α -Tocopheryl Succinate Activates Protein Kinase C in Cellular and Cell-Free Systems

Kentaro KOGURE¹, Susumu HAMA¹, Satoru GOTO¹, Tatsuo MUNAKATA², Akira TOKUMURA¹ and Kenji FUKUZAWA^{1,*}

¹Faculty of Pharmaceutical Sciences, The University of Tokushima, Shomachi-1, Tokushima 770–8505, Japan

²Department of Industrial Chemistry, Faculty of Engineering, Tohwa University, 1–1–1 Chikushigaoka, Minami-ku, Fukuoka 815–8510, Japan

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Summary The effect of α -tocopheryl succinate (TS) on protein kinase C (PKC) activity was examined. TS increased the auto-phosphorylation of PKC in vascular smooth muscle cells. Furthermore TS activated isolated PKC-like phorbol 12-myristate 13-acetate (PMA), although it was required at a significantly higher concentration than PMA for PKC activation. Molecular superimposition of the TS on PMA by computation suggested that TS took an active binding conformation to the PKC-like PMA, but that the conformational population was about 1/1,000. Consequently, we conclude that TS interacts directly with PKC, and activates it by taking an active conformation like PMA.

Key Words α -tocopheryl succinate, protein kinase C, phorbol ester, molecular superimposition

α -Tocopheryl succinate (TS, chemical structure shown in Fig. 1) is the succinyl ester of a well-known antioxidant, α -tocopherol. Although TS lacks antioxidative activity due to the masking of its phenolic OH moiety, which is an active site for antioxidation, it is known to have various biological activities such as inhibition of the function of nuclear factor-kappa B (1), enhancement of lipopolysaccharide/interferon- γ -induced nitric oxide production on vascular smooth muscle cells (VSMC) (2) and induction of apoptosis in various cell lines (3–9). The apoptotic toxicity of TS to cancer cells is attracting special attention as a new type of anti-cancer agent. Thus, many researchers have studied the mechanism of TS effects to obtain information for the development of a novel type of anti-cancer drug, but theories of the mechanism are still controversial. Recently, we found that TS-enhanced nitric oxide production in VSMC was prevented by inhibitors of protein kinase C (PKC) (2). Similarly, Bang et al. reported that TS treatment induced a gradual increase in PKC activity in HL-60 cells (10). In addition, we reported that TS-induced apoptosis in VSMC was caused due to superoxide exogenously generated at the outside of the plasma membrane by activated NADPH oxidase, the activation of which is known to be caused through the activation of

PKC (8). On the contrary, Gopalakrishna et al. reported that TS directly inhibited the activity of purified PKC (11), while Neuzil and his co-workers suggested that TS indirectly inhibited PKC through the activation of protein phosphatase 2A like α -tocopherol (12–15). Thus, the effect of TS on PKC activity is still not clear. In addition, it has been reported that PKC plays an important role in growth regulation (16), and is related to the inhibition or induction of apoptosis (17–19). Furthermore, TS has been reported to activate various factors in the cellular signal transduction pathway such as extracellular signal-regulated kinase (ERK), c-jun-NH₂-terminal kinase (JNK) and mitogen-activated protein kinase kinase (MEK) (7, 20). Since PKC has been suggested to act in the upstream in the signal transduction pathway above MEK, ERK and JNK (21–23), it was thought to be the key kinase in the process of the TS effects. In this study, to clarify the relation between TS and PKC, we examined the effect of TS on PKC in cellular and non-cellular systems.

MATERIALS AND METHODS

Materials. RRR- α -Tocopheryl succinate (TS), phorbol 12-myristate 13-acetate (PMA, chemical structure shown in Fig. 1) and PKC isolated from rat brain (mixture of α , β and γ isoenzymes) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Myristoylated alanine-rich C-kinase substrate (MARCKS) protein phosphorylation site domain-derived peptide was purchased from Calbiochem (Darmstadt, Germany). Other reagents were of the highest grade commercially available.

Treatment of VSMC with TS. VSMC were isolated from rat thoracic aorta using the proteases elastase and collagenase as described previously (24). The VSMC

* To whom correspondence should be addressed.

E-mail: fukuzawa@ph.tokushima-u.ac.jp

Abbreviations: TS, α -tocopheryl succinate; VSMC, vascular smooth muscle cells; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; MARCKS, myristoylated alanine-rich C-kinase substrate; β PAA, 4 β -OH-phorbol 12 β ,13 α -diacetate; α PAA, 4 α -OH-phorbol 12 β ,13 α -diacetate; TECS, 2,5,7,8-tetramethyl-2-ethyl-chroman-6-yl succinate; p-PKC, phosphorylated PKC α .

