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Minimal Structural Requirements for Diglyceride-Site Directed Activators of Protein Kinase C

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Abstract: The important regulatory enzyme protein kinase C is physiologically activated by the interaction of (S)-diglycerides with its regulatory domain. This interaction can be mimicked by the structurally diverse tumor promoters, which share, along with the diglycerides, the common structural feature of three hydrophilic atoms at the vertices of a triangle with sides of approximately 6 Å. It is shown in this article that molecules with the same triangular arrangement of hydrophillic atoms but with shorter sides can also activate PKC. S-Farnesylthiotriazole (FTT) is a heterocyclic molecule previously shown to specifically activate PKC. In the work reported here, structure-activity studies in the FTT series reveal that three hydrophilic atoms are required for activation, and that the minimal activation unit is close to an equilateral triangle with sides of between 2.4 - 2.7 Å. This demonstrates that there is an unanticipated flexibility at the PKC regulatory site. The intermolecular activation model based on structural analysis of the tumor promoters may represent the maximum distances allowed between the hydrophilic atoms of a PKC activator. © 1997 Elsevier Science Ltd.

INTRODUCTION

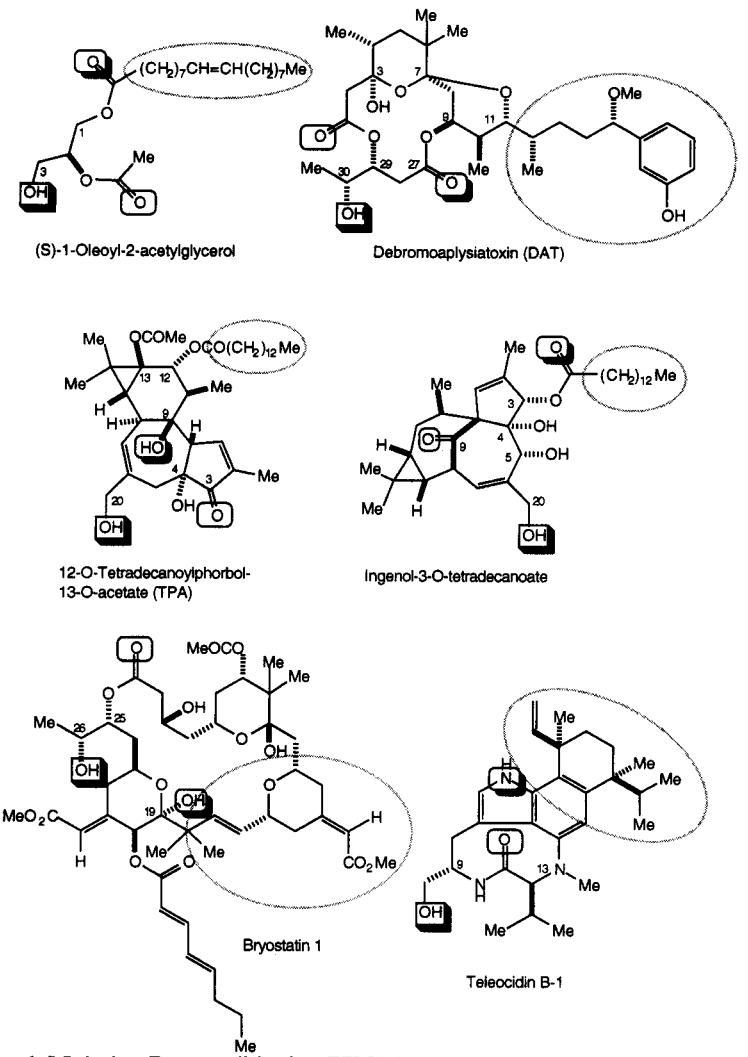
Protein Kinase C (PKC) is an important regulatory enzyme in signal transduction pathways and is found in all cell types[1]. PKC is composed of a single polypeptide chain of approximately 78 kD, and has two functional domains which can be separated by proteolytic cleavage[2]. The catalytic domain contains the ATP and substrate binding-sites, and the regulatory domain contains an effector (diglyceride) binding-site as well as binding-sites for Ca^{+2} and an acidic lipid such as phosphatidylserine. The enzyme exists as a family of isoforms which often are distributed in a tissue-specific manner[3]. PKC is normally quiescent in cells, but, in most isoforms, is temporally activated by the presence of 1,2-

