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Evaluation of series of isobenzofuranone dimers as PKCα ligands: implication for the distance between the two ligand binding sites

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Abstract—Protein kinase C (PKC) is a family of enzymes, which play important roles in intracellular signal transduction. To examine the distance between the two ligand binding sites (C1A and C1B) of PKC, we designed and synthesized two series of isobenzofuranone dimers. Peak binding activities were observed for the C3-acyl chain dimers having a C_{10}–C_{12} linker and for the C7 dimers having a C_{14}–C_{16}.

Protein kinase C (PKC) is a family of Ser/Thr kinases involved in important intracellular signaling pathways.[1] The isozymes are divided into three classes, conventional PKCs (α, βI/βII, γ), novel PKCs (δ, ε, η, θ), and atypical PKCs (ζ, ι/λ) according to the structure of the regulatory domains. DAG (1,2-diacyl-*sn*-glycerol) is a physiological ligand for the former two classes of isozymes. Phorbol esters such as TPA are strong activators of PKCs. In the case of the conventional PKCs, the regulatory domain includes the C1 domain, which is composed of tandem ligand-binding domain C1A and C1B, and the C2 calcium-binding domain. The novel PKCs have a similar C1 domain structure, but their C2 domain does not bind calcium ion. A single ligand-binding domain, which lacks DAG binding affinity exists in the atypical PKCs. The molecular mechanisms of activation of the isozymes are of particular interest, but have not yet been fully clarified.[2] Crystal and NMR structures of the C1B domain and C2 domain have been reported,[3] but there is no structural information about the active form of the whole PKC molecule so far. This is because of the complex nature of active PKC, involving many activators, such as DAG, Ca^{2+}, and phospholipid-containing membrane. Since no large structural difference was observed between the free and phorbol 13-acetate-bound C1B domain of PKCδ, it is likely that a drastic change of the relative arrangement of the C1A and C1B domains is the key to the conformational change of PKC upon activation. Although several models of the relative C1A–C1B arrangement have been proposed on the basis of molecular modeling studies,[4] experimental studies are required to determine which model is most appropriate. Thus we planned to synthesize a series of dimeric ligands to obtain information about the inter-site distance and relative orientation of the two ligand binding sites, C1A and C1B.

As described in the previous paper, we have developed a new isobenzofuranone-type PKC ligand **1** (Fig. 1).[5] This isobenzofuranone **1** has a rigid core structure and two separate variable sites at the C3-acyl group and C7 position. In contrast, the two variable sites of phorbol ester are located at similar positions (C12 and C13). Thus, we selected isobenzofuranone as a probe ligand to examine the distance between the two ligand binding sites, even though the binding affinity of this isobenzofuranone skeleton itself is weaker than that of the phorbol skeleton (Fig. 2).

Two series of dimers (*R*,*R*)-**4** and (*R*,*R*)-**7** were synthesized from the optically active isobenzofuranone

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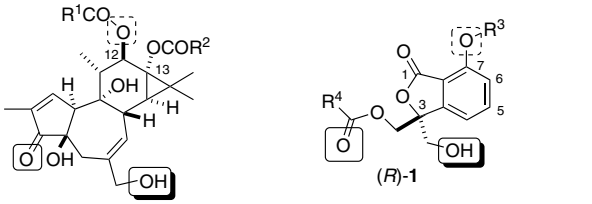


Figure 1. Structure of the phorbol esters and the isobenzofuranone ligands.

