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2,6-Diamino-*N*-([1-(1-oxotridecyl)-2-piperidinyl] methyl)hexanamide (NPC 15437): A Novel Inhibitor of Protein Kinase C Interacting at the Regulatory Domain

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SUMMARY

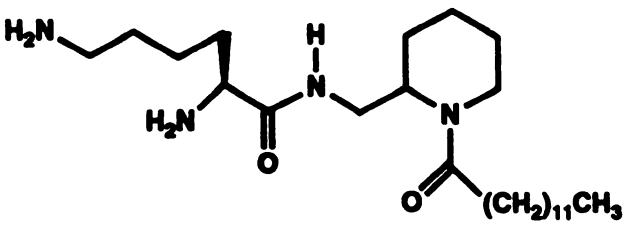
NPC 15437 is a prototype member of a new class of synthetically derived protein kinase C (PKC) inhibitors. PKC activity and binding of phorbol ester to the enzyme were inhibited by NPC 15437, with IC_{50} values of 19 ± 2 μM and 23 ± 4 μM, respectively. No inhibition of cAMP-dependent or calcium/calmodulin-dependent protein kinases was observed at concentrations of NPC 15437 up to 300 μM. To investigate the mechanism by which NPC 15437 exerts its effect, a kinetic analysis of the inhibition with respect to three activators of the enzyme, phosphatidylserine, calcium, and phorbol ester, was performed. NPC 15437 was a competitive inhibitor of the activation of PKC by phorbol ester (K_i = 5 ± 3 μM). Stimulation of PKCα by phosphatidylserine was competitively inhibited by NPC 15437 (K_i = 12 ± 4 μM). The inhibition was mixed with respect to activation by calcium. These results suggest that NPC 15437 is a selective inhibitor of PKC, interacting at the regulatory region of the enzyme. NPC 15437 inhibited PKC in intact cells, dose-dependently antagonizing the phorbol ester-induced phosphorylation of a 47-kDa protein in human platelets.

The pivotal role of the phospholipid/calcium/1,2-*sn*-DAG-dependent protein kinase (PKC) in regulating a wide variety of transmembrane signal transduction events is now widely accepted (1, 2). Extracellular agents, including hormones, transmitters, and growth factors, bind to their respective cell surface receptors and elicit hydrolysis of phosphatidylinositol, phosphatidylcholine, or phosphatidylethanolamine (3, 4), to yield DAGs that stimulate activity of the enzyme. The discovery that phorbol esters bind to and activate the enzyme by an interaction at the DAG site implicated PKC in the action of tumor promoters, regulation of cell differentiation, and oncogenesis (5–7).

PKC is a family of closely related proteins; seven isozymes have been cloned and expressed to date. Each has been shown to have distinct activation properties, ontogeny, and tissue distribution (8). Limited tryptic hydrolysis of the enzyme yields a 51-kDa carboxyl-terminal catalytic fragment that is fully active in the absence of the three activators, phospholipid, Ca^{2+}, and DAG/phorbol ester, and a 32-kDa amino-terminal regulatory fragment that contains the phorbol ester-binding region (C1 domain) and the calcium-binding site (C2 domain) (9–11). The function of this regulatory region appears to be the maintenance of the enzyme in an inactive state in the absence of Ca^{2+}, phospholipid, and DAG. Inactive PKC is largely cytosolic; upon activation by DAG or phorbol ester, the enzyme is rapidly translocated to membranes, where it interacts with the lipid bilayer (12).

The pleiotropic effects of PKC activation make a detailed understanding of its mechanism of regulation essential. Inhibitors of PKC have been used extensively to define the physiological function of this enzyme and to confirm and extend knowledge of the molecular mechanisms of enzyme activation. These inhibitors can be classified into two groups, depending on their site of interaction with the enzyme. Inhibitors that interact at the regulatory region of PKC include sphingosine (13), dibucane (14), trifluoperazine (15), calphostin (16), chremophor EL (17), tamoxifen (18), several bioactive polypeptides (19), and aminoacridines (20). A second group, interacting at the catalytic domain of the molecule, includes the isoquinolesulfonamide H-7 (21), K252 (22), staurosporine (23), sangivamycin (24), and several antineoplastic agents (25). Unfortunately, most of these molecules display some activity towards other protein kinases. Herein we report the discovery of a novel PKC inhibitor, NPC 15437 (2,6-diamino-*N*-{[1-(1-oxotridecyl)-2-piperidinyl]methyl}hexanamide) (Fig. 1), which interacts with the regulatory portion of the enzyme and displays marked selectivity towards PKC, compared with either PKA or myosin light chain kinase. In addition, we show that NPC 15437 is an inhibitor of PKC in intact cells.