diglycerides, which are themselves products of the phospholipase C mediated hydrolysis of phosphoinositides, a specific class of membrane phospholipids[4]. Activation of most isoforms of the enzyme requires the simultaneous presence of a diglyceride, phosphatidyl serine, and low concentrations (approx. 1 μM) of Ca^{+2} [3]. The diglyceride stoichiometrically binds to the regulatory domain of PKC and assists in the translocation of the enzyme from the cytoplasm to the membrane, where PKC becomes catalytically active.

The interaction of diglyceride with PKC is, as expected, highly specific, with only the (S)-diglyceride being active[5]. Small structural modifications of the natural diglycerides generally lead to molecules incapable of activating PKC[6]. PKC is also activated by a structurally diverse group of tumor promoting natural products (Scheme 1), including the phorbol esters, the aplysiatoxins, and the teleocidins[7-10]. These molecules bind to the diglyceride binding-site of the regulatory domain. Important issues to resolve include how the various PKC activators can all interact with the same binding site in PKC, what the structure of the minimal activating unit might be, and how this information could be used to design specific regulatory-site directed antagonists of PKC.

An experimental study of the structurally rigid debromoaplysiatoxins (DATs) demonstrated that tumor promoters contain a core moiety responsible for PKC activation[8-11]. The core pharmacophore, which is necessary and sufficient for PKC activation, is structurally similar to the active (S)-diglycerides (Scheme 1), which require three spatially defined hydrophilic atoms for fruitful interaction with PKC[12, 13]. Other tumor promoters function by maintaining a similar arrangement of three hydrophilic atoms in space[12, 14, 15, 16]. The mean distances measured between the hydrophilic atoms in DAT are 5.4 Å, 4.6 Å, and 5.5 Å. One can find approximate (within 1 Å) matches for these distances in all of the analyzed tumor promoters (Scheme 1)[12, 17], suggesting that PKC possesses cognate binding elements at its regulatory domain binding site. Recent studies on the X-ray derived structure of a portion of the PKC regulatory domain, co-crystallized with a phorbol ester, suggest that this binding site lies quite near the surface of the protein[18]. The hydrophobic moieties of the activator appear to protrude from the enzyme surface; this allows interaction with the membrane to help anchor PKC.

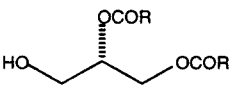
A question that arises is whether there are other structural arrangements of hydrophillic atoms through which an effector can activate PKC. The answer is yes, because we demonstrate here that the minimal activation unit for PKC is found in small, planar molecules with three heteroatoms at the vertices of a very nearly equilateral triangle with sides approximately 2.4-2.7 Å in length.



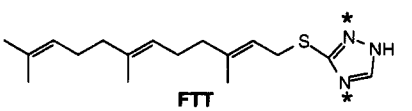
Scheme 1 Moieties Responsible for PKC Activation. For each tumor promoter, boxed atoms correspond to the spatially similar hydrophilic atoms of diglyceride and the circled region corresponds to the hydrophobic group of the molecules.

RESULTS

The Minimal Structure of an Activator: We had previously shown that the farnesylated heterocycle S-farnesyl-thiotriazole (FTT) (Scheme 2) activated PKC both *in vitro* and *in vivo* [19]. This finding revealed the first member of a new class of PKC activators which are structurally unlike any other activator class previously described. An important question to answer here is which of the heteroatoms in FTT are necessary for interactions with PKC. It had previously been deduced that three heteroatoms, arranged at set distances from one another, are essential for the activation of the enzyme by



