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Importance of Interaction between C1 Domain and Lipids in Protein Kinase Cα Activation: Hydrophobic Side Chain Direction in Isobenzofuranone Ligands Controls Enzyme Activation Level

Go Hirai,[a] Tadashi Shimizu,[a] Toru Watanabe,[a, b] Yosuke Ogoshi,[b] Megumi Ohkubo,[a, b] and Mikiko Sodeoka*[a]

Protein kinase C (PKC) isozymes play important roles in intracellular signal transduction related to various cellular events including proliferation, differentiation, and apoptosis.[1] PKCs are serine-threonine protein kinases, and the mammalian PKC family comprises 11 isozymes grouped into three classes, known as conventional, novel, and atypical PKC isozymes. All members have in common a conserved catalytic domain at the C-terminal and regulatory domains at the N-terminal. The regulatory domains contain an autoinhibitory pseudosubstrate sequence and one or two membrane-targeting C1 and C2 domains. The activity of conventional PKC isozymes is stimulated by the physiological ligand diacylglycerol (DAG), calcium ions, and phosphatidylserine (PS). The natural products, phorbol ester, bryostatin, aplysiatoxin, and teleocidins, are strong activators of the conventional and novel classes of PKC isozymes.[2]

The activation mechanism of PKCα has been proposed to be as follows: PKCα is localized in cytosol as an inactive conformer, in which the catalytic domain is capped by the pseudosubstrate sequence (inactive state I, Figure 1). An increase of the calcium level causes translocation of PKCα to the membrane by Ca^{2+}-dependent interaction of PS with the C2 domain. At this point, the equilibrium still favors the inactive state II, rather than the active state III. Binding of DAG or phorbol ester to the C1 domain stabilizes the active conformer, and PKC becomes fully activated (active state IV, Figure 1).[3,4] The structure of the whole PKC molecule has not yet been clarified,[5] and the precise molecular mechanism of PKC activation is also not well understood. The crystal structures of the PKCδC1B domain and its complex with phorbol 13-acetate have been solved,[6] and no significant structural difference between the two was observed, suggesting that structural alteration of the C1B domain upon ligand binding is not a major driving force for the conformational change of the whole PKC molecule. Rather, it is likely that interaction with the lipid membrane plays a critical role in the conformational change leading to activation of PKC. Phorbol 12-myristate-13-acetate (PMA, **1**) is a powerful PKC activator, whereas phorbol esters having shorter alkyl side chains at the C12/C13 positions are less effective activators, indicating that the hydrophobic side chain is important both for binding to PKC and for the activation of PKC.[7] Various synthetic C1 domain ligands, which were designed based on DAG or natural products, have also been reported.[8] Almost all of these ligands have a hydrophobic side chain and the fact that they act as strong PKC activators supports the importance of the C1 domain–lipid membrane interaction.[9,10] In this communication, we focus on the role of the C1 domain–lipid membrane interaction in the conformational change of PKCα from the inactive state to the active state. We hypothesized that the direction of the hydrophobic side chain would be critical for the conformational change. To test this hypothesis, we required PKC ligands having differently directed hydrophobic side chains.

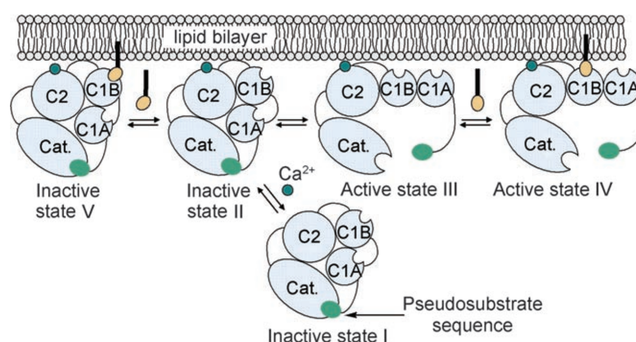

Figure 1. Proposed activation mechanism of PKCα.