ABBREVIATIONS: DAG, diacylglycerol; PKC, protein kinase C; PS, phosphatidylserine; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; EGTA, [ethylenebis(oxyethylenenitrilio)]tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PKA, protein kinase A.



NPC 15437

Fig. 1. Structure of NPC 15437.

Experimental Procedures

Materials. Calf thymus histone (type III-S), ATP, soybean trypsin inhibitor, cAMP-dependent protein kinase, and dibutyryl-cAMP were purchased from Sigma; [^{32}P]orthophosphate, [γ-^{32}P]ATP, and [^{3}H]PDBu were from DuPont-New England Nuclear; PS was from Avanti Polar Lipids (Birmingham, AL); PMA and PDBu were from LC Services Corp. (Woburn, MA); L-(tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin was from Worthington Chemicals; DEAE-cellulose (DE52) was from Whatman; Ultrogel AcA 44 and threonine-Sepharose were from LKB; and protein determination reagents were from Bio-Rad. A purified preparation of PKC isolated from rat brain was obtained from Calbiochem.

Isolation of PKCα. PKCα was isolated from the cytosolic fraction of confluent cultures of SV-T$_2$ cells (which contain only this isozyme of PKC) (26), by a slight modification of the method of Huang *et al.* (27). The cells were homogenized in 25 mM Tris·HCl, pH 7.5, 0.25 M sucrose, 2 mM EDTA, 2 mM EGTA, 5 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and the homogenate was centrifuged at 100,000 × *g* for 60 min. The supernatant was applied to a DEAE-cellulose column, which had previously been equilibrated in buffer A (25 mM Tris·HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 5 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 10 mM phenylmethylsulfonyl fluoride). The column was washed with 2 column volumes of the same buffer containing 20 mM NaCl. PKC activity was eluted from the column using a gradient of NaCl (0–0.3 M) in buffer A. Fractions containing PKC activity were pooled, concentrated by ultrafiltration, and applied to a phenyl-Sepharose column equilibrated with buffer A. The kinase was eluted with a gradient of 1.5–0 M NaCl in buffer A. Fractions containing kinase activity were pooled, concentrated, and applied to a Sephacryl S-200 column equilibrated with buffer A. PKCα was further purified on polylysine-agarose, using a 0–0.8 M NaCl gradient, and finally on an hydroxylapatite column, using a 0–200 mM gradient of potassium phosphate in buffer A without Tris·HCl. Purified PKCα had a specific activity of 800 units/mg of protein, where 1 unit of kinase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of phosphate from ATP into substrate protein/min. Immunoblot analysis of the purified kinase using monoclonal antibodies specific for the α, β, and γ subtypes indicated that the purified 76-kDa protein was PKCα (data not shown).

Preparation of the regulatory and catalytic fragments of PKC. PKC was partially digested with trypsin (28). The catalytic fragment was isolated by Ultrogel AcA 44 gel permeation chromatography, and the regulatory fragment was isolated by threonine-Sepharose chromatography (29).

PKC activity. PKC activity was determined using histone III-S. The standard reaction mixture (250 μl) contained 20 mM Tris·HCl, pH 7.5, 10 mM magnesium acetate, 400 μM $CaCl_2$, 0.1 mM EGTA, 10 μM [γ-^{32}P]ATP (1200 cpm/pmol), 100 μg/ml PS, 10 μM histone, 1 μM PMA, 25 μl of inhibitor or water, and 10 ng of PKCα or 20 ng of the purified rat brain PKC preparation. The mixture was incubated at 30° for 5 min. Mixtures were removed to an ice-bath and a 50-μl aliquot of each was added to Whatman P81 paper and washed in 10% trichloroacetic acid for 20 min. PKC activity was quantitated by liquid scintillation counting. PKC-independent protein kinase activity was determined by including 2 mM EGTA in the assay mixture described above.

