Farnesyl Thiotriazole, A Potent Neutrophil Agonist and Structurally Novel Activator of Protein Kinase C†

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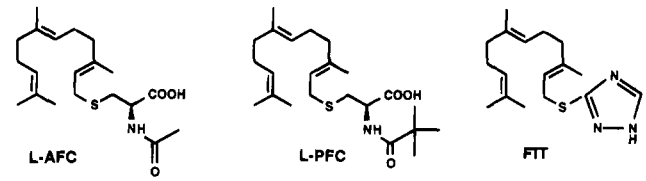
ABSTRACT: Farnesylcysteine derivatives can initiate or inhibit superoxide (O_2^-) release in neutrophils. The mechanism by which one of these derivatives, farnesyl thiotriazole (FTT), initiates O_2^- release in neutrophils is the subject of this paper. Treatment of guinea pig neutrophils with FTT results in the rapid release of O_2^- by a route shown to be independent of the chemotactic peptide *N*-formyl-Met-Leu-Phe (fMLP) receptor. The signal transduction pathway utilized by the chemoattractant fMLP is generally accepted as the paradigm for receptor-mediated stimulation of O_2^- production. Antagonists of fMLP had no effect on FTT-induced O_2^- release, and pretreatment of neutrophils with fMLP had no effect on the ability of FTT to trigger further O_2^- generation. In fact, FTT behaves like a typical protein kinase C (PKC) activator. It promotes phosphorylation of the 47-kDa subunit of the NADH oxidase complex (p47-phox) in neutrophils, and this phosphorylation is specifically blocked by 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), an antagonist of PKC. FTT is also shown to activate PKC *in vitro* in a specific and saturable fashion. FTT is approximately equipotent with (*S*)-diolein, a physiologically relevant activator of this kinase. FTT represents a new, and quite novel, structure for a PKC activator. PKC activators include diglycerides and the structurally diverse tumor promoters.

Farnesylcysteine (FC)[1] derivatives have profound effects on signal transduction events in a variety of cells. For example, AFC (Scheme 1) blocks all of the following processes: fMLP-induced O_2^- release from neutrophils (Philips et al., 1993; Ding et al., 1994), platelet aggregation induced by prostanoids or thrombin (Akbar et al., 1993; Ma et al., 1994), macrophage chemotaxis (Volker et al., 1991), and insulin release from pancreatic cells (Metz et al., 1993).

While FC analogs were originally designed as substrates and/or inhibitors of the isoprenylated protein methyltransferase (Tan et al., 1991a,b; Gilbert et al., 1992; Pérez-Sala et al., 1992; Shi & Rando, 1992), recent studies have indicated that this enzyme cannot be the only pharmacological target for these molecules. For example, FC analogs having bulky moieties attached to the amino group, such as PFC (Scheme 1), are neither substrates nor inhibitors of the methyltransferase, yet they can possess the same cellular activities as FC analogs which are methyltransferase substrates or inhibitors (Ding et al., 1994; Ma et al., 1994). Moreover, a recent report has shown that L-AFC can also inhibit receptor-mediated activation of G-proteins in isolated membranes by a mechanism independent of protein carboxymethylation (Sheer & Gierschik, 1993). The nature of the molecular receptors for FC analogs is of substantial interest because these receptors may represent new pharmacological targets.

Scheme 1: Farnesylcysteine Analogs



In studies on the stimulation of O_2^- release in neutrophils by the chemoattractant fMLP, two modes of FC action were noted (Ding et al., 1994). One class of molecules, illustrated by AFC, blocked the release of O_2^- initiated by fMLP (Ding et al., 1994). A second class of molecules, illustrated by PFC, initiated O_2^- formation in the absence of fMLP (Ding et al., 1994). The pattern of O_2^- release with the agonist PFC was very similar to that observed for fMLP (Ding et al., 1994). This stimulation process proved to undergo homologous desensitization with PFC and heterologous desensitization with fMLP (Ding et al., 1994), suggesting a molecular target close to the receptor [cf. Ali et al. (1993) and Lefkowitz (1993)]. The fMLP binding site on the chemotactic receptor is not the target for PFC (Ding et al., 1994).