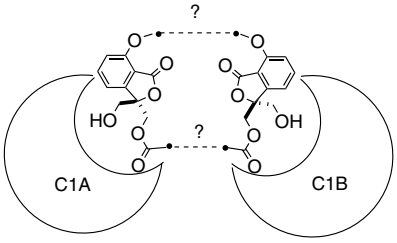


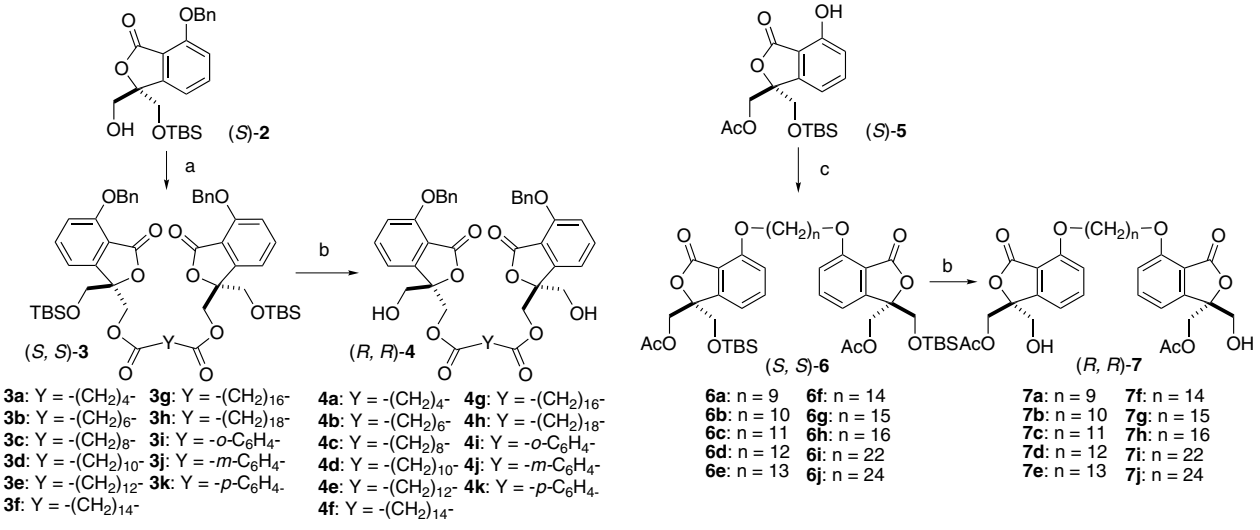
Figure 2. Distances between two different points of a dimeric ligand bound to PKC.

intermediates (*S*)-**2** and (*S*)-**5**, respectively.[5] Various C3-acyl chain dimers (*R*,*R*)-**4a**–**k** were synthesized by the reaction of (*S*)-**2** with the corresponding dicarboxylic acid chlorides followed by deprotection with HF·pyridine complex. For the preparation of a series of C7 dimers, two molecules of (*S*)-**5** were connected with various ether linkers by means of the Mitsunobu reaction, followed by deprotection to give (*R*,*R*)-**7a**–**j** (Scheme 1).[6]

Next, the binding affinity of the isobenzofuranone dimers to PKCα was examined by measurement of competitive inhibition of the binding of tritium-labeled phorbol dibutyrate ([^{3}H]PDBu) to PKCα at 100 μM.[5] Details of the assay method were reported previously.[5] As shown in Fig. 3A, the binding affinity of the dimers **4a**–**h**, in which the acyl group at the C3 position is connected with a methylene linker, exhibited a clear bell-shape relationship depending on the length of the methylene linker. Compounds **4d** and **4e** having C_{10} and C_{12} linkers bound most strongly to PKCα, being much more potent than the corresponding acetate monomer **1a**. The dimers with either longer or shorter methylene linkers showed much weaker binding. The dimers with the rigid benzene linkers, **4i**–**k**, showed negligible binding, indicating that these linkers prevent effective interaction with PKC. Since a hydrophobic side chain is known to enhance binding of a monomer ligand, presumably through interaction with the membrane, part of the positive effect of increasing the number of methylene groups may be due to this hydrophobic effect. Indeed, the monomer **1b** having C_{12} side chain indicated higher binding affinity compared with the acetate **1a**. However, the drastic decrease in the affinity of the dimers having a more hydrophobic, longer methylene linker such as **4g** and **4h** strongly suggests that the second isobenzofuranone group of the dimers **4d** and **4e** should, at least, have a positive interaction with the second ligand binding site, though it is still difficult to obtain a direct evidence of the binding to the second site.

The binding profile of the C7 methylene dimers **7a**–**j** is shown in Fig. 3B. Compounds **7f**–**h** having C_{14}–C_{16} linkers exhibited the strongest binding to PKCα. As the length of the linker is increased from C_9 to C_{15}, the binding is also increased (**7a**–**g**), and the binding strength of the C_{14}–C_{16} dimers **7f**–**h** seems to be the maximum. The dimers **7i** and **7j** with much longer C_{22} and C_{24} linkers showed much weaker binding to PKCα.

