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NPC 15437 interacts with the C1 domain of protein kinase C

An analysis using mutant PKC constructs

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We recently demonstrated that 2,6,diamino-*N*-([1-(oxotridecyl)-2-piperidinyl]methyl)-hexanamide (NPC 15437) is a selective inhibitor of PKC interacting at the regulatory domain of the enzyme. To further investigate the interaction of NPC 15437 with PKC we expressed a series of cDNAs encoding mutant PKC molecules in COS7 cells. NPC 15437 had no effect on the protein kinase activity of mutants lacking the N-terminal region of the C1 domain. Further, NPC 15437 was a competitive inhibitor of the activation of PKCα by phorbol ester and attenuated the binding of phorbol ester to the enzyme in intact cells. The present study demonstrates that mutant enzyme constructs can be used to localize the site of interaction of NPC 15437 with PKC to residues 12–42, which encodes the pseudosubstrate binding domain and part of the first cysteine-rich repeat sequence.

Protein kinase C; NPC 15437; Inhibitor; Phorbol ester

1. INTRODUCTION

The pivotal role of protein kinase C (PKC) in regulating a wide variety of transmembrane signal transduction events is now widely accepted [1,2]. At least seven subtypes of the enzyme exist, each has been shown to have distinct properties, ontogenic and tissue distributions [3]. The amino acid sequences deduced from the cDNAs cloned from various species have revealed that PKC has three conserved (C1–C3) and four divergent regions [3]. The availability of recombinant mutant PKCs has extended earlier biochemical findings about the role of these domains [4–6]. Thus, mutants lacking the C1 domain demonstrate kinase activity independent of phospholipid, calcium, and phorbol ester [4,5]. Protein kinase activity of mutants lacking the C2 region is much less dependent on calcium compared to the wild-type PKC [5]. A PKC mutant lacking the C3 region has no protein kinase activity under any conditions [4]. Such mutants are also useful tools for studies on the sites of interaction of inhibitors with the enzyme.

We have recently reported that 2,6,diamino-*N*-([1-(oxotridecyl)-2-piperidinyl]methyl)-hexanamide (NPC 15437) inhibits PKC by an interaction at the regulatory domain [7]. Further, we demonstrated that NPC 15437 is more selective in its inhibition of PKC than either H7, staurosporine or sphingosine. In the present study we have expressed a series of cDNAs coding for mutant PKCs (Fig. 1) in COS7 cells in order to further investigate the inhibitory mechanism of NPC 15437 against PKC.

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2. MATERIALS AND METHODS

2.1. *Materials*

cDNA encoding bovine PKCα and a series of cDNAs coding for mutant PKCs, cloned into the expression vector pcDSR [5], were kindly provided by Dr. Masa-Aki Muramatsu (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). EGF-R peptide (Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu) was synthesized using an automated solid phase synthesizer. An anti-PKC monoclonal antibody and ^{125}I-labeled anti-mouse F(ab)$_2$ fragment were purchased from Amersham Corporation. [γ-^{32}P]ATP and [^{3}H]phorbol 12,13-dibutyrate (PDBu) were obtained from DuPont-New England Nuclear. Phosphatidylserine (PS) was purchased from Avanti Polar Lipids, Birmingham, AL, phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate were from LC Services Corporation, Woburn, MA, DEAE-sephacel (DE) was from Whatman.

2.2. *Transfection of DNA to COS7 cells*

Exponentially growing COS7 cells (1.5×10^6/10 cm plate, 4 plates/plasmid) were seeded for 48 h prior to transduction with plasmids. COS7 cells were transfected with plasmids using calcium precipitation [8] and cultured at 37°C for 72 h prior to isolation of PKC.

2.3. *Purification and analysis of PKC*

Transfected cells were harvested, washed and homogenized as described [5]. Supernatants were applied to DEAE-Sephacel columns equilibrated with 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 20 mM 2-ME, 100 μM PMSF, 10 μg/ml leupeptin and 10% glycerol. Wild-type PKCα was eluted with 100 mM NaCl, the mutant PKC activities were eluted with 200 mM NaCl [5]. To identify the PKC mutants, extracts from COS cells transfected with these con-

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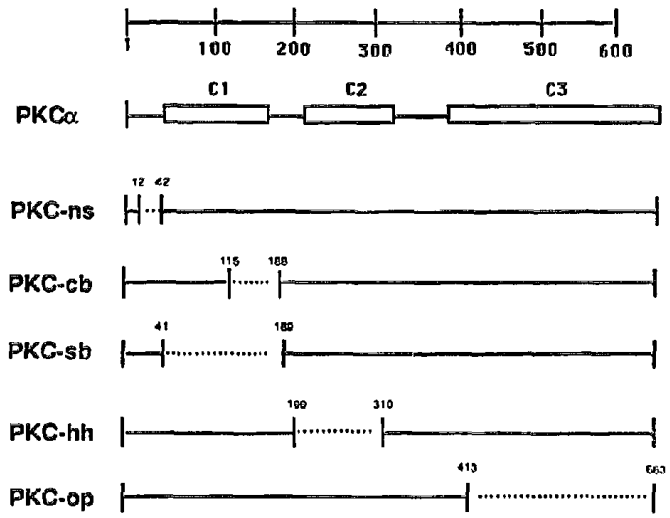