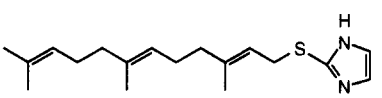
(S)-Diacylglyceride



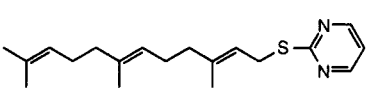
FTT

Scheme 2 Known PKC Activators

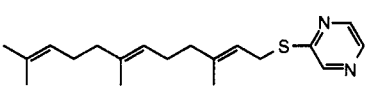
diglycerides and tumor promoters[12-16] .In the case of FTT, this would mean that the sulfur atom and two of the three nitrogen atoms, or just the three nitrogen atoms by themselves, are involved in the activation process[19]. Which nitrogen atoms are important is testable by studying the activation of PKC by the molecules shown in Scheme 3. Molecules (FTI and FTPyrm, Scheme 3) in which one nitrogen atom is deleted were studied first. Neither FTI nor FTPyrm possesses the third nitrogen atom (the NH of FTT, Scheme 2), and the study



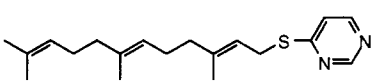
FTI



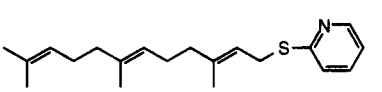
FTPyrm



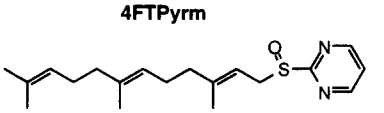
FTPyrz



4FTPyrm



FTPyr



FSOPyrm

Scheme 3 Analogs Studied to Probe the Role of the Hydrophilic Atoms of FTT.

of PKC activation by these analogs allows for a determination of the possible role of this nitrogen in PKC activation by FTT. As shown in Table 1, both of these analogs are

Table 1. Activation of PKC with Analogs

Analog	K_d (μM)	V_{max} (nmol mg^{-1} min^{-1})
S-Diolein	1.12±0.20	333±29
FTT	0.80±0.08	366±30
FTPyrm	1.71±0.20	452±22
FTPyr	inert	
FTI	0.94±0.03	479±22
FTMT	0.58±0.03	255±15
HFT	inert	
FAT	0.96±0.10	177±11
HFTT	0.35±0.03	264±10
FT	inert	

Data shown in this table are the average values of three independent assays. Assays were performed as indicated in Materials and Methods. The apparent K_d is determined kinetically.

approximately as potent and efficacious as FTT itself. These studies reveal a minimal molecular structure capable of activating PKC, and suggest that N(1) and N(3) (the starred Ns in Scheme 1), along with sulfur, are the hydrophilic atoms of FTT responsible for activation. That the three hydrophilic atoms are essential for activation can be demonstrated by determining that FTPyr and FSOPyrm are not activators of PKC, while FTPyrm is an activator (Scheme 3) (Table 1, Figure 1). In addition, the relative position of the two nitrogen atoms of FTPyrm is important for PKC activation. This can be seen by comparing the results from a series of positional isomers- FTPyrm, FTPyrz and 4FTPyrm

(Scheme 3). The potency of the agonist is reduced as the nitrogen-sulfur distance is increased, with FTPyrm (N-S distances: 2.74 Å and 2.72 Å) being almost as active as FTT

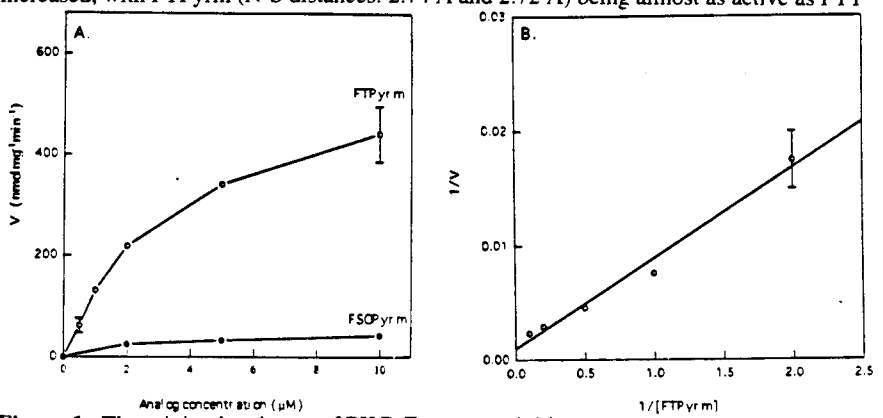
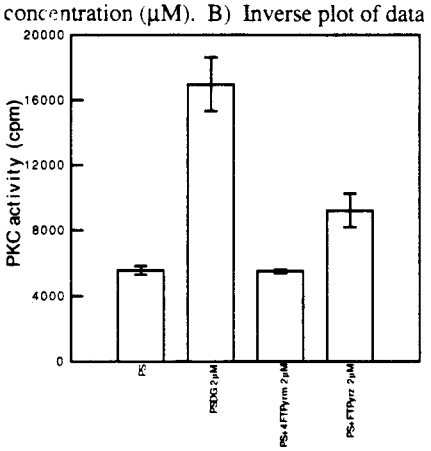