Farnesyl thiotriazole (FTT) (Scheme 1) also stimulated neutrophils to release O_2^- formation (Ding et al., 1994), but it is shown here that this response is fundamentally different from that observed with PFC. This third class of molecules, illustrated by FTT, does not exhibit homologous or heter-

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[1] Abbreviations: Ac-MBP(4-14), acetylated peptide corresponding to residues 4-14 of myelin basic protein; AFC, *N*-acetyl-*S*-farnesyl-L-cysteine; DAG, diacylglyceride; FC, *S*-farnesyl-L-cysteine, FTT, *S*-(farnesyl-3-thio)-1*H*,1,2,4-triazole; PFC, *N*-pivaloyl-*S*-farnesyl-L-cysteine; PKC, protein kinase C; O_2^-, superoxide; PMA, 4β-phorbol 12-myristate 13-acetate; fMLP, *N*-formyl-Met-Leu-Phe; BocPLPLP: *N*-*t*-boc-Phe-D-Leu-Phe-D-Leu-Phe; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; HA1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide; Me_2SO, dimethyl sulfoxide.

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ologous desensitization. This suggests that the molecular target for FTT is at a distal part of the transduction cascade. The nature of this class of farnesylcysteine receptors is the subject of this paper. It is shown here that FTT is a potent activator of protein kinase C *in vivo* and *in vitro* and that this activation process completely explains how FTT stimulates O_2^- release in neutrophils. Moreover, FTT defines a novel class of PKC activators which could be of substantial pharmacological importance.

EXPERIMENTAL PROCEDURES

Materials

FTT was synthesized as described in Ding et al. (1994). Bovine phosphatidylserine (PS), egg phosphatidylcholine (Type XI-E, PC), and 1,2-dioleoyl-*sn*-glycerol [(*S*)-diolein] were obtained from Sigma Chemical Co. [γ-^{32}P]ATP of specific activity ~6000Ci/mmol (1 Ci = 37 GBq) was from Dupont. ATP was purchased from Boehringer Mannheim. Protein kinase Cα from rabbit brain, Ac-MBP(4-14) substrate peptide, and phosphocellulose disks were products of Gibco–BRL. Filtron X was from National Diagnostics Co, Atlanta, GA. Wortmannin was purchased from Kamiya Biomedical Co., Thousand Oaks, CA. 1-Butanol was obtained from Fisher Scientific Inc. Sources of all other materials are described elsewhere (Robinson et al., 1985; Heyworth et al., 1989).

Methods

Preparation of Neutrophils. Guinea pig peritoneal neutrophils were prepared as described previously (Badwey & Karnovsky, 1986). These cell preparations contained ≥90% neutrophils with viabilities always ≥90%.

Superoxide Release. Superoxide release was measured as described earlier (Robinson et al., 1985). Cells [(1.0–2.0) × 10^6/mL] were incubated in the reaction mixture at 37 °C with or without the inhibitor for 3 or 5 min before O_2^- release was initiated by the addition of the stimulus.

Stock solutions of FTT (20 mM), PMA (2.0 mg/mL) fMLP (10 mM), wortmannin (4.0 mM), and BocPLPLP (20 mM) were prepared in Me_2SO and stored at −20 °C. These compounds were diluted with Me_2SO so that the final amount of solvent in the assays was 0.25% or 0.50% (*v*/*v*) in all cases (this includes the 0.25% added with the stimulus). These amounts of solvent did not cause any of the effects noted. H-7 (10 mM) and HA1004 (10 mM) were dissolved in H_2O and stored in the dark at 4 °C.

Labeling of Neutrophils with $^{32}P_i$ and SDS–Polyacrylamide Gel Electrophoresis for Studies on Protein Phosphorylation. These techniques were performed as described previously (Heyworth et al., 1989), except the amount of radioactivity was increased to 1.0 mCi of $^{32}P_i$/mL.