Fig. 1. Structure of mutant PKC constructs (modified from [5]).

structs were subjected to SDS-polyacrylamide gel electrophoresis [9], followed by immunoblot analysis using an anti-PKC antibody (the epitope is mapped around residues 296–317 of the wild-type enzyme). The mutant enzymes were identified by their altered electrophoretic mobilities [5].

2.4. *PKC activity*

PKC activity was measured using EGF-R as a substrate. The reaction mixture (250 μl) contained 20 mM Tris-HCl, pH 7.5; 10 mM $Mg(CH_3COO)_2$; 100 μM $CaCl_2$; 100 μM EGTA; 50 μg/ml phosphatidylserine (PS); 1 μM PMA; 10 μM [γ-^{32}P]ATP (1200 cpm/pmol); 100 μM EGF-R and 100 ng PKC mutant. The mixture was incubated at 30°C for 15 min. Mixtures were removed to an ice-bath, a 50 μl aliquot of each was added to Whatman P81 paper and washed in 10% TCA for 20 min. PKC activity was quantitated by liquid scintillation counting. Activator independent kinase activity was determined by including 1 mM EGTA in the above assay mixture.

2.5. *Phorbol ester binding to intact cells*

Following transfection with the PKC constructs, COS7 cells were washed three times with phosphate buffered saline and then incubated with 200 μl assay buffer (50 mM Tris, pH 7.4, and 1 mg/ml bovine serum albumin), 100 μl [^{3}H]PDBu (20 nM final concentration) and 50 μM of NPC 15437 or water. The reaction was carried out at 37°C for 30 min. Non-specific binding was determined under the same conditions in the presence of 100 μM non-radioactive PDBu.

3. RESULTS

3.1. *PKC activity in extracts from COS7 cells transfected with cDNAs for mutant PKCs*

Following partial purification, the PKC activity of the mutants was determined using a peptide substrate that is specific for PKC [10]. In agreement with previous studies [5], transfection of COS7 cells with SRαPKCα resulted in a 5-fold increase in PKC activity which was dependent upon added phospholipid, Ca^{2+} and phorbol ester (Table I). Transfection with SRαPKAC (a mutant in which amino acids 1–253 of PKCα are replaced with amino acids 1–17 of the N-terminal region of PKA), SRαPKCαΔNS or SRαPKCαΔSB conferred an increase in protein kinase

Table I

Protein kinase activities of PKC mutant constructs

PKC construct	PKC activity (cpm)	
	PS + Ca^{2+} + TPA	EGTA
SRαPKCα	86 494	2 086
SRαPKAC	22 484	26 494
SRαPKCαΔNS	19 685	21 764
SRαPKCαΔCB	23 934	14 984
SRαPKCαΔSB	24 864	27 492
SRαPKCαΔHH	33 094	4 125
SRαPKCαΔOP	3 628	2 413

Aliquots (25 μl) of each mutant were assayed for kinase activity using EGF-R (100 μM) as a substrate and in the presence of 1 μM PMA, 100 μM Ca^{2+}, 40 μg/ml phosphatidylserine (PS), 10 μM [γ-^{32}P]ATP and 100 μM EDTA. Activator-independent kinase activity was determined in the presence of 1 mM EGTA. PKC activity in the COS-7 cells was 14 580 cpm. Above values are representative of a typical experiment.

activity independent of phospholipid, Ca^{2+}, and phorbol ester. However, SRαPKCαΔCB was still partially dependent on the presence of the activators for full activity (Table I). SRαPKCαΔHH demonstrated protein kinase activity that was dependent on phospholipid, Ca^{2+}, and phorbol ester. However, this protein kinase required a much lower concentration of Ca^{2+} for full activity than wild-type PKC.