Figure 1. The minimal activator of PKC. Enzyme activities were assayed in triplicate as described in Materials and Methods. Data represent the mean±S.E.M. of three independent assays. A) PKC activity (nmol mg^{-1} min^{-1}) plotted versus FTPyrm and FSOPyrm concentration (μM). B) Inverse plot of data presented in A



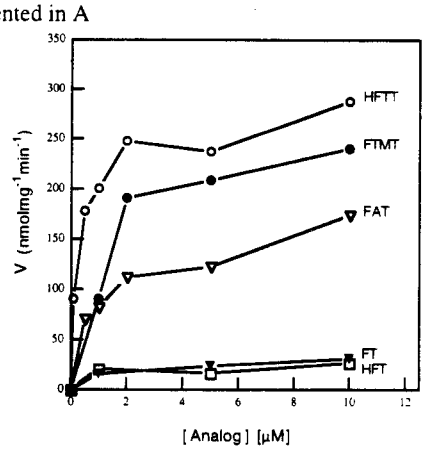
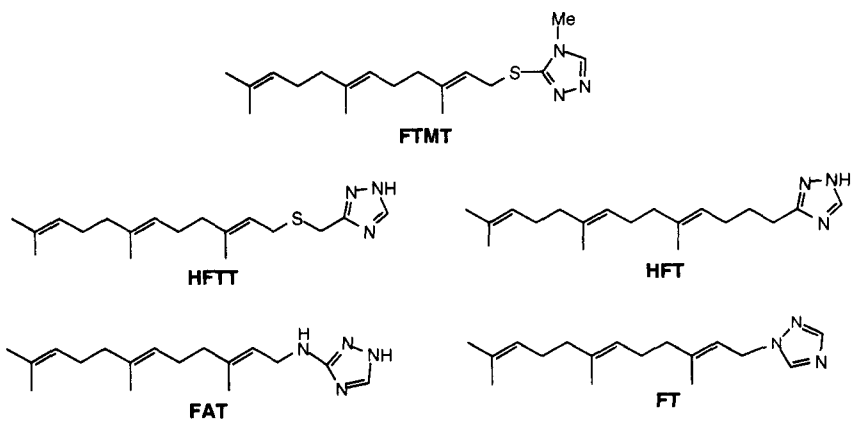


Figure 2 (left). PKC activity in the presence of FTPyrz or 4FTPyrm. Assays were performed as indicated in Materials and Methods. Data represent the mean±S.E.M. of three independent assays. All compounds were tested at a concentration of 2 μM.