We have already reported that the isobenzofuranone derivative **2a** is a good ligand of PKCα.[11] The isobenzofuranone derivative **2a** (Type A) was designed as a conformationally fixed DAG analogue based on the reported interactions between phorbol ester and PKCδC1B.[5] Figure 2 shows binding models of the PKCαC1B domain with PMA (**1**) and with the isobenzofuranone derivative **2a** generated by using homology modeling and docking programs. (see Supporting Information) These models suggest that three hydrogen bonds are formed between the two oxygen atoms (hydroxyl group and carbonyl oxygen of the pivaloyl group) of **2a** and the C1B domain (Thr 113/Leu 122/Gly 124) in the same fashion as observed in the binding of the phorbol ester, and the *t*Bu group interacts with the two leucines (Leu 121, Leu 125). According to these models the long alkyl chain at the C7 position of **2a** is expected to be located at a similar position to the C12/C13 alkyl chains of PMA, and should interact with the lipid membrane in a similar manner to that of PMA. Herein, we report the synthesis of the regioisomers of the isobenzofuranone ligand, **2b** (Type B), **2c** (Type C), and **2d** (Type D), having a hydrophobic chain at different positions (Figure 2), and the result of a com-

[a] Dr. G. Hirai, Dr. T. Shimizu, T. Watanabe, M. Ohkubo, Dr. M. Sodeoka
Synthetic Organic Chemistry Laboratory, RIKEN, 2-1, Hirosawa, Wako-shi, Saitama 351-0198 (Japan)
Fax: (+81) 48-462-4666
E-mail: sodeoka@riken.jp

[b] T. Watanabe, Y. Ogoshi, M. Ohkubo
Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, 2-1-1 Katahira, Aoba, Sendai, Miyagi 980-8577 (Japan)

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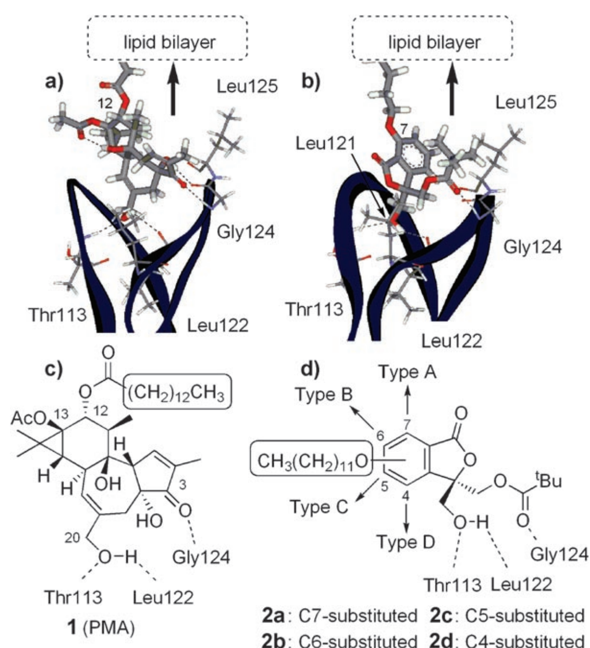
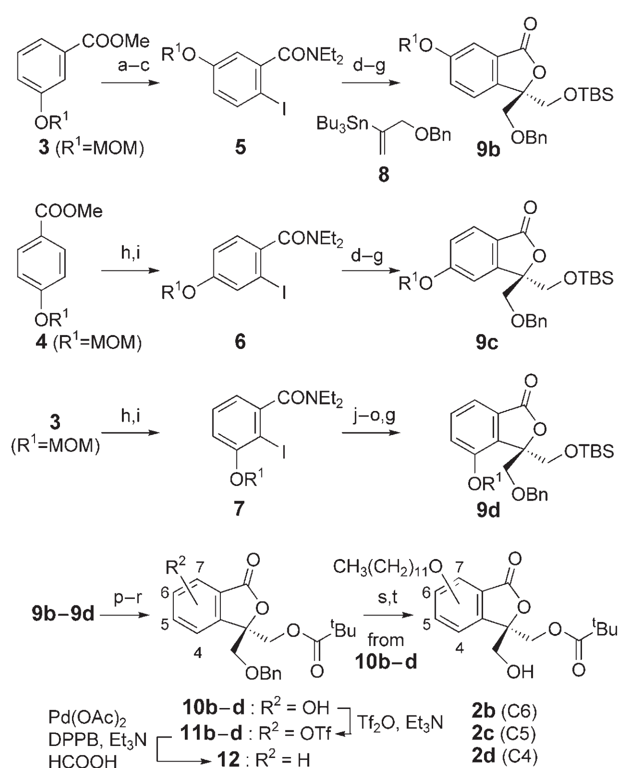