[^{3}H]PDBu binding. PDBu binding to PKC and the regulatory fragment of the enzyme was determined in the presence of 200 μl of assay buffer (50 mM Tris, pH 7.4, 100 μg/ml PS, 0.4 mM $CaCl_2$, 1 mg/ml bovine serum albumin), 100 μl of [^{3}H]PDBu (8 nM final concentration), 25 μl of NPC 15437 or water, and 20 ng of enzyme. The reaction was carried out at 37° for 30 min. Bound [^{3}H]PDBu was separated from free ligand by filtering through GF/C glass fiber filters and was washed five times with 1 ml of ice-cold 20 mM Tris·HCl buffer, pH 7.5. Nonspecific binding was determined under the same conditions in the presence of 100 μM nonradioactive PDBu.

Myosin light chain kinase activity. Myosin light chain kinase was purified from chicken gizzards as described (30). The assay mixture (200 μl) contained 25 mM Tris, pH 7.5, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 60 mM KCl, 15 μg/ml calmodulin, 0.5 mg/ml myosin, 5 μCi of [^{32}P]ATP (500 μM), and enzyme. The reaction was allowed to continue for 5 min at 25°.

PKA activity. PKA activity was measured in the presence of 150 μl of assay buffer (see PKC activity assay), 20 mg/ml histone III-S (10 μl), 25 μl of NPC 15437 or water, 10 μl of enzyme (4 ng of catalytic subunit or 8 ng of intact enzyme), 100 μM dibutyryl-cAMP, and 100 μM [^{32}P]ATP. The reaction was carried out at 30° for 5 min and stopped as described for PKC activity assays. cAMP-dependent protein kinase activities were corrected for cAMP-independent activity by assaying in the absence of cAMP.

Phosphorylation of a 47-kDa polypeptide in human platelets. Phosphorylation of a 47-kDa protein in intact human platelets was carried out as described (31). Human blood (50 ml) was collected in 5 mM EDTA (final concentration), pH 7.5. The blood was centrifuged at 500 × *g* for 10 min at room temperature. The platelet-rich plasma (20 ml) was removed and incubated with 2.5 mCi of [^{32}P]orthophosphate at 37° for 60 min. Platelets were pelleted by centrifugation at 900 × *g* for 10 min at room temperature and were then washed with 25 ml of 140 mM NaCl, 3 mM KCl, 12 mM $NaHOO_3$, 5.5 mM glucose, 1 mM EDTA, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.2. The platelets were resuspended in the same buffer, to a concentration of 10^9 platelets/ml. Aliquots of 10^8 platelets were resuspended in the same buffer, to a concentration of 10^9 platelets/ml. Aliquots of 10^8 platelets were incubated with the appropriate concentration of NPC 15437 for 10 min and then incubated with 100 nM PDBu for 3 min at 37°. Reactions were terminated by the addition of 50 μl of sodium dodecyl sulfate sample buffer. Polyacrylamide gel electrophoresis of the sample was performed as described (32). After exposure of the gel to an X-ray film for 12–24 hr, the 47-kDa and 20-kDa bands were excised, and radioactivity in the slices was determined by liquid scintillation counting.

Calculation of the free calcium concentration in solution was determined using the EQCAL program (Biosoft, Milltown, NJ). Enzyme kinetic data were analyzed using classical methods (33, 34). Data were fitted using linear regression or spline curve-fitting programs.

Results

Inhibition of PKC, PKA, and myosin light chain kinase by NPC 15437. Rat brain contains a mixture of all seven PKC isozymes (1). The effect of NPC 15437 on a purified preparation of these isozymes is shown in Fig. 2. Under assay conditions eliciting maximal kinase activity, NPC 15437 inhibited enzymic activity with an IC_{50} value of 19 ± 2 μM. Significantly, NPC 15437 had no effect on the catalytic activity of either cAMP-dependent protein kinase or calcium/calmodulin-dependent myosin light chain kinase at concentrations up to 300 μM (Fig. 2). In contrast, staurosporine, sphingosine, and H-7 inhibited both PKA and myosin light chain kinase at these concentrations (Table 1), in close agreement with previous reports.

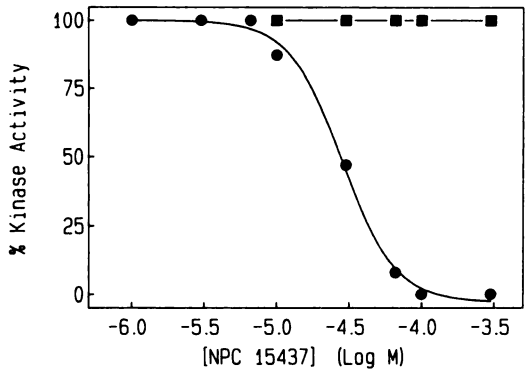


Fig. 2. NPC 15437 selectively inhibits PKC activity. PKC, PKA, and myosin light chain kinase activities were determined as described in Experimental Procedures. ●, PKC activity; ■, PKA and myosin light chain kinase activities.

