Pergamon

Tetrahedron Letters, Vol. 36, No. 48, pp. 8795-8798, 1995
Elsevier Science Ltd
Printed in Great Britain
0040-4039/95 $9.50+0.00

0040-4039(95)01864-6

Photoaffinity Labeling of PKC with a Phorbol Derivative: Importance of the 13-Acyl Group in Phorbol Ester-PKC Interaction

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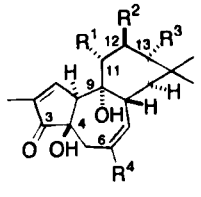
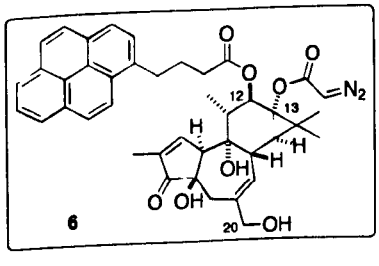
Abstract: We report the design and synthesis of a novel photoaffinity ligand, phorbol 12-(1-pyrenebutyrate) 13-diazoacetate (PPDA, **6**). This photolabile phorbol derivative labeled with tritium was used for photocrosslinking experiments with a sample of PKC partially purified from rat brain, and specific binding of **6** to PKC was observed.

The phorbol esters such as phorbol 12-myristate 13-acetate (PMA, **1**) are known as powerful tumor promoters and activators of protein kinase C (PKC).[1] First discovered by Nishizuka *et al.*, PKC is a phospholipid- and calcium-dependent serine/threonine kinase,[2] physiologically activated by 1,2-diacyl-*sn*-glycerol (DAG). PKC, which has now been characterized as a family of closely related enzymes, plays a central role in cellular signal transduction, controlling many cellular processes including proliferation and differentiation.[3] PKC is also known to be an important target for other structurally diverse tumor promoters such as the ingenols, teleocidins, and aplysiatoxins. Structure-activity analyses on a variety of analogs of DAG and these tumor promoters have been carried out. Although many pharmacophore models for PKC have been proposed from molecular modeling,[4] no information about specific amino acid residues that interact with these ligands is available. Photoaffinity labeling is thus expected to be a useful method for the characterization of the ligand-binding site of PKC, and, indeed, the syntheses of several photoaffinity ligands and their crosslinking experiments have been reported.[5] To our knowledge, however, no crosslinking to PKC has been observed. Here we report the design and synthesis of a novel photoaffinity ligand **6** and its specific photocrosslinking to PKC.





	R^1	R^2	R^3	R^4	
1	CH_3	$OCO(CH_2)_{12}CH_3$	$OCOCH_3$	CH_2OH	
2	CH_3	$OCO(CH_2)_{12}CH_3$	OH	CH_2OH	
3	CH_3	OH	$OCO(CH_2)_{12}CH_3$	CH_2OH	
4	CH_3	$OCO(CH_2)_2CH_3$	$OCO(CH_2)_2CH_3$	CH_2OH	
5	H	$OCO(CH_2)_{12}CH_3$	H	CH_2OH	
7	CH_3	OH	OH	CH_2OH	a
8	CH_3	OH	OH	CH_2OTr	b
9	CH_3	OH	$OCOCH_2NHCOO^tBu$	CH_2OTr	c
10	CH_3	$OCOC_{19}H_{15}$	$OCOCH_2NHCOO^tBu$	CH_2OTr	d
11	CH_3	$OCOC_{19}H_{15}$	$OCOCH_2NH_2$	CH_2OH	e
6	CH_3	$OCOC_{19}H_{15}$	$OCOCHN_2$	CH_2OH	f
12	CH_3	$OCOC_{19}H_{15}$	$OCOCHN_2$	CHO	g
[^{3}H]-**6**	CH_3	$OCOC_{19}H_{15}$	$OCOCHN_2$	CH_2(T)OH	

Figure 1.
(a) TrCl (3 eq), pyridine, 23 °C, 56%; (b) ^{t}BuOCONHCH$_2$COOH (5 eq), DCC (2.5 eq), NEt_3 (1 eq), THF, 0-23 °C, 76%, (c) 1-pyrenebutyric acid ($C_{19}H_{15}COOH$, 2 eq), EDCI•HCl (2 eq), DMAP (1 eq), THF, 0-23 °C, 91%; (d) TFA, 0 °C, 99%; (e) ^{t}AmONO (30 eq), AcOH (1 eq), 10%- ^{t}AmOH-$CHCl_3$, 0-23 °C, 53%; (f) MnO_2, CH_2Cl_2, 60 %; (g) [^{3}H]$NaBH_4$ (13.6 Ci/mmol), 65% based on the [^{3}H]$NaBH_4$