Figure 2. Proposed binding models of PKCαC1B domain with a) **1** and b) **2a**. Structures and key hydrogen bonding interaction of c) **1** and d) isobenzofuranone derivatives **2a**–**2d**.



Scheme 1. Preparation of Type B–D derivatives. a) *n*BuLi, $HNEt_2$ then *s*BuLi then TMSCl; b) *s*BuLi, TMEDA then I_2; c) TBAF, DMPU (56 %, 3 steps); d) $PdCl_2(PPh_3)_2$, LiCl, **8**; e) OsO_4, NMO; f) TBSCl, imidazole (for **5**, 73 %; for **6**, 73 %, 3 steps); g) separation by HPLC (CHIRALPAK AD-H); h) *n*BuLi, $HNEt_2$; i) *s*BuLi then I_2 (for **4**, 67 %; for **3**, 82 %, 2 steps); j) $PdCl_2(PPh_3)_2$, LiCl, **8**; k) conc.HCl aq. MeOH (38 %, 2 steps); l) OsO_4, NMO; m) TBSCl, imidazole (39 %, 2 steps); n) K_2CO_3, MeOH; o) MOMCl, *i*Pr_2NEt (90 %, 2 steps); p) TBAF, THF; q) *t*BuCOCl, DMAP; r) HCl, MeOH (for **10b**, 74 %; for **10c**, 96 %; for **10d**, 77 %, 3 steps); s) $CH_3(CH_2)_{11}OH$, PPh_3, DEAD; t) $Pd(OH)_2$, H_2 (for **2b**, 79 %; for **2c**, 97 %; for **2d**, 52 %, 2 steps); TMEDA = *N,N,N′,N′*-tetramethylethylenediamine; DMPU = 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone; NMO = 4-methylmorpholine *N*-oxide; DEAD = diethyl azodicarboxylate; Tf = trifluoromethanesulfonyl; DPPB = 1,4-bis(diphenylphosphino)butane.

parison of the activity of all four regioisomers. Interestingly, **2a** and **2b** were found to activate PKCα strongly, whereas **2c** was a relatively weak activator and **2d** showed essentially no activation.

For efficient synthesis of these regioisomers we took a different strategy from that reported for the synthesis of Type A compound **2a**.[11] Key steps for the new synthetic route are regioselective introduction of a C3 carbon unit by Stille coupling and construction of the lactone ring by dihydroxylation (Scheme 1). The critical intermediates, the methoxymethyloxy-2-iodo-benzamide derivatives, **5**–**7** were synthesized by selective *ortho*-lithiation of the amide prepared from **3** and **4**, followed by reaction with iodine. Preparation of the iodide **5** was achieved according to Snieckus's procedure.[12] The coupling reaction of **5** with alkenylstannane **8** gave the desired alkenylated product in good yield. OsO_4-catalyzed dihydroxylation and silylation proceeded smoothly to give the type B compound **9b**. Type C compound **9c** was synthesized from **6** using essentially the same reaction procedures, in good yield. Simple application of these reaction sequences to the sterically hindered type D compound was not successful. Coupling reaction of **7** with **8** gave the desired product, although in low yield, but dihydroxylation did not occur. Fortunately, after removal of the MOM group by acid hydrolysis, the dihydroxylation reaction proceeded to give the desired product having an isobenzofuranone skeleton. Next, **9d** was prepared by disilylation, deprotection of the phenolic TBS group, and its protection with a MOM group. As *R* stereochemistry of the quaternary carbon center in isobenzofuranone derivatives **2** is important for binding to PKCα, optical resolution of **9b**–**9d** was performed by HPLC using a chiral-phase column, and both enantiomers were obtained in optically pure form. Synthesis of **2b**–**2d** was achieved through a conventional five-step sequence from intermediates **9b**–**9d**. The absolute configuration of these regioisomers was determined by comparing the optical rotation of the deoxy derivative **12**, which was prepared by Pd-catalyzed reduction of the corresponding triflates **11b**–**11d**, and that derived from the type A compound (see Supporting Information).