TABLE 1

Inhibition of PKA, myosin light chain kinase, and PKC by sphingosine, staurosporine, H-7, and NPC 15437

Kinase activities were determined as described in Experimental Procedures. Results represent the mean ± standard error of five experiments.

Inhibitor	IC_{50}		
	PKC	PKA	MLCK*
		μM	
NPC 15437	19 ± 2	≫300	≫300
Sphingosine	57 ± 12	98 ± 8	145 ± 10
Staurosporine	0.08 ± 0.02	0.17 ± 0.03	0.11 ± 0.01
H-7	60 ± 4	167 ± 20	184 ± 9

* MLCK, myosin light chain kinase.

TABLE 2

Effect of NPC 15437 on the regulatory and catalytic domains of PKC

PKC activity was determined in the presence of 20 mg/ml histone, 50 μM Ca^{2+}, 100 μg/ml PS, 1 μM PMA, and 10 μM ATP. Activator-independent protein kinase activity was measured in the presence of 2 mM EGTA. [^{3}H]PDBu binding to the intact enzyme and the regulatory and catalytic fragments was determined as described in Experimental Procedures. Results represent the mean ± standard error of four experiments.

Inhibitor	Inhibition			
	PKC activity		PDBu binding	
	Intact enzyme	Catalytic fragment	Intact enzyme	Regulatory fragment
	%			
NPC 15437 (50 μM)	80 ± 6	0 ± 3	75 ± 4	69 ± 4
H7 (50 μM)	65 ± 4	60 ± 8	29 ± 6	0 ± 2

Interaction of NPC 15437 with the regulatory domain of PKC. Inhibitors of PKC may interact either with the substrate binding sites in the catalytic domain of the enzyme or with the regulatory region. To determine the site of interaction of NPC 15437 with PKC, the catalytic domain of the enzyme was generated by limited tryptic hydrolysis. The activity of this fragment, which does not associate with membranes, was not inhibited by NPC 15437 (Table 2). Under similar conditions, H-7, a competitive inhibitor of PKC with respect to ATP (21), did inhibit the catalytic fragment (Table 2). Thus, NPC 15437 appears to be interacting at the regulatory region of PKC.

To demonstrate a more direct association between NPC 15437 and the regulatory domain of the enzyme, the effect of NPC 15437 on binding of [^{3}H]PDBu was investigated. NPC 15437 was found to inhibit phorbol ester binding to the intact enzyme preparation (IC_{50} = 23 ± 4 μM) and to the 32-kDa regulatory fragment generated by limited tryptic hydrolysis (Table 2). These data confirm that NPC 15437 inhibits PKC activity by interacting at the regulatory domain of the enzyme.

Mechanism of inhibition of PKCα by NPC 15437. To investigate the interaction of NPC 15437 with the regulatory region of PKC, a detailed kinetic analysis of the inhibition with respect to activation by PS, Ca^{2+}, and PMA was performed. To ensure that in all studies a homogeneous preparation of PKC was present, PKCα isolated from murine fibroblasts was used. Previous studies have demonstrated that this cell line expresses the α subtypes but not the β or γ (26). The PS dependence of PKC activation was studied at fixed PMA (1 μM) and free calcium (400 μM) concentrations, using several fixed concentrations of NPC 15437. The potency of inhibition was found to be highly dependent on the concentration of PS (Fig. 3). Double-reciprocal analysis of the interaction revealed an essentially competitive type of inhibition, with a K_i value of 12 ± 4 μM (Fig. 3).