Figure 4 shows the dose-dependent inhibition curves of the monomers **1a** and **1c** and the dimers **4d** and **7g** for



Scheme 1. Synthesis of the optically active isobenzofuranone dimers. (a) ClCO-Y-COCl, NEt_3, DMAP, CH_2Cl_2 (**3a**, 84%; **3b**, 91%; **3c**, 89%; **3d**, 72%; **3e**, 88%; **3f**, 75%; **3g**, 33%; **3h**, 51%; **3i**, 62%; **3j**, 86%; **3k**, 84%); (b) HF·Py, pyridine–CH_2Cl_2 (**4a**, 47%; **4b**, 88%; **4c**, 94%; **4d**, 91%; **4e**, 82%; **4f**, 94%; **4g**, 54%; **4h**, 48%; **4i**, 90%; **4j**, 97%; **4k**, 96%; **7a**, 87%; **7b**, 84%; **7c**, 91%; **7d**, 89%; **7e**, 78%; **7f**, 45%; **7g**, 75%; **7h**, 53%; **7i**, 92%, **7j**, 56%); (c) $HO(CH_2)_nOH$, DEAD, PPh_3, THF (**6a**, 98%; **6b**, 87%; **6c**, 85%; **6d**, 82%; **6e**, 77%; **6f**, 93%; **6g**, 74%; **6h**, 85%; **6i**, 88%; **6j**, 95%).

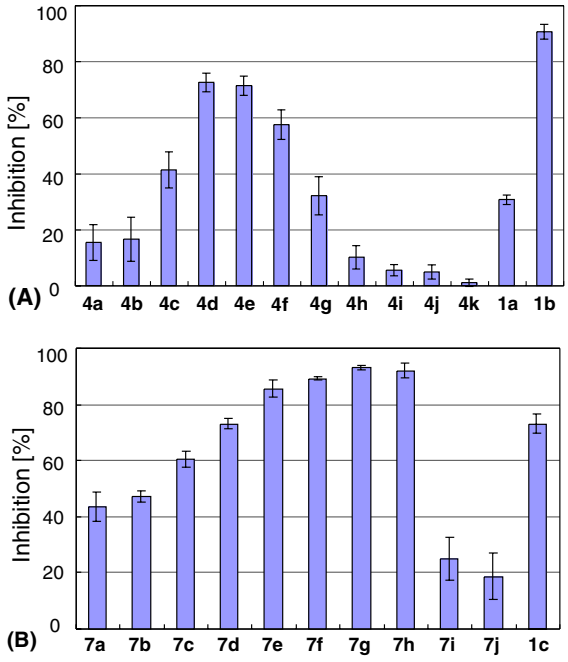


Figure 3. Inhibition of binding of [^{3}H]PDBu to PKCα by the isobenzofuranone dimers (100 μM). PKCα 4.3–4.8 nM, [^{3}H]PDBu 10 nM, $CaCl_2$ 4 mM, PS 100 μg/mL, BSA 4 mg/mL, 50 mM Tris–HCl (pH 7.5).

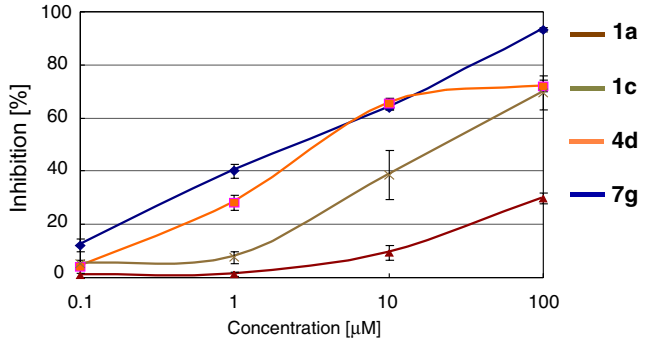


Figure 4. Dose-dependent inhibition curves of **1a**,**c**, **4d**, and **7g** for [^{3}H]PDBu binding to PKCα.

