhibition by staurosporine:

$$\text{PKC} + \text{ATP} + \text{histone} + \text{staurosporine} \rightleftharpoons \text{PKC}\cdot\text{ATP} \rightleftharpoons \text{PKC}\cdot\text{ATP}\cdot\text{histone} \rightleftharpoons \text{PKC}\cdot\text{ATP}\cdot\text{histone}\cdot\text{staurosporine} \quad (1)$$

(in which the final complex does not break down to products). Consistent with the mechanism implied by the kinetics observed with the catalytic fragment of PKC-γ when the histone III-S concentration was varied, a mixed competitive/noncompetitive pattern of inhibition was observed when the ATP concentration was varied, providing evidence that saturating concentrations of ATP neither prevented nor enhanced the binding of staurosporine to the catalytic fragment of PKC-γ. This interpretation of the kinetics of PKC inhibition by staurosporine (eq. 1) is also supported by the observations that histone (10–100 μg/ml) enhances the binding of [^{3}H]dimethylstaurosporine to PKC and that, although ATP does inhibit [^{3}H]dimethylstaurosporine binding to PKC with a K_i of 2.5 mM, saturating concentrations of this substrate are not sufficient to prevent the binding of [^{3}H]dimethylstaurosporine to PKC (14). However, the mixed kinetics observed when ATP concentrations were varied do suggest that staurosporine affects the affinity of ATP for PKC and, therefore, that its mechanism of inhibition is not fully described by eq. 1. Finally, mixed competitive/noncompetitive inhibitory kinetics are consistent with a mechanism in which the inhibitor binds to a site on the enzyme that overlaps with the binding site for the varied substrate, and the staurosporine analog K252a appears to bind to the ATP substrate binding site of PKC (15). Therefore, the kinetic results presented here and the kinetics of PKC inhibition by K252a (15), taken together, suggest that staurosporine may bind to PKC at a site that overlaps with its ATP substrate binding site.

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