The effect of NPC 15437 on PKC activity was modulated markedly by phorbol ester, and inhibition was largely overcome by increasing PMA concentrations (Fig. 4). Lineweaver-Burk and Dixon analysis of the data indicated that NPC 15437 was a competitive inhibitor with respect to PMA activation, with an apparent K_i value of 5 ± 3 μM. These studies suggest that

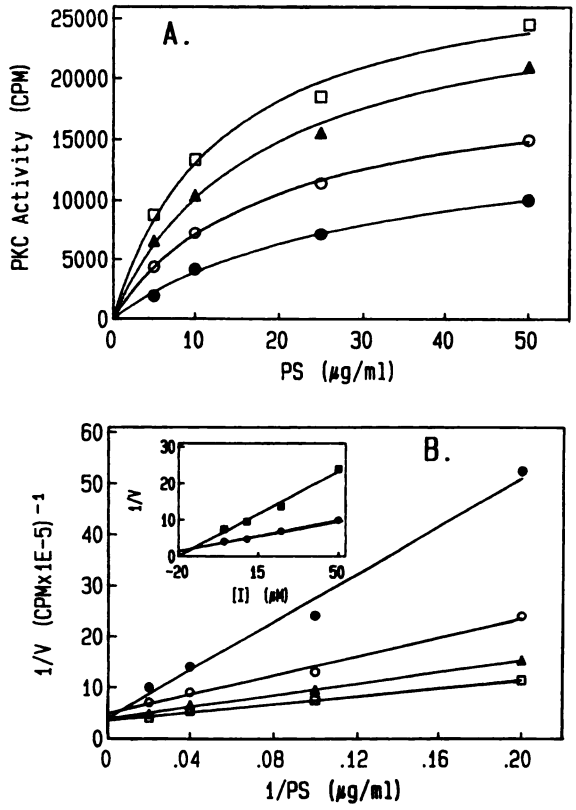


Fig. 3. Effect of PS on the inhibition of PKCα by NPC 15437. A, PKC activity was determined in the presence of 400 μM free Ca^{2+}, 1 μM PMA, 10 μM ATP, and the indicated concentrations of PS. NPC 15437 was present at 0 μM (□), 10 μM (▲), 25 μM (○), and 50 μM (●). B, Double-reciprocal analysis of the data in A. *Inset*, Dixon analysis of the inhibition. K_i, determined from the point of intersection of the two lines, is 12 ± 4 μM. PS concentrations were 10 μg/ml (■) and 50 μg/ml (●).

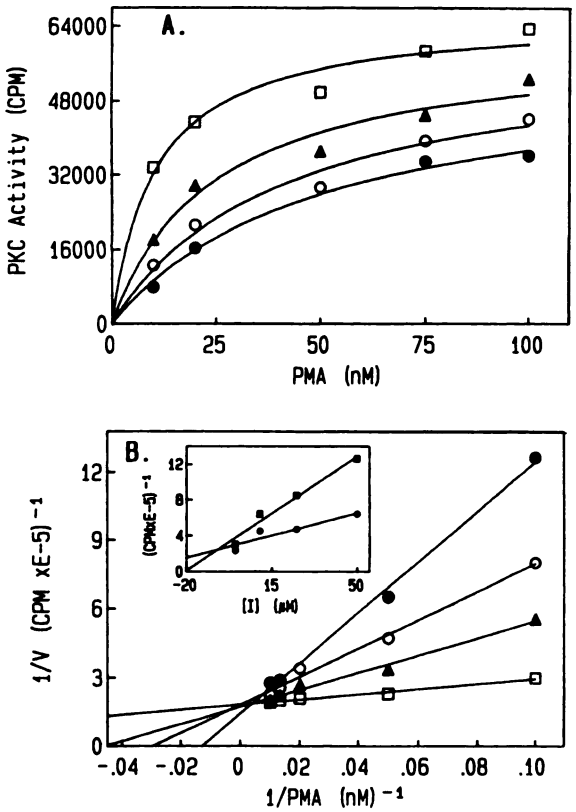
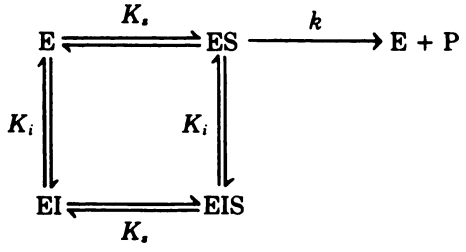


Fig. 4. Effect of PMA on the inhibition of PKCα by NPC 15437. A, PKC activity was determined in the presence of 400 μM free Ca^{2+}, 100 μg/ml PS, 10 μM ATP, and the indicated concentrations of PMA. NPC 15437 was present at 0 μM (□), 10 μM (▲), 25 μM (○), and 50 μM (●). B, Double-reciprocal analysis of the data in A. *Inset*, Dixon analysis of the inhibition in the presence of 10 nM PMA (■) and 50 nM PMA (●). K_i, determined from the point of intersection of the two lines, is 5 ± 3 μM.