With the four regioisomers in hand, we next evaluated their activity. PKC binding was estimated in terms of competitive inhibition of binding of tritium-labeled phorbol dibutylate ([3H]PDBu) (Figure 3, black line).[13] Potency as a PKCα activator was estimated by comparison of the phosphorylation levels in the absence and presence of the compound (Figure 3, red line). All four compounds were found to show significant binding to PKCα at high concentration, and the affinities of **2a** and **2b** were higher than those of **2c** and **2d**. The K_i values were 122 nM (**2a**), 359 nM (**2b**), 1115 nM (**2c**), and 1259 nM (**2d**). In contrast, activation levels of PKCα induced by these compounds differed dramatically. As expected, the stronger binders **2a** and **2b** showed strong activation of PKCα in a dose-depen-

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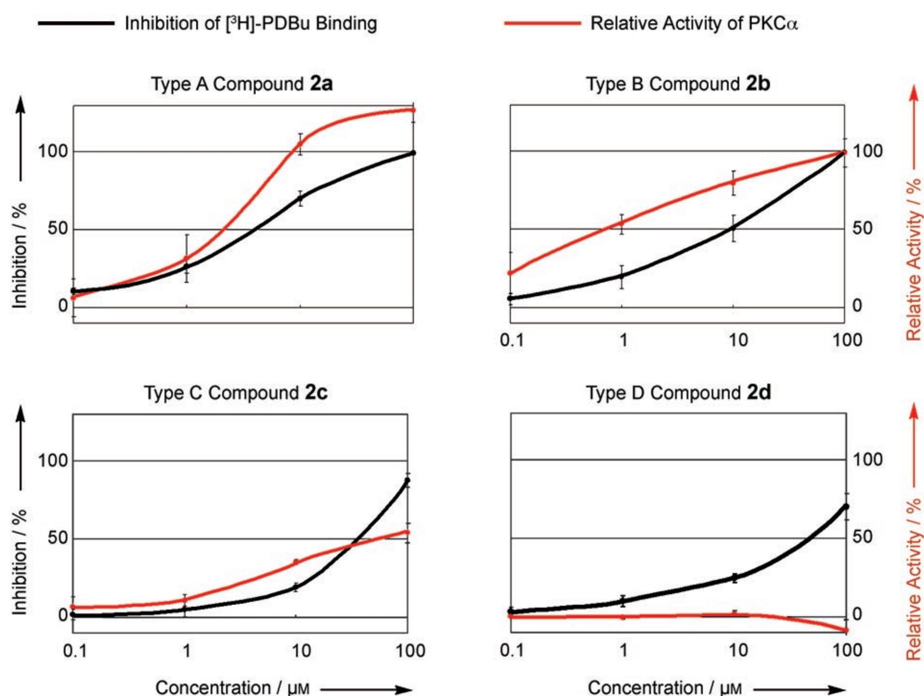

Figure 3. Evaluation of the activity of the isobenzofuranones (**2a**–**2d**). Black line: Dose-dependent inhibition of [3H]PDBu binding to PKCα. Red line: Relative activity of PKCα (100% = activity with 10 μM PMA).

stabilization of the membrane-bound active or inactive conformer. Insertion of the long alkyl chain into the phospholipid membrane would affect the C1 domain–membrane interaction (see Supporting Information). Interaction of PKCα–**2a** (or **2b**) complex with the lipid membrane might stabilize the active conformer (Figure 1, active state IV), whereas that of PKCα–**2D** may stabilize the inactive conformer (Figure 1, inactive state V).