Assay for PKC. PKC was assayed as previously described (Boni & Rando, 1985), except that Ac-MBP(4-14) was used as the substrate (Ysuda et al., 1990). The method for assaying for PKC activity is based on measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into Ac-MBP(4-14) peptide (Ysuda, et al., 1990).

RESULTS

Effects of FTT on Neutrophils. Progress curves for O_2^- release from neutrophils stimulated with FTT (10 μM) or fMLP (1.0 μM) are presented in Figure 1. Cells treated with FTT release O_2^- at a linear rate that persists for time periods > 10 min (curve a). Neutrophils stimulated with the tumor promoter PMA also release O_2^- for similar periods of time [e.g., Robinson et al. (1987)]. In contrast, cells stimulated with fMLP exhibit a transient burst of O_2^-, with the majority of the O_2^- being released within the first 1–3 min (*curve b*) [e.g., Okamura et al. (1989)]. Neutrophils treated with fMLP do not respond to the addition of a second batch of fMLP (homologous desensitization) (curve b, broken arrow) but can be restimulated by the addition of PMA (curve e) (Ali et al., 1993; Wilde et al., 1989) or FTT (curve d). The tumor promoter PMA bypasses surface receptors and heterotrimeric G-proteins and directly activates PKC (Castagna et al., 1982). The rates of O_2^- release from neutrophils stimulated with optimal amounts of FTT (10 μM; Figure 1 inset) and fMLP were 74 ± 16 (SD, n = 5) and 46 ± 9 nmol of O_2^- min^{-1} 10^7 $cells,^{-1}$ respectively. The rate observed with FTT was superior to that observed with 50 nM PMA [53 ± 5 nmol of O_2^- min^{-1} $(10^7$ $cells)^{-1}$] (Badwey et al., 1989), the most potent stimulus in general use. It is noteworthy that the FC analog PFC is similar to fMLP (and different from FTT) in that it stimulates transient release of O_2^- from neutrophils and cannot restimulate cells previously treated with fMLP (Ding et al., 1994).

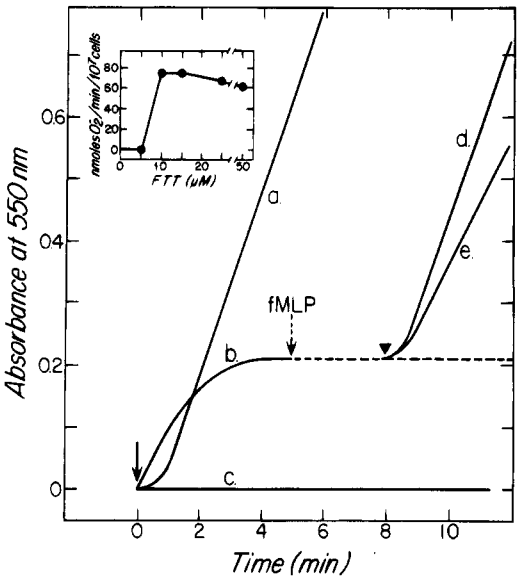


FIGURE 1: Stimulation of superoxide release by FTT. Reaction progress curves demonstrate O_2^- release by guinea pig neutrophils (10^6/mL) stimulated with 10 μM FTT (curve a) or 1.0 μM fMLP (curve b), which were added last to initiate the reactions (solid arrow). Curve c is for unstimulated cells. Addition of 1.0 μM fMLP (broken arrow, curve b) to cells previously treated with this agonist did not result in the restimulation of O_2^- release. In contrast, the addition (arrowhead) of either 10 μM FTT (curve d) or 50 nM PMA (curve e) to these assay mixtures resulted in normal rates of O_2^- release. The *inset* shows the dose–response curve for FTT in stimulating O_2^- release.