NPC 15437 inhibits PKCα by a direct interaction with the phorbol ester binding site.

To further explore the mechanism of NPC 15437 inhibition, the effect of the concentration of calcium used was examined at fixed PS (50 μg/ml) and phorbol ester (1 μM) concentrations and at 0, 25, and 50 μM NPC 15437. Double-reciprocal plots of the calcium dependencies were linear (Fig. 5). The data suggest that, with respect to activation by calcium, there appear to be both partially competitive and fully noncompetitive components to the inhibition by NPC 15437. A mechanism for this mixed type of inhibition has been described (34):



where

$$v = \frac{V_{max}}{\frac{K_s}{S}(1 + I/K_i) + (1 + I/K_i')}$$

The kinetic inhibition constants, K_i and K_i', for this type of

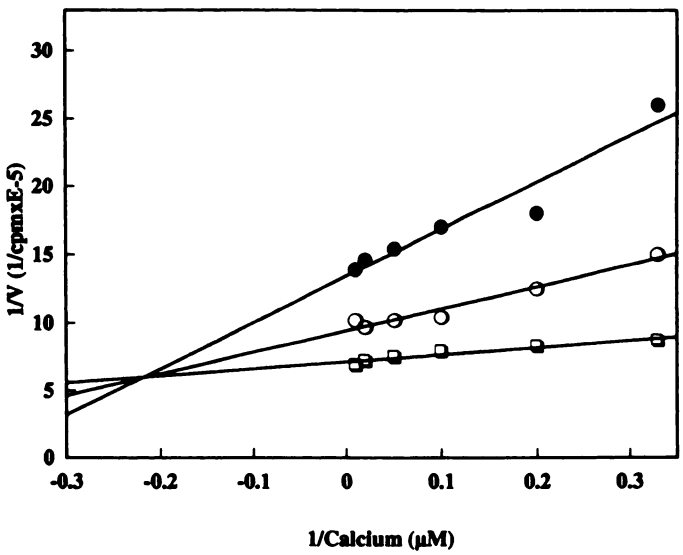


Fig. 5. Lineweaver-Burk analysis of the effect of calcium on the inhibition of PKCα by NPC 15437. PKC activity was determined in the presence of 1 μM PMA, 100 μg/ml PS, 10 μM ATP, and the indicated concentrations of free calcium. NPC 15437 was present at 0 μM (□), 25 μM (○), and 50 μM (●).

inhibition were determined by Dixon (33) and Cornish-Bowden analyses (34) (Fig. 6). These resulted in values of 25 μM and 60 μM for K_i and K_i', respectively.

Inhibition of PKC activity by NPC 15437 in intact cells. Finally, the ability of NPC 15437 to exert an inhibitory effect on PKC-mediated processes in intact cell systems was assessed. Activation of PKC by phorbol esters in human platelets results in a rapid increase in the phosphorylation of pleckstrin, a 47-kDa protein (31). Stimulation of platelets with PDBu increased phosphorylation of pleckstrin by 350 ± 15%. NPC 15437 dose-dependently antagonized the PDBu-induced phosphorylation of this protein (Fig. 7), with an IC_{50} of 34 ± 9 μM. Interestingly, phosphorylation of myosin light chain (20 kDa) was also increased (60%), in agreement with reports that this protein can be phosphorylated by PKC as well as myosin light chain kinase in platelets (35). NPC 15437 attenuated the phosphorylation of myosin light chain. The ability of NPC 15437 to inhibit phosphorylation demonstrates that this compound can readily penetrate the cell membrane.

Discussion

In this study, NPC 15437 was shown to inhibit PKC activation and binding of phorbol ester. Significantly, NPC 15437 was also selective in its inhibition of PKC, compared with both cAMP-dependent protein kinase and the calcium/calmodulin-dependent myosin light chain kinase. Detailed studies of the effect of NPC 15437 on the regulatory and catalytic domains of the enzyme support a specific interaction with the regulatory region. These results were confirmed by a kinetic analysis of the interaction between the inhibitor and a purified preparation of the α isozyme of PKC. NPC 15437 was a competitive inhibitor of PS and phorbol ester activation of the enzyme and demonstrated a mixed type of inhibition (predominantly non-competitive) with respect to activation by calcium.