In conclusion, the isobenzofuranone regioisomers **2a**–**2d** have been synthesized and evaluated for ability to activate PKCα. All four regioisomers showed significant binding affinity to PKCα. The type A and B compounds, **2a** and **2b**, strongly activated PKCα, whereas **2c** was a weak activator, and **2d** showed negligible activation. The observed differences in activation profile depending on the position of the hydrophobic side chain suggest that the directional interaction between the ligand and lipid membrane plays a key role in the conformational change of PKCα. As the binding affinity of **2d** to PKCα is low compared with that of a strong ligand such as PMA, the potency of **2d** as a PKCα inhibitor is low. Nevertheless, our finding indicates that it should be possible to design PKCα inhibitors based on this new concept of tuning the interaction between the C1 domain and the membrane.

dent manner. According to the results of inhibition assays of [3H]PDBu binding (Figure 3), all four ligands are expected to bind most of the PKCα molecules at a concentration of 100 μM under the activation assay conditions. There were good correlations between the expected binding and the activation profiles of **2a** and **2b**, indicating that these two compounds are potent PKCα agonists. In contrast, PKCα activation with 100 μM of **2c** was much lower, and negligible activation was observed in the presence of 100 μM **2d**, indicating that **2c** and **2d** are a partial agonist and antagonist, respectively. These observed partial agonist and antagonist activities for **2c** and **2d** are distinct from those observed for phorbol analogues having a hydrophilic side chain.[9] In the latter cases, the hydrophilic side chain may interfere with the interaction between the ligand and the lipid membrane. In contrast, all four isobenzofuranone derivatives described here have the same hydrophobic side chain—the only difference is the position of the side chain.

It is possible that substituents at different positions on the isobenzofuranone ring may greatly alter the ligand binding mode or the structure of C1B domain. To examine this possibility, binding models of **2b**–**2d** with the PKCαC1B domain were constructed using the same procedure as described for the type A compound **2a** (derivatives with a propyl group as the side chain were used for calculation). As shown in Figure 4, the isobenzofuranone part of all four regioisomers seems to bind the PKCαC1B domain in essentially the same fashion: only slight differences were observed. As expected, the directions of the alkyl chain were different from each other in the different regioisomers. These results support our hypothesis that the direction of the hydrophobic side chain is important for

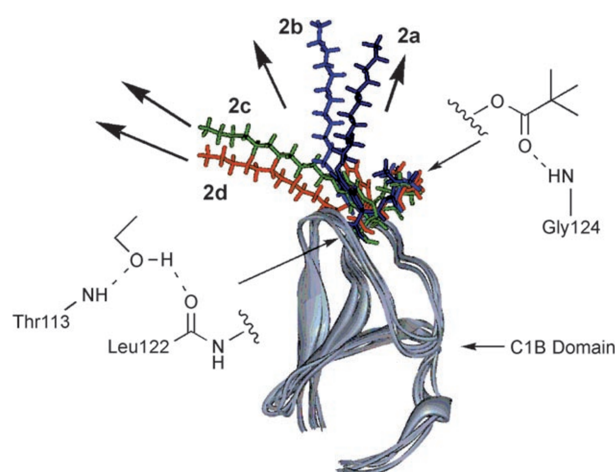

Figure 4. Superimposition of the proposed binding models of **2a**–**2d** with the PKCαC1B domain. (see Supporting Information) Although it is not possible to determine the conformation of the long hydrophobic chain located outside PKC, a possible direction of the side chain of each ligand is indicated.

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