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# Dramatic Switching of Protein Kinase C Agonist/Antagonist Activity by Modifying the 12-Ester Side Chain of Phorbol Esters

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Protein kinase C (PKC) is a family of core enzymes (serine/threonine kinases) in intracellular signal transduction cascades and is intimately involved in the regulation of a variety of cellular functions such as gene expression, cellular growth, and differentiation.[1] Decades of intensive studies revealed a sophisticated picture for PKC activation:[2] increase of intracellular $Ca^{2+}$ concentration and/or generation of the lipid second messenger, diacylglycerol (DAG), results in the recruitment of PKC from the cytosol, where the PKC is maintained in an inactive conformation, to the membrane, where the PKC becomes allosterically activated by interactions with DAG and phosphatidyl-L-serine (PS) in the hydrophobic environment. Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA: **1**) and phorbol 12,13-dibutyrate (PDBu: **2**), are naturally occurring extremely potent PKC agonists that strongly activate PKC (at least 3 orders of magnitude higher than DAG) through the same mechanism as that of the endogenous activator, DAG.[3] In addition, although DAG is metabolized immediately through phosphorylation or hydrolysis, phorbol esters persistently activate PKC because there is no natural mechanism to terminate their action in an animal cell. Because of this ability, phorbol esters can produce malignant cell growth and are the most potent tumor promoters known to date. In this communication, we report that the strong PKC agonist activity of phorbol esters is completely suppressed by a slight modification of the ester moiety at the 12-position, but still retains the ability to bind to PKC. Moreover, the compound demonstrated significant PKC inhibitory activity.

Our ongoing focus is to develop a PKC specific inhibitor as a potential pharmaceutical compound for anticancer therapies.[4] Generally, inhibitors that bind to the regulatory domain of PKC are thought to be more advantageous than catalytic domain binders with respect to kinase selectivity, because the catalytic domain of PKC shares many features with the catalytic sites of other kinases, such as protein kinase A (PKA).[5] The strong and selective binding affinity of phorbol esters to the regulatory domain of PKC led us to design a PKC inhibitor containing the phorbol moiety. Crystallographic[6] and molecular modeling[7] studies of phorbol ester−PKC complexes suggested that the molecular basis of action of phorbol esters is ascribed to their bifunctional nature: on one hand, with their hydrophilic part, phorbol esters bind PKC by a hydrogen bond network, mainly through oxygen atoms at the 20- and 3-positions. On the other hand, the hydrophobic ester chain at the 12-position helps to retain the phorbol ester−PKC complex in the membrane where PKC is activated. Thus, both sets of oxygen atoms at the hydrophilic part and the hydrophobic ester chain are essential for phorbol esters to activate PKC.[8] We designed a PKC inhibitor on the basis of this mechanism of action of phorbol esters. The

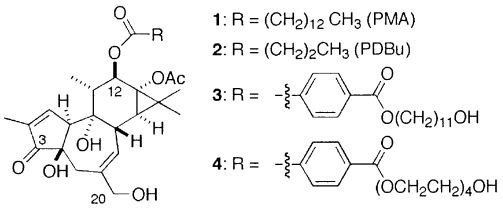


***Figure 1.*** Phorbol derivatives.

hydrophilicity of the 12-ester chain of phorbol esters would modify the stability of the phorbol ester−PKC−membrane complex, which is required for PKC activation. A hydrophilic 12-ester chain would greatly decrease the affinity of the phorbol ester−PKC complex and the membrane, while maintaining an affinity between the phorbol ester and PKC. Because PKC activation does not occur in the absence of an interaction with the membrane, a phorbol ester containing a hydrophilic ester chain would be a competitive inhibitor of PKC activation by an agonist such as PMA. Phorbol esters derived from aliphatic carboxylic acids of various carbon lengths at the 12-position (R in Figure 1) are known; however, there are no previous studies on the structure−activity relationship concerning the nature (e.g., hydrophilicity, charge, rigidity, etc.) of the 12-ester chain. Thus, we synthesized **3** and **4**, containing an alkanol and tetra(ethylene glycol) at the 12-ester chain.[9]

The PKC-binding affinity of PMA (**1**), **3**, and **4** was first assessed by competitive inhibition of the binding of tritium-labeled **2** to human recombinant PKCα in the presence of a PS vesicle as a membrane mimic (Figure 2a).[10] Although **1** and **3** had a similar strong binding affinity to PKC ($K_d \approx 0.01\ \mu M$), the affinity of **4** was ca. 2 orders of magnitude lower ($K_d > 1\ \mu M$) than that of **1** and **3**.[11] As compared with the physiologic ligand DAG, however, the binding affinity of **4** was still higher, reflecting the high binding affinity of the phorbol moiety.