Stoichiometry experiments (36), structure-function analysis (36, 37), and the availability of amino acid sequence data for the different isozymes (8) have allowed the mechanism of

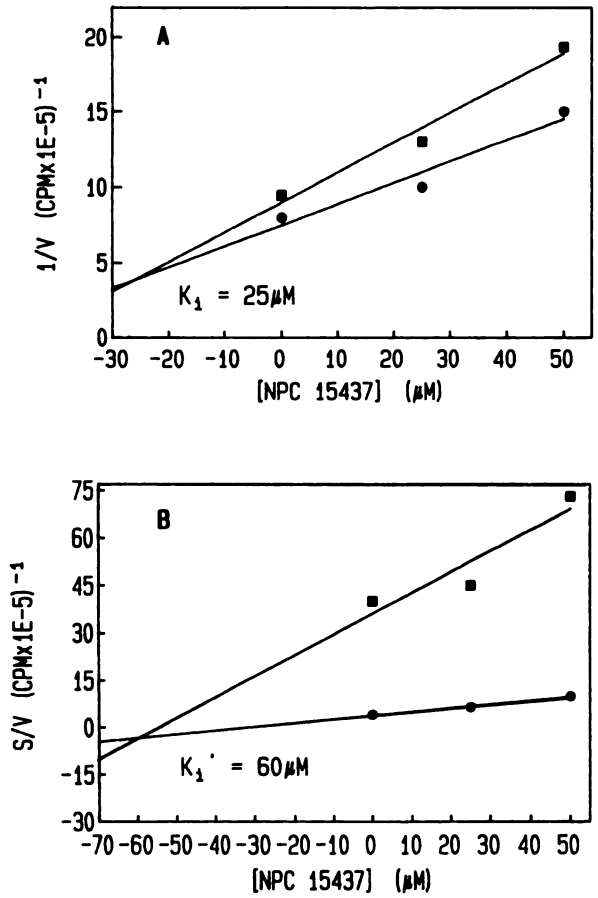


Fig. 6. Dixon and Cornish-Bowden analysis of the interaction of calcium with NPC 15437. A, Dixon analysis of the inhibition of PKC by NPC 15437 in the presence of 5 μM Ca^{2+} (■) and 50 μM Ca^{2+} (●). K_i, determined from the point of intersection of the two lines, is 25 μM. B, Cornish-Bowden analysis of the inhibition in the presence of 5 μM Ca^{2+} (■) and 50 μM Ca^{2+} (●). K_i', determined from the point of intersection of the two lines, is 60 μM.

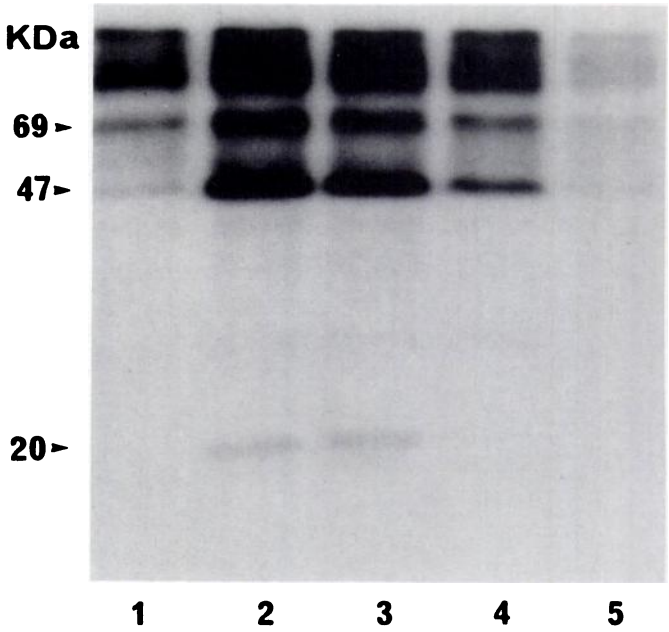


Fig. 7. Inhibition of protein phosphorylation in human platelets by NPC 15437. Platelets were pretreated with NPC 15437 for 10 min before stimulation with PDBu for 3 min. *Lane 1*, 0 PDBu; *lane 2*, 100 nM PDBu; *lane 3*, 100 nM PDBu and 5 μM NPC 15437; *lane 4*, 100 nM PDBu and 30 μM NPC 15437; *lane 5*, 100 nM PDBu and 100 μM NPC 15437.