Neutrophils exhibit rapid incorporation of $^{32}P_i$ into a 47-kDa protein upon stimulation with PMA (Figure 2A, lane b) or FMLP (Heyworth et al., 1989; Okamura et al., 1988). This 47-kDa protein (p47-phox) is a subunit of the NADPH–oxidase complex and a substrate for PKC [e.g., Heyworth and Badwey (1990)]. Cells stimulated with 10 μM FTT also exhibitied marked phosphorylation of the 47-kDa protein (Figure 2A, lane c). The amount of phosphorylation of this protein with 10 μM FTT was estimated by densitometry

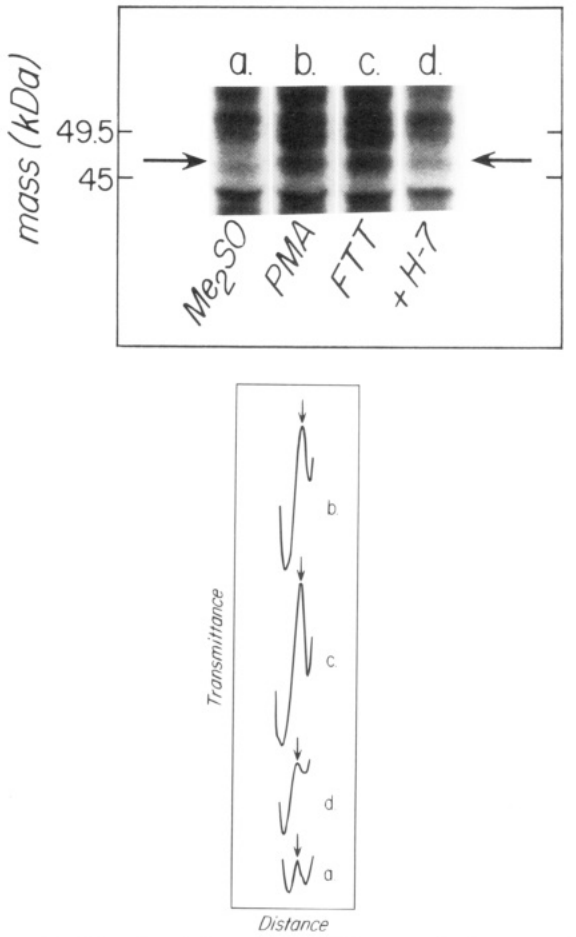


FIGURE 2: Phosphorylation of the 47-kDa protein in neutrophils during cell stimulation. The top panel compares portions of autoradiograms from ^{32}P-labeled neutrophils (1.5×10^6/mL) treated with (*a*) 0.25% (*v*/*v*) Me_2SO for 1 min (unstimulated cells), (*b*) 50 nM PMA for 1 min, (*c*) 10 μM FTT for 1 min, and (*d*) 200 μM H-7 for 3 min followed by 10 μM FTT for 1 min. The reaction times represent periods at which the cells exhibited maximal rates of O_2^- release under the conditions specified (e.g., Figure 1). The 47-kDa protein is indicated by the arrow. The bottom panel presents the densitometric scans of the 47-kDa protein under the various conditions in the top panel. This pattern was observed in three separate experiments performed on different preparations of cells.

(Figure 2B) and found to be 98% ± 20% (SD, $n = 3$) of that observed with PMA. Cells stimulated with FTT also exhibited an increased incorporation of $^{32}P_i$ into several uncharacterized proteins (lane c). It is noteworthy that all of these bands also responded to PMA (lane b), an observation consistent with a common target for both FTT and PMA. Along these lines H-7, a known PKC antagonist (see below), blocked phosphorylation induced by FTT (Figure 2A, lane d).