Figure 3 (right). PKC activation analogs shown in Scheme 4. PKC activity was measured as described in Materials and Methods. Data represent the mean of two independent assays. Standard errors in these experiments were below 6%.

or DAG, FTPyrz (N-S distances: 4.05 Å and 2.78 Å) being a weak agonist, and 4FTPyrm (N-S distances: 4.60 Å and 2.79 Å) being inert (Figure 2). Furthermore, the observation that both FTPyrm and FTMT (Scheme 3) are agonists means that none of the hydrophilic atoms of an activator need be an H-bond donor. The requirement of a hydrophilic atom at the sulfur position for activity can be established by comparing the activity of HFTT with that of its C for S substitution analog HFT (Figure 3 and Table 1). HFT is inert as an activator of PKC, demonstrating the importance of the sulfur atom for agonist activity in this series. The fact that a sulfur atom can substitute for oxygen and nitrogen heteroatoms draws into question whether H-bonding capacity is essential for activator function at all hydrophilic atoms since a sulphur atom is not a good hydrogen bond acceptor. Along these



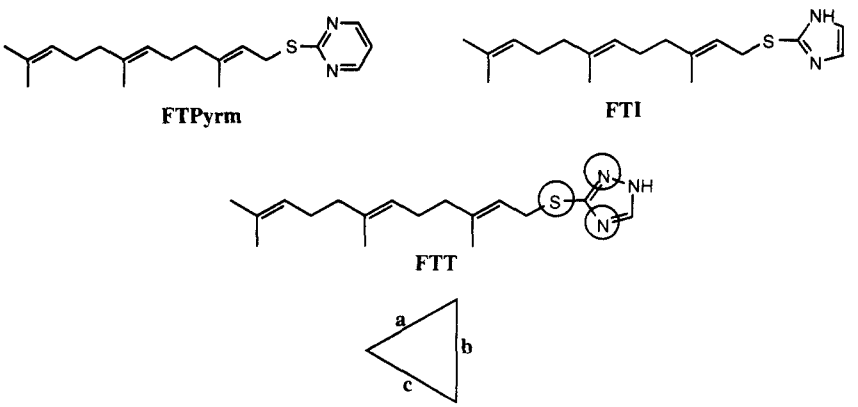
Scheme 4 Analogs Studied to Probe the Role of the Sulfur Atom.

lines, it is interesting to note that the sulfoxide (FSOPyrm, Figure 1, Scheme 3) is not an activator of PKC. This again reveals the specificity of the activation process. The sulfoxide is significantly different both sterically and electronically from FTPyrm (Scheme 3).

It is interesting to note that, the sulfur atom per se is not essential for activity in this series, because FAT (Scheme 4) is a weak agonist (Figure 3 and Table 1). Further clear specificity in the activation process is evidenced by the finding that FT is inert as a PKC activator (Figure 3 and Scheme 4). It should be noted that FT, FTPyr, 4FTPyrm and HFT, none of which is a PKC agonist, do not antagonize activation in the presence of diglyceride, when tested at a concentration of 5 μM.

DISCUSSION

The results described here unambiguously define the minimal activation unit for a PKC activator. The fact that these molecules are cyclic and planar removes ambiguities due to conformational preferences. The minimal activation unit can be deduced from the activation of PKC by FTT, FTI, and FTPyrm, as shown in Scheme 5. In these molecules, the three hydrophilic atoms, which are the sine qua non for activation,



Scheme 5 Minimal Activators of PKC. The circled atoms of FTT indicate those critical for activation. The triangle indicates minimal distances between essential atoms. The mean distances between the vertices are as follows: (a) 2.74 ± 0.04 Å, (b) 2.28 ± 0.09 Å, and (c) 2.81 ± 0.03 Å.

are approximately at the vertices of equilateral triangles. The distances between the atoms are substantially shorter than those measured for DAT, in which the mean distances measured between the hydrophilic atoms are 5.4 Å, 4.6 Å, and 5.5 Å respectively[12, 13]. In fact, using the published general pharmacophore model[12, 13] as a guide, average distances between the hydrophilic triads of the various tumor promoters are 6.00 ± 0.03 Å , 5.70 ± 0.60 Å , and 6.40 ± 0.60 Å, respectively [17]. Moreover, a recent X-Ray structural study on the binding of a phorbol ester to the PKC regulatory domain definitely shows that the 3-keto and 20-methylene hydroxyl groups of the phorbol ester are involved in hydrogen bonding to the peptide backbone of PKC [18]. A weak hydrogen bond may also occur between the 4-hydroxyl group of the phorbol ester and protein [18]. However, this interaction cannot be essential, because 4-deoxyphorbol esters are active as tumor promoters [20]. The distance between the 3-keto and 20-methylene hydroxyl groups of the