activation of PKC by the allosteric modulators to become more clearly understood. The cytoplasmic form of PKC is inactive, due to a pseudosubstrate region in the amino-terminal end of the regulatory region blocking the substrate-binding region in its catalytic domain. The pseudosubstrate region (amino acids 19–29) of PKCα has five basic and no acidic residues. To account for the ability of calcium ions and DAG to cause both translocation of PKC to the plasma membrane and activation of the enzyme, it has been suggested that when these activators bind to the enzyme the pseudosubstrate region is exposed and then binds to negative lipids in the membrane (38, 39). Stoichiometric analysis of this interaction indicates that PKC binds to a complex composed of n PS molecules (where n = 2–4, depending on the isoform being examined), calcium, and DAG. The points of contact with PS are inferred to be with the carboxyl, amino, and phosphate groups. A three-point stereospecific interaction between DAG and the PKC-PS-Ca^{2+} complex has been proposed (36, 37), with two bonds being directly with the enzyme and the third directly with calcium.

According to this model and from the kinetic analysis, NPC 15437 interacts with PKC at either the phorbol ester- or PS-binding regions. An interaction with the phorbol ester site may alter the conformation of the enzyme such that it is unable to bind to the PS-Ca^{2+} complex via the pseudosubstrate-binding domain. The competitive inhibition observed with respect to PS may be due to NPC 15437 acting as an "allosteric" inhibitor of the enzyme. Thus, binding of the inhibitor to the enzyme results in a conformational change in the enzyme that precludes binding of phospholipid to its site. Significantly, recent studies using recombinant mutant PKC molecules provide support for an interaction of NPC 15437 with the phorbol ester-binding region (40). In these studies, NPC 15437 had no effect on the protein kinase activity of mutants lacking the first of the cysteine-rich repeat sequences that are believed to be indispensable for the binding of phorbol ester to the enzyme (40, 41). However, a direct interaction with the PS-binding region(s) on the enzyme cannot be discounted entirely. Although the very basic nature of the pseudosubstrate-binding region makes it an unlikely site for interaction with a cationic species like NPC 15437, several putative PS binding sites in the DAG/phorbol ester-binding region have recently been reported (42). By making a series of point mutations in the C1 domain, it may be possible to define further the site of interaction of NPC 15437 with PKC.

Three classes of PKC inhibitors currently exist. A large group of molecules, including dibucaine (14), chlorpromazine (14), quercetin (43), amiloride (44), Adriamycin (15), and verapamil (14), act on the lipid-binding domain of the enzyme in a manner not yet fully understood but believed to involve perturbation of the cell membrane bilayer. Bioactive amphipathic polypeptides, such as mellitin, polymyxin B, and cardiotoxin (19), and antineoplastic lipid analogs, such as ET-18-OCH_3 (45), inhibit the enzyme either by binding to the PS binding site(s) on PKC or by directly interacting with the phospholipid to prevent enzymic activation. Aminoacridines also inhibit the enzyme by acting at the regulatory domain (20), presumably by bridging the PS-Ca^{2+}-enzyme complex to block DAG binding. Sphingosine and related sphingolipids inhibit PKC by competing with the phorbol ester binding site (13) and thus have the potential

for specificity. However, sphingosine also inhibits Ca^{2+}/calmodulin-dependent protein kinases (46) (Table 1) and other biological processes in a PKC-independent manner (46).

A second class of inhibitors, exemplified by H7 (21), suramin (47), and staurosporine (23) and its derivatives (48), act on the enzyme by competition at the ATP binding site. These inhibitors exhibit low specificity, because most serine/threonine kinases share similar catalytic structures (49). Furthermore, whereas staurosporine and its derivatives may be potent inhibitors of the enzyme *in vitro*, the high concentrations of ATP in cells (millimolar, compared with micromolar levels *in vitro*) lead to a significant reduction in potency in intact cell systems (48). Peptides that bind to the pseudosubstrate region are the most potent inhibitors of the enzyme *in vitro* (50) and constitute a third class of inhibitor. Unfortunately, peptides are unstable in biological environments, are excluded from the cytoplasm, and, therefore, are of limited value *in vivo*. As a selective inhibitor of PKC both *in vitro* and *in vivo*, with a direct site of action on the C1 domain of the enzyme, NPC 15437 represents a new class of synthetically derived PKC inhibitor.

Acknowledgments

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