A variety of inhibitors were employed to identify components of the stimulatory pathway utilized by FTT (Table 1). Superoxide release inhibited by FTT was not appreciably affected by a specific antagonist of the fMLP-binding site on the chemotactic receptor (BocPLPLP) (Naccache et al., 1979), by an inhibitor of phosphatidylinositol 3-kinase (wortmannin) [e.g., Okada et al. (1994)], or by a substrate for phospholipase D (1-butanol) [e.g., Bonser et al. (1989)]. 1-Butanol can also directly block the activation of PKC by diacylglycerol but not activation by PMA (Slater et al,. 1994). All of these inhibitors were effective when fMLP was the stimulus [e.g., Naccache et al. (1979), Okada et al. (1994), Bonser et al. (1989), and Slater et al. (1994); Table 1). By contrast, H-7 substantially blocked O_2^- release in neutrophils stimulated with FTT, whereas HA1004 had little effect. The drug H-7 inhibits both PKC and the cyclic nucleotide-dependent protein kinases with similar affinity (Hidaka et al., 1984). HA1004 inhibits the latter kinases as effectively as H-7 but has much less activity against PKC and is thus useful as a control for implicating PKC in certain cellular phenomena (Hidaka et al., 1984). H-7 also significantly reduced the phosphorylation of the 47-kDa protein in FTT-stimulated neutrophils (Figure 2, lane d). The amount of phosphorylation of this 47-kDa protein with H-7 was estimated by densitometry and found to be inhibited by 58% ± 13% (SD, $n = 3$). The sensitivity of O_2^- release from FTT-stimulated neutrophils to the inhibitors listed in Table 1 was very similar to that observed with PMA but strikingly different from that for fMLP.

Table 1: Effects of Inhibitors on Superoxide Release by Neutrophils[a]

	% inhibition[b]		
		stimulus[a]	
inhibitor	FTT (10 μM)	PMA (50 nM)	fMLP (1.0 μM)
BocPLPLP (25 μM)	11 ± 8	nd	nd
BOCPLPLP (10 μM)	6 ± 4	nd	87 ± 3
wortmannin (1.0 μM)	4 ± 6	8 ± 8	84 ± 9
1-butanol (55 mM)	3 ± 3 ($n = 2$)	26 ± 7	70 ± 5
H-7 (200 μM)	74 ± 10	86 ± 8	86 ± 3
HA1004 (200 μM)	0 ± 0 ($n = 2$)	11 ± 8	8 ± 4

[a] Neutrophils (10^6/mL) were incubated with the inhibitors in the standard assay mixture for 3 min (BocPLPLP, H-7, HA1004) or 5 min (1-butanol, wortmannin) prior to stimulation with the agonists. The concentrations of inhibitors employed did not affect cell viability as measured by the exclusion of trypan blue, nor did they affect O_2^- release in an O_2^--generating system (i.e., xanthine oxidase plus purine). [b] Data are expressed as the mean % inhibition ± SD for 3−10 different preparations of cells, except where indicated otherwise. nd, not determined. The data for PMA with H-7 and HA1004 are from Badwey et al. (1989).

Isoprenoids other than FTT (e.g., retinal) have previously been reported to stimulate neutrophils (Robinson et al., 1987; Badwey et al., 1989). However, cells stimulated with those agents are largely insensitive to H-7 and do not exhibit phosphorylation of the 47-kDa protein (Badwey et al., 1989). Finally, neither *all-trans*-farnesylthio-2-pyridine (10−50 μM), *all-trans*-farnesol (10 μM), thiotriazole (10 μM), nor these latter two agents in combination was capable of initiating O_2^- release from neutrophils (data not shown), further suggesting a specific mode of action for FTT.

Activation of PKC by FTT. The experiments described above are consistent with a mechanism in which FTT functions by activating PKC *in vivo*. To demonstrate this directly, FTT was studied as an activator of pure PKC *in vitro*. In these experiments, the activation of PKC by FTT was compared to the activation of the enzyme by (*S*)-diolein, a natural regulator of PKC (Nishizuka, 1992; Boni & Rando, 1985). Results of these experiments are shown in Figure 3. Curves showing counts per minute incorporated into PKC substrate as a function of DAG and FTT concentrations are shown in Figure 3 (top panel). Inverse plots of these data are shown in Figure 3 (bottom two panels). FTT shows saturable activation of PKC, just like other specific PKC agonists. The measured K_D and V_{max} values for FTT are 2.5 ± 0.1 μM and 296 ± 11.84 nmol mg^{-1} min^{-1}, and for