The acyl groups of phorbol esters were initially thought to be important for a specific interaction with PKC because of their structural similarity to DAG;[6] however, the majority of recent molecular modeling studies have concluded that these acyl groups are not crucial for specific interactions of the ligand with PKC. Since binding of the ligand to PKC requires a phosphatidylserine-containing membrane, the hydrophobic acyl groups contribute to association of the ligand with the membrane and thus to PKC in a non-specific manner.[4] Most of these models concur that the C20-OH, C9-OH, and C3-C=O groups (and C4-OH group) in phorbol esters are crucial sites for specific interactions. None of the three point (or four point) models, however, can explain all of the experimentally observed structure-activity relationships. The dramatic difference in binding of the phorbol 12- and 13-monoesters to PKC illustrates this point. For example, Blumberg *et al.* have reported[7] that phorbol 12-myristate (**2**) shows significantly weaker binding to PKC (K_i for [^{3}H]PDBu (**4**) binding = 100 nM) than phorbol 13-myristate (**3**) (K_i = 0.5 nM) indicating that removal of the acetyl group at the C13 position from **1** (K_i = 0.06 nM) causes more than a thousand-fold drop in binding. We have also found that optically active 13-deacetoxy-11-demethyl-PMA (**5**), a compound we recently synthesized, binds to PKC with two orders of magnitude less affinity than **1**.[8] These facts suggest that the hydrophobic group at the C12 position is not sufficient for high affinity binding to PKC, implicating an additional role for the 13-*O*-acyl group possibly *via* the carbonyl oxygen. Based on this hypothesis we have designed phorbol 12-(1-pyrenebutyrate) 13-diazoacetate (PPDA, **6**) as a novel photoaffinity ligand with the photoreactive functional group α to the carbonyl at C13.[9]

Synthesis of **6** was achieved as shown in Figure 1. After selective protection of the C20-OH of phorbol (**7**) as a trityl ether,[10] the C13-OH was selectively acylated with *N*-Boc-glycine (DCC, NEt_3, THF, 0 - 23 °C) to give **9** in 76% yield. The C12-OH was acylated with 1-pyrenebutyric acid (DCC, DMAP, THF, 0 - 23 °C) affording **10** (91%), and removal of the trityl and Boc groups was then accomplished on treatment with TFA (0 °C, 99%). Final conversion of the amino group to a diazo group was achieved on reaction of **11** with isoamyl nitrite-acetic acid (in 10% isoamyl alcohol-$CHCl_3$, 0 - 23 °C) giving the desired **6** in 53% yield.[11] Tritiated PPDA ([^{3}H]**6**, 1.6 Ci/mmol) was prepared from **6** by oxidation of the C20-OH to the corresponding aldehyde **12** with MnO_2 (60%) and subsequent reduction with [^{3}H]sodium borohydride (65%).

Binding of photoaffinity ligand **6** to PKC was measured in the presence of $CaCl_2$ and phosphatidylserine according to the reported procedure with minor modifications.[12,13] As shown in Table 1, PPDA (**6**) strongly inhibits the specific binding of [^{3}H]PDBu to PKC even at low concentrations indicating that the affinity of **6** to PKC is comparable to **1**.

	Inhibition (%)	
Concentration	PMA (**1**)	PPDA (**6**)
10 nM	40	60
100 nM	80	90
1 μM	100	100
10 μM	100	100

Table 1 Inhibition of [^{3}H]PDBu Binding to PKC. Various concentrations of **1** or **6** were incubated with [^{3}H]PDBu (10 nM) and PKC (0.8 μg/mL) in 300 μL of buffer containing Tris•HCl (50 mM, pH 7.5), $CaCl_2$ (4 mM), phosphatidylserine (100 μg/mL), bovine serum albumin (4 mg/mL), and DMSO (0.5 %) on ice for 2 h, and then the PKC-bound [^{3}H]PDBu was counted using a scintillation counter after separating the complex by filtration through a polyethyleneimine-treated glassfiber filter paper. Data are shown as % inhibition of the [^{3}H]PDBu binding relative to that without cold competitor.

This photolabile phorbol derivative **6** was used for photocrosslinking experiments with a sample of PKC partially purified from rat brain according to the reported procedure.[14] Thus, [^{3}H]**6** (200 nM) was incubated with the crude PKC sample (~ 100 pmol) in 300 μL of buffer containing Tris•HCl (50 mM, pH 7.5), $CaCl_2$ (1 mM), phosphatidylserine (100 μg/mL) and DMSO (1.5 %) at 30 °C for 30 min and then irradiated at 254 nm using a hand lamp at 0 °C for 5 min[15]. After concentration of this reaction mixture by lyophilization, the protein fraction was separated from the organic fraction by repeated MeOH-$CHCl_3$ extractions. An SDS-PAGE gel of the protein fraction is shown in Figure 2A. Quantitation of the radioactivity bound to the proteins was done by scintillation counting of each gel slice (Figure 2B). The slice containing PKC (No. 5) was observed to be labeled (lane a and b).[16,17] The major band in the slice No. 5 corresponding to ca. 82 kDa[18] was immunostained with antibodies specific for PKC α, β, γ, and δ. The labeling was suppressed by addition of a 50-fold excess of the competitive lignd PMA (**1**) indicating that the photolabeling is specific (lane c). It is

noteworthy that slice No. 6 containing unknown proteins was also photolabeled but that this labeling was not prevented by PMA showing that this is a non-specific labeling.