3.2. *Interaction of NPC 15437 with the mutant PKC molecules*

The interaction of NPC 15437 with SRαPKCα and SRαPKAC is shown in Fig. 2. Under conditions that maximally stimulate kinase activity, NPC 15437 inhibited the protein kinase activity of PKCα with an IC_{50} value of 22 μM and a Hill coefficient value (n_H) of 2, in close agreement with our previous study [7]. Significantly, NPC 15437 had no effect on the protein

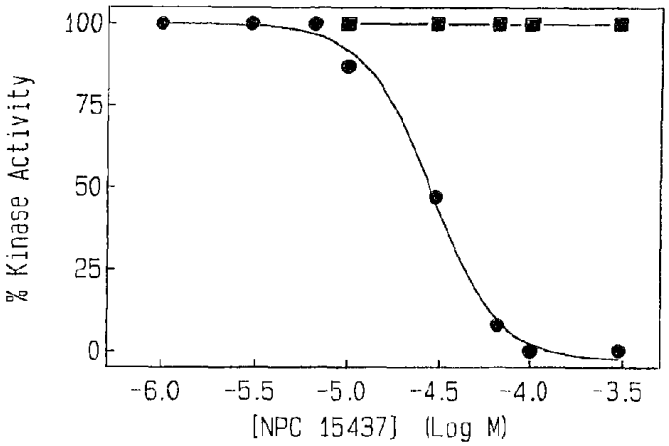

Fig. 2. Interaction of NPC 15437 with SRαPKCα and SRαPKAC. Kinase activities were determined in the presence of 100 μM EGF-R, 1 μM PMA, 50 μg/ml PS, 100 μM Ca^{2+}, 100 μM EGTA, 10 μM [γ-^{32}P]ATP and 100 ng PKCα (•) or PKAC (■). Activity of PKAC was also determined in the presence of 1 mM EGTA.

Table II

Interaction of NPC 15437, staurosporine, and H-7 with SRαPKCα and SRαPKAC

Inhibitor	IC_{50}	
	PKCα	PKAC
NPC 15437	22 ± 4 μM	-
Staurosporine	30 ± 6 nM	10 ± 4 nM
H-7	15 ± 4 μM	18 ± 5 μM

PKC activity was determined as described in Materials and Methods. Values represent mean ± SE of triplicate determinations in at least 3 separate experiments.

kinase activity of SRαPKAC. In contrast, H-7 and staurosporine, non-selective inhibitors of PKC [11,12], inhibited both kinase activities (Table II). These studies indicate that NPC 15437 interacts with the regulatory domain of the enzyme which would account for the selectivity observed in our earlier studies. Mutants SRαPKCαΔNS, SRαPKCαΔSB, SRαPKCαΔCB and SRαPKCαΔHH provided more information concerning the site of interaction of NPC 15437 with the regulatory domain.

Protein kinase activity of mutants lacking either the entire C1 domain (ΔSB) or a mutant lacking the pseudosubstrate binding region and part of the phorbol ester binding domain (ΔNS) was unaffected by the presence of NPC 15437 at concentrations up to 200 μM (Fig. 3) indicating an interaction between the inhibitor and the C1 region of the enzyme deleted in these mutants. In contrast, H-7 inhibited the protein kinase activity of these mutants with IC_{50} values of 15 μM and 22 μM, respectively. NPC 15437 readily inhibited the kinase activity of ΔHH (IC_{50} = 30 μM) but was a weak inhibitor of ΔCB (IC_{50} = 130 μM).

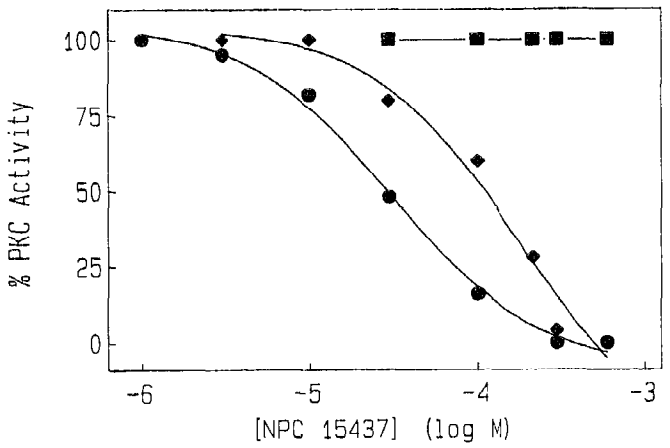

Fig. 3. Effect of NPC 15437 on the kinase activity of SRαPKCαΔNS and SRαPKCαΔSB (■), SRαPKCαΔCB (♦) and SRαPKCαΔHH (•). Kinase activities were determined as described in the legend to Fig. 2. The n_H values for the inhibition of SRαPKCαΔCB and SRαPKCαΔHH were 1.1 and 1.4, respectively.