We then investigated the agonist activity of **3** and **4**.[12] As shown in Figure 2b, **3** had agonist activity comparable to that of PMA ($EC_{50} < 0.01\ \mu M$). In sharp contrast, the agonist activity was completely eliminated in the glycol ester **4** ($EC_{50} \gg 10\ \mu M$). The large difference from the expected binding affinity to PKC in the agonist activity of **4** suggested that activation of PKC is a multistep process and that **4** does not promote any of these steps other than the binding to PKC. One possible explanation for the sharp contrast in agonist activity between **3** and **4** might be the difference in the affinity of the agonist−PKC complex to the membrane (Figure 3). Thus, the **4**−PKC complex might be stable in solution due to the hydrophilicity of the poly(ethylene glycol) ester chain, and thus translocation to the membrane where PKC is activated might be prevented. Results of the following partition experiments appear to support this explanation. The distribution of **3** and **4** in the water

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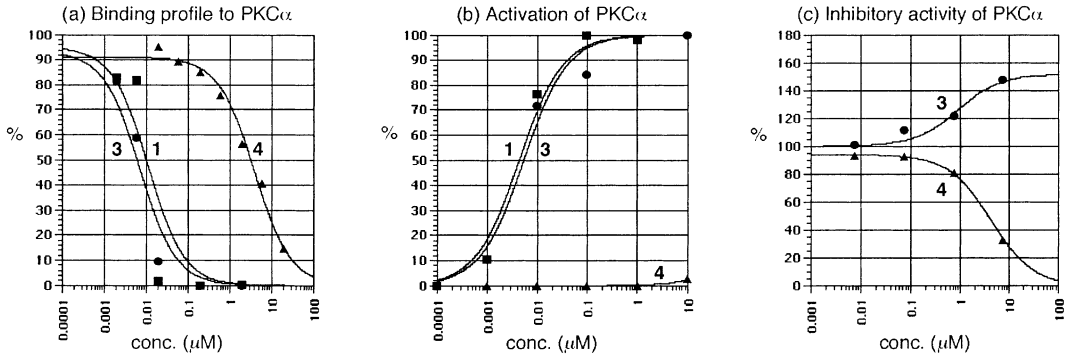


***Figure 2.*** Evaluation of new phorbol derivatives containing the 12-ester chain of different hydrophilicity. (a) The PKCα binding profile of PMA (**1**), **3**, and **4** evaluated by competitive experiments with [$^3$H]PDBu. ■, **1**; ●, **3**; ▲, **4**. (b) The activation profile of PKCα evaluated by the phosphorylation of EGF-R in the presence of [$\gamma$-$^{32}$P]ATP. ■, **1**; ●, **3**; ▲, **4**. (c) The inhibitory activity of **3** and **4** against PKCα activation by 10 nM PMA. ●, **3**; ▲, **4**.

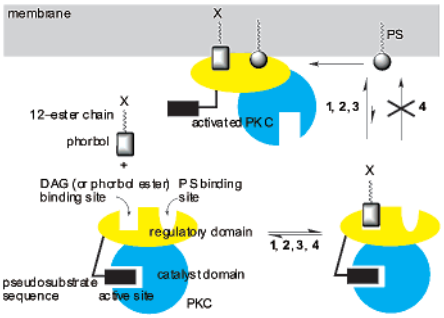


***Figure 3.*** Cartoon for PKC activation mechanism by phorbol derivatives.

layer was measured in the Hela cell/PBS buffer system using HPLC analysis (detection with UV).[13] Whereas **4** mainly existed in the water layer (water layer:cell = 11:1), **3** was efficiently concentrated in the Hela cell membrane (water layer:cell = 1:3). This contrasting distribution is probably due to the hydrophilicity difference of the 12-ester chain, and the difference might be an important factor in determining the PKC agonist activity.

As expected from the binding ability and completely eliminated agonist activity of **4**, the inhibitory assays demonstrated that **4** is a significant inhibitor of PMA activation of PKCα. PKC activity was suppressed up to 30%, depending on the inhibitor concentration (Figure 2c). In the case of **3**, however, PKCα activity increased in a concentration-dependent manner, reflecting the agonistic character of **3**. Therefore, the function of the phorbol ester derivatives could be dramatically switched from a potent agonist to a pure antagonist with no partial agonist activity by tuning the hydrophilicity of the 12-ester chain.

The described results demonstrated a possibility for a rational design of a phorbol-based PKC inhibitor by modification of the 12-ester side-chain hydrophilicity, thus affecting the membrane affinity of the inhibitor−PKC complex. Because the phorbol is easily available from commercial sources and esterification of the 12-hydroxyl group is synthetically trivial, a wide range of diverse inhibitor candidates can be synthesized using this approach. Systematically changing the nature (hydrophilicity, charge, and rigidity) of the ester moiety to develop a more potent and selective PKC inhibitor is currently under investigation.

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**Supporting Information Available:** Experimental procedures and characterization of the products (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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