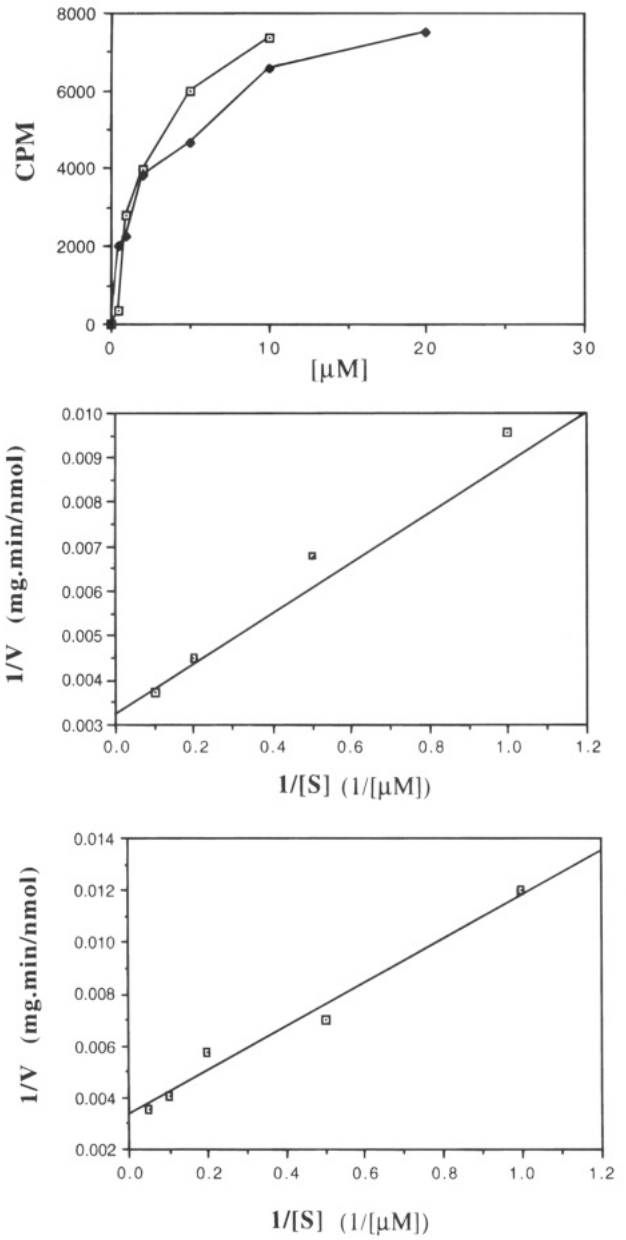
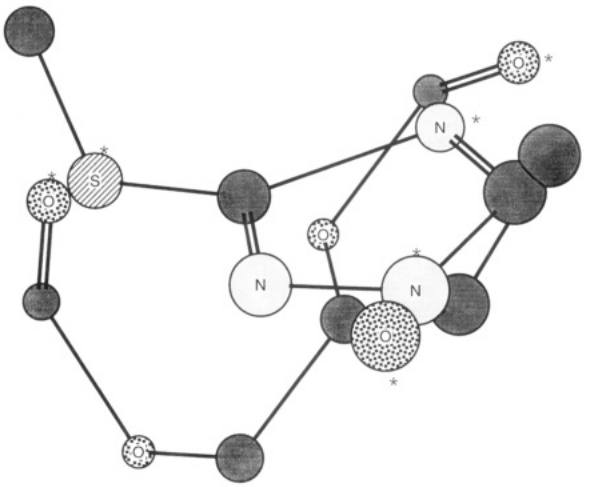