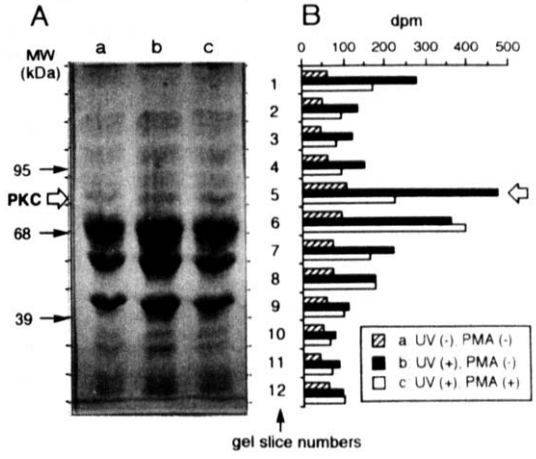


Figure 2. Photocrosslinking of [^{3}H]PPDA with PKC.
[^{3}H]PPDA (200 nM) was incubated with the crude PKC sample (~ 100 pmol) in 300 μL of buffer containing Tris•HCl (50 mM, pH 7.5), $CaCl_2$ (1 mM), phosphatidylserine (100 μg/mL) and DMSO (1.5 %) with (lanes b and c) or without (lane a) irradiation of UV in the absence (lanes a and b) or presence (lane c) of cold PMA (10 μM). After SDS-PAGE of the protein fraction (**A**), each lane of the gel was sliced into 12 pieces (5 mm), and the radioactivity was quantitated using a scintillation counter (**B**). Positions of the molecular size marker and PKC (ca. 82 kDa) are indicated on the left side.

To our knowledge, this is the first example of the specific photolabeling of PKC with a phorbol derivative. The crosslinking of **6** to PKC indicates that the diazoacetyl group at C13 is located in close proximity to the protein in the ligand-PKC-phospholipid complex. In addition to the three oxygen functional groups at C20, C9 and C3, the C13-acyl group is strongly implicated as important in PKC-ligand interactions. This information should be useful for making a better pharmacophore model. The tertiary structure of a portion of the regulatory domain of PKC was recently solved by NMR studies,[19] and the crystal structure of the small fragment of PKC δ in complex with phorbol 13-acetate, a very low affinity ligand, was reported after completing this manuscript.[20] In the X-ray crystal structure having no lipid component, C13-acyl group only makes intramolecular hydrogen bonding with C9-OH group. However, it may be still possible that additional hydrogen bonding of C13-acyl group with PKC is formed upon interaction with lipid membrane and plays an important role in high affinity binding. Identification of the crosslinked amino acid residues will pave the way for clarifying interactions in the ligand-PKC-phospholipid complex. This photolabile ligand may also be a useful tool for identifying novel unknown phorbol receptors.[21] The results described in this paper on the photocrosslinking of a phorbol deriavtive to PKC are a step toward these goals. Further studies along this line are in progress.

Acknowledgment. We thank Dr. T. C. Saido of the Department of Molecular Biology at Tokyo Metropolitan Institute of Medical Science for helpful discussions on the purification of PKC. We also thank Dr. Y. Hatanaka of the Faculty of Pharmaceutical Sciences at Toyama Medical & Pharmaceutical University for a discussion on photoaffinity labeling. This work was supported by the Grant-in Aid for Scientific Research on Priority Areas No. 062-4012 from the Ministry of Education, Science and Culture, Japan, and Fellowship of the Japan Society for the Promotion of Science for Japanese Junior Scientists.

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15. Since half-life for photolysis of PPDA under these conditions determined by UV absorption analysis was about 1 min, photolysis for 5 min is sufficient for complete photolytic decomposition of PPDA

16. Based on the total radioactivity of [^{3}H]PPDA used and dpm for slice No.5, ca. 0.2 % of the ligand bound irreversibly to proteins. This yield may be improved by modification of photoreactive functional group at C13.

17. Photolabeling of proteins corresponding to ca. 82 kDa was reproduced in several independent experiments.

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(*Received in Japan* 29 *August* 1995; *revised* 25 *September* 1995; *accepted* 27 *September* 1995)