[^{3}H]PDBu binding to PKCα. The binding affinity of the dimer **4d** was about two orders of magnitude higher than that of the monomer **1a**. The dimer **7g** also binds to PKCα about 10 times more strongly than the monomer **1c**.

According to our binding model of isobenzofuranone derivatives,[5] the C7 oxygen of the isobenzofuranone would occupy a similar position to the C12 oxygen atom of phorbol in the phorbol 13-acetate-PKCδ complex. Synthesis and evaluation of several phorbol ester dimers with methylene linkers were also reported.[4b,7,8] The phorbol dimers with C_{10}–C_{14} methylene ester linkers (corresponding to C_{12}–C_{16} linkers if the carbonyl carbons are also counted) showed much stronger binding to PKC compared with the dimers with C_6 and/or C_{20} methylene linkers. These results are consistent with our findings on the isobenzofuranone derivatives.

Recently C1A and C1B domains of PKCα were reported to have different ligand preference. Binding affinity of phorbol esters with C1B is much higher than that with C1A. In contrast, DAG binds C1A more strongly than C1B.[9] Although it is difficult to know the binding affinity to C1A by the assay using [^{3}H]PDBu, the isobenzofuranone derivatives are likely to have affinity not only to C1B but also to C1A.

In conclusion, we have synthesized two series of isobenzofuranone dimers **4** and **7**, and evaluated their PKCα binding affinity. Peak binding activity was observed for the C3-acyl chain dimers **4** with C_{10}–C_{12} linkers and for the C7 dimers **7** with C_{14}–C_{16} linkers. These experimental facts should provide useful information for modeling studies of the whole PKC molecule and for the design of novel agonists or antagonists.

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6. All compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR, and mass spectroscopy. Spectral data of the selected compounds are as follows: (*R*,*R*)-**4d**: colorless oil; $[\alpha]_D^{20}$ −18.0 (*c* 0.14, $CHCl_3$); IR ($CHCl_3$) 3620, 3540, 2950, 2860, 1770, 1740, 1610, 1490, 1460, 1380, 1050, 1030 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.20 (m, 12H), 1.45 (m, 4H), 2,23 (m, 4H), 3.90 (s, 4H), 4.48 (d, *J* = 12.0 Hz, 2H), 4.50 (d, *J* = 12.0 Hz, 2H), 5.31 (s, 4H), 6.98 (d, *J* = 8.0 Hz, 2H), 7.05 (d, *J* = 8.0 Hz, 2H), 7.31 (t, *J* = 8.0 Hz, 2H), 7.38 (t, *J* = 8.0 Hz, 4H), 7.49 (d, *J* = 8.0 Hz, 4H), 7.55 (t, *J* = 8.0 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.69, 28.85, 29.01, 29.15, 33.94, 63.94, 64.40, 70.53, 85.79, 113.58, 114.25, 126.77, 128.06,

128.31, 128.69, 135.87, 136.34, 150.11, 157.75, 167.08, 173.44; FABMS (m/z) 817 [M+Na$^+$]. (*R*,*R*)-**7g**: colorless oil; $[\alpha]_D^{20}$ −40.6 (c 0.25, $CHCl_3$); IR ($CHCl_3$) 3446, 2925, 1748, 1603, 1488, 1386, 1219, 1033 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.30 (m, 18H), 1.49 (m, 4H), 1.88 (m, 4H), 2.03 (s, 6H), 2.36 (t, $J = 6.9$ Hz, 2H), 3.89 (m, 4H), 4.14 (m, 4H), 4.42 (d, $J = 11.9$ Hz, 2H), 4.51 (d, $J = 11.9$ Hz, 2H), 6.96 (d, $J = 8.3$ Hz, 2H), 7.02 (d, $J = 7.6$ Hz, 2H), 7.59 (dd, $J = 8.3$, 7.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.63, 25.78, 28.79, 29.29, 29.46, 29.52, 29.55, 29.58, 64.19, 64.46, 69.10, 85.38, 112.69, 113.55, 113.97, 136.41, 149.98, 158.45, 166.96, 170.67; FABMS (m/z) 713 [M+H$^+$].

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