FIGURE 3: Activation of PKC by FTT *in vitro*. Pure PKCα was used as the enzyme and acetylated myelin basic protein (AcMBP) (Yasuda et al., 1990) was used as the substrate. Reactions were run in 50 μL total volume of 20 mM Tris-HCl at pH = 7.4 containing 5 mM $MgCl_2$, 50 μM $CaCl_2$, 1 mM EDTA, 50 μM AcMBP, 0.005 μg of PKCα, 8 μg of egg lecithin (PC), 2 μg of phosphatidylserine (PS), and 18 μM ATP plus 84 nM [γ-^{32}P]ATP. Assays were performed at 30 °C as previously published (Boni & Rando, 1985). Increasing concentrations of either DAG [(*S*)-diolein] or FTT at the indicated concentrations were added to the PC/PS mixtures before sonication (Boni & Rando, 1985). After incubation for 10 min at 30 °C, the reaction was stopped by spotting a 25-μL aliquot of the reaction mixture onto a phosphocellulose disk, which was immediately placed in 1% H_3PO_4. The phosphocellulose disks were washed three times for 5 min with the same acid solution and washed two times for 5 min with distilled water, followed by counting the ^{32}P in Filtron X scintillation fluid on a Beckman LS 330 scintillation counter. Counts per minute incorporated into substrate as a function of activator concentration are shown in the upper panel and inverse plots for the two analogs are shown in the lower two panels. The middle panel is for DAG and the lower one is for FTT.

DAG [(*S*)-diolein] they are 2.02 ± 0.18 μM and 310 ± 27.9 nmol mg^{-1} min^{-1}. Therefore, FTT and (*S*)-diolein are remarkably similar in their abilities to act as activators of PKC. These data demonstrate that FTT is a potent activator of PKC.

Chart 1: Overlay of FTT and the Deduced Diglyceride Conformer Which Binds to PKC



DISCUSSION

In this article it is shown that FTT stimulates O_2^- production in neutrophils by a mechanism identical to that observed with PMA but quite different from that reported for PFC. In the latter case, the mechanism of stimulation involves a signal transduction pathway similar to that observed with fMLP (Ding et al., 1994). The situation found with FTT is quite different. For example, both FTT and PMA chronically stimulate O_2^- release (Figure 1, curve a), whereas fMLP and PFC stimulate transient generation of O_2^- (Ding et al., 1994). Furthermore, after stimulation of neutrophils with fMLP, further stimulation with fMLP or PFC does not occur (Ding et al., 1994). By contrast, pretreatment of neutrophils with fMLP had no effect on the abilities of either PMA or FTT to trigger further O_2^- generation (Figure 1, curves d and e).

A linkage between FTT and PKC activation was further suggested by the demonstration that FTT-stimulated cells exhibited phosphorylation of p47-phox and that this phosphorylation was blocked by H-7, a PKC antagonist (Figure 2). Along these lines, studies with other antagonists showed that only H-7 blocked O_2^- release induced with FTT. By contrast, wortmannin and 1-butanol were potent antagonists when fMLP was used as the stimulating agent (Table 1). These experiments strongly suggest that PKC is a target for FTT. This possibility was demonstrated directly in a quantitative way by showing that pure PKC is activated in a saturable and specific way by FTT. In fact, the apparent K_D for FTT was close to that of (*S*)-diolein (Figure 3), a natural regulator of PKC (Nishizuka, 1992; Boni & Rando, 1985).

As noted above, diglycerides are the physiologically relevant activators of PKC (Nishizuka, 1992). While the structures of FTT and diolein seem unrelated, they are in fact not unrelated with respect to PKC activation. PKC is activated not only by diglycerides but also by the structurally diverse tumor promoters (Castagna et al., 1982). A successful pharmacophore model has been developed in which structurally similar activating moieties can be identified in the various PKC activators (Rando & Kishi, 1992a,b).

Basically, this model involves the apposition in space of three crucial H-bonding atoms in all of the PKC activators. From this analysis, the active conformation of diglyceride could be used as a template for the discovery of novel PKC activators. When this template is used as an overlay with FTT using CS Chem 3D, the structures shown in Chart 1 are generated. Two nitrogen atoms and the sulfur atom of FTT can effectively overlay the three critical oxygen atoms (all highlighted by asterisks) of the deduced active conformation of diglyceride (the alkyl moieties of the diglyceride have been left out for ease of exposition) (Rando & Kishi, 1992a,b). Thus, the PKC-activating activity of FTT is readily understood using the described pharmacophore model for PKC activation (Rando & Kishi, 1992a,b). The discovery of a new class of PKC agonists, which are presumably nonmetabolizable, is of interest. The design of specific, regulatory-site-directed PKC antagonists based on these molecules would be of great importance.

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