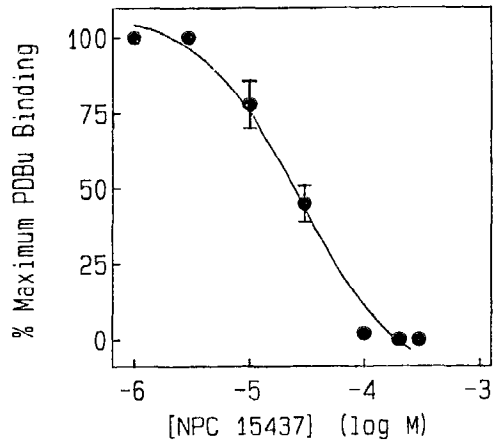

Fig. 4. NPC 15437 inhibits [^{3}H]PDBu binding to PKCα in intact cells (n_H = 1.8). Studies were carried out as described in Materials and Methods.

Further evidence for the involvement of the C1 region in the interaction of NPC 15437 with PKC came from a kinetic analysis of the inhibition with respect to activation of the enzyme by phorbol ester. Inhibition was largely overcome by increasing concentrations of phorbol ester, Lineweaver-Burke and Dixon analysis indicated that NPC 15437 was a competitive inhibitor of the activation by phorbol ester with a K_i value of 5 ± 2 μM.

3.3. *NPC 15437 inhibits [^{3}H]PDBu binding to PKC in intact cells*

Finally, the ability of NPC 15437 to exert an inhibitory effect on phorbol ester binding to PKC in intact cells was assessed. NPC 15437 dose-dependently antagonized phorbol ester binding activity in COS7 cells transfected with either ΔOP, a mutant lacking the entire catalytic domain of the enzyme, or PKCα (Fig. 4) with IC_{50} values of 19 μM and 27 μM, respectively. H-7 had no effect on the binding of phorbol ester to ΔOP.

4. DISCUSSION

The mechanism underlying the selective inhibition of PKC by NPC 15437 is now becoming better understood. In a previous report, we demonstrated that this synthetically derived compound is a selective inhibitor of PKC activity [7]. Mechanistic studies had indicated a direct interaction with the regulatory domain most likely at either the phorbol ester or phospholipid binding sites. Significantly, recent evidence indicates that NPC 15437 is not inhibiting the enzyme via a lipid perturbation effect (Sullivan, J.P. and Connor, J.R., unpublished data). Herein, using recombinant mutant PKC molecules, we have shown that NPC 15437 is interacting with the N-terminal region of the C1 domain.

Indirect evidence for this conclusion came from studies using ΔHH and ΔOP. First, recent observations that nPKC, PKCδ and PKCε, all of which lack the C2 domain [13], require phospholipid and diacylglycerol/phorbol ester but not Ca^{2+} for maximal activation of the enzyme have suggested that this region may regulate the affinity of PKC for Ca^{2+} requirement. Since NPC 15437 inhibited the protein kinase activity of ΔHH it is reasonable to assume that the activity of this mutant is not dependent on the calcium concentration. Secondly, NPC 15437 attenuated the binding of phorbol ester to PKCα and ΔOP, demonstrating that the presence of the catalytic domain is not critical to the inhibitory activity of the compound. The Hill coefficient for phorbol ester binding to PKCα was 1.8, confirming that the DAG/phorbol ester binding site regulates the enzyme allosterically [1,3,16]. Not surprisingly, H-7, a non-selective inhibitor that interacts at the ATP binding site, had no effect on the phorbol ester binding to ΔOP. Deletions in the C1 domain of the enzyme provided more conclusive evidence on the site of interaction of NPC 15437 with PKC.

The C1 region of PKCa has a tandem repeat characteristic of a sequence that resembles the cysteine-rich zinc-finger motif. Such a sequence motif has been identified in many DNA binding proteins that appear to be active in transcriptional regulation [14]. While there is presently no indication that PKC interacts directly with DNA, there is evidence to indicate that these sequences are indispensable for the binding of phorbol ester to the enzymes [5,6] and that binding of phorbol ester to either of the sequences is sufficient for PKC activation [6]. In addition, the C1 domain contains the pseudosubstrate binding region. This stretch of amino acids is believed to play a crucial role in the conformational changes that result in activation of the enzyme [15,16]. We have shown that NPC 15437 has no effect on the kinase activity of (i) a mutant lacking both of the cysteine-rich sequences, ΔSB, and (ii) a mutant lacking the pseudosubstrate region and part of the first of the cysteine repeat sequences, ΔNS. Interestingly, NPC 15437 did have some effect on the protein kinase activity of ΔCB, a mutant lacking the second of the cysteine-rich sequences that lies between amino acids 104 and 161 [6]. Taken together with the kinetic analysis these results would suggest that NPC 15437 is interacting with the N-terminal region of the C1 domain, a stretch of amino acids that includes the pseudosubstrate region and part of the first of the cysteine-rich sequences [5,6]. Such an interaction may induce conformational changes in the enzyme that prevent DAG/phorbol ester binding to the enzyme or, more likely, binding of the inhibitor may block the ability of DAG/phorbol ester to alter the conformation of PKC in order to activate it.

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