phorbol ester is anywhere between 4.88 and 6.40 Å, depending on the conformation of the methylene hydroxyl group[17]. Clearly, where distance measurements between important hydrophilic moieties have been made on tumor promoters, the distances in question are substantially longer than is evident in the molecules described here. It would be tempting to suggest that there are only two classes of activator binding sites; those with distances defined by the tumor promoters, and those with the minimal distances described here. This appears not to be the case, however, since HFTT is quite active as a PKC activator (Figure 3 and Table 1). In this case the approximate distances between the hydrophilic moieties in the energy minimized structures are 3.4 Å, 3.6 Å, and 2.2 Å, respectively.

The results described here demonstrate that there must be flexibility at the PKC regulatory effector binding-site, which allows for the different classes of activator molecules to function. How is this to be understood, given the relatively strict spatial requirements found in the case of the tumor promoters? It is reasonable to assume that the distances between hydrophilic atoms derived from the tumor promoters actually define the maximal distances allowed between these atoms, rather than the exact distances. The scaffolding of the structurally diverse tumor promoters are such that the hydrophilic atoms are not close to each other. Therefore, studying the tumor promoters does not allow one to sample structures in which the three essential hydrophilic atoms are as close to one another as in these planar, heterocyclic activator molecules. It is also worth remembering that important conclusions derived from studies on the tumor promoters are in evidence in the studies described here. The idea that three hydrophilic atoms are placed at the vertices of an approximate equilateral triangle is still of central importance.

EXPERIMENTAL SECTION

Assay of PKC Activity

The PKC activity assay was based on a published procedure[21], which measures the incorporation of ^{32}P from [γ-^{32}P]ATP into lysine rich histone type III-SS (Sigma). The typical reaction mixture contained 200 µL total volume of 20 mM Tris-HCl, pH 7.5, buffered with 2.5 mM EDTA, 5 mM $Mg(OAc)_2$, 200 µM $CaCl_2$ (free concentration of Ca^{+2} ~ 10 µM after buffering), 800 µg/mL histone, 8.5 µg of phosphatidylserine in the presence or in the absence of lipids (as described below), 10 µU of purified rat brain PKC (Boehringer Mannheim), 10 µM ATP and 10 nM [γ-^{32}P]ATP (1 µCi/mL). Assays were performed at 30 °C for 10 min. The reaction was stopped by the addition of 10% trichloroacetic acid, followed by collection of the acid-precipitable materials on a Millipore HA type filter, and counted in Filtron-X scintillation fluid on a Beckman LS-330 scintillation counter.

The lipid mixtures were prepared as follows: phosphatidylserine (PS), diacylglycerol ((S)-diolein) (Sigma), or FTT analogs in chloroform were first mixed and

the solvent was evaporated under a stream of N_2 for 30 min, then dried *in vacuo*. The vesicles were formed by the addition of 20 mM Tris-HCl pH 7.5 containing 2.5 mM EDTA to the lipids. The mixture was then sonicated for 2 min on ice.

The syntheses of all analogs are provided in the Supplemental Section.

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Abbreviations Used: DAG: diacylglyceride; FAT: 3-Farnesylamino-1,2,4-triazole; FT: 1-Farnesyl-1,2,4-triazole; FTI: 2-Farnesylthioimidazole ; FTMT: 3-Farnesylthio-4-methyl-1,2,4-triazole FTPyr: 2-Farnesylthiopyridine ; FTPyrm: 2-Farnesylthiopyrimidine ; FTPyrz: 2-Farnesylthiopyradizine ; FSOPyrm: Farnesyl, pyrimidine-sulfoxide ; 4FTPyrm: 4-Farnesylthiopyrimidine; HFT : 3-Homofarnesyl-1,2,4-triazole ; HFTT : 3-Homothiofarnesyl-1,2,4-triazole ; PKC: Protein kinase C; PMA: 4β-phorbol 12-myristate 13-acetate; Me_2SO: dimethylsulfoxide

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