Fig. 1. Chemical structures of TS, TECS and phorbol esters PMA and βPAA .

(1×10^6 cells) were seeded into 35 mm dishes, and cultured for 24 h in Dulbecco's modified Eagle's medium with 10% fetal calf serum in a CO_2 -incubator at 37°C with CO_2 in humidified air. Then, the medium containing serum was removed and the cells were washed with phosphate-buffered saline (PBS). Next, 1 mL of medium containing TS without serum was added to the dishes, and after a certain period, the cells were subjected to Western blot analysis.

Analysis of PKC activation in VSMC. To detect the effect of test reagents on the activation of PKC in VSMC, we performed Western blot analysis of phosphorylated $\text{PKC}\alpha$ in the cells. After removal of the culture medium, cells were collected from the dishes into sample tubes using a cell scraper. Buffer (2% SDS, 20% glycerol, 50 mM Tris-HCl, pH 6.8) for cell dissolution was added to the cells, and then the cell suspension was sonicated in a bath-type sonicator for 5 min. The protein content of the solubilized cells was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard. The solubilized cells were subjected to 7% SDS-polyacrylamide gel electrophoresis, and proteins were transferred electrophoretically to a polyvinylidene difluoride membrane. The membrane was treated with a rabbit anti-phosphorylated $\text{PKC}\alpha$ polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA), which is specific for rat $\text{PKC}\alpha$ phosphorylated at its Ser657, at a dilution of 1:1,000 and a goat anti-rabbit horseradish peroxidase conjugated antibody at a dilution of 1:2,500 as the primary and secondary antibodies, respectively. The blots were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, UK) and exposed to photographic films. Images of the exposed films were scanned with a computerized image scanner and the areas of the positive bands were calculated with the analyzing software Scion Image (Scion Co., Frederick, MD, USA).

Estimation of PKC activity in vitro. To estimate the PKC activity in vitro, we performed enzyme-linked immunosorbent assay (ELISA) of phosphorylation of the

model substrate MARCKS by PKC. For binding of the substrate peptide of PKC to the well surfaces of the ELISA plates, 50 μL of 0.05 M sodium carbonate buffer (pH 9.6) containing 100 $\mu\text{g}/\text{mL}$ MARCKS was added into each well, and incubated at 4°C overnight. After removal of the substrate solution, the wells were treated overnight at 4°C with PBS containing 0.05% Tween 20 and 5% BSA for blocking. Then, the wells were washed with PBS containing 0.05% Tween 20 and treated at room temperature with a phosphorylation solution [0.858 units/mL PKC, 0.1 mM ATP, 50 $\mu\text{g}/\text{mL}$ phosphatidylserine, 0.3 mM MgCl_2 , 0.2 mM CaCl_2 and 2.5 mM Tris-HCl buffer (pH 7.0)] to induce kination of the substrate by PKC. After 5 min, 20% H_3PO_4 was added to the wells to terminate the reaction. For the detection of phosphorylated peptide, the wells were treated with a mouse anti-phosphoserine monoclonal antibody (Calbiochem-Novabiochem Corp., San Diego, CA, USA) at a dilution of 1:1,000 and a rabbit anti-mouse IgG horse-radish peroxidase conjugated antibody at a dilution of 1:10,000 as the primary and secondary antibodies, respectively. The extent of adsorption of the primary antibody was estimated colorimetrically with a color developing solution [0.4 mg/mL *o*-phenylene-diamine, 0.175% H_2O_2 and 0.1 M citrate buffer (pH 5.0)], and the absorbance at 490 nm was measured with a microplate reader (Bio-Rad Laboratories, Tokyo, Japan).

Theoretical computations. We developed a FORTRAN95 program, "MGP504.exe," for completing a comprehensive conformational search for medicinal and phytochemical compounds based on our modified method of the corner-flipping/edge-flipping algorithm (25). In our program, the conformations generated were optimized by molecular mechanism calculations with AMBER force fields (26). Atomic charges of the computed molecules were determined by semi-empirical molecular orbital calculation by the Hamiltonian parameterization method 3 (PM3) in the MOPAC program (27).

RESULTS AND DISCUSSION

First, to study whether or not TS has the ability to activate PKC, we examined its effect on the auto-phosphorylation of $\text{PKC}\alpha$, which is an isoform expected to participate in the effects of TS from previous studies (2, 10), in VSMC using Western blot analysis. As shown in Fig. 2, TS induced significant auto-phosphorylation of $\text{PKC}\alpha$ in a concentration ($\sim 50 \mu\text{M}$)- and time (~ 6 h)-dependent manner. However, the amount of $\text{PKC}\alpha$ protein did not change even at 6 h after the addition of TS (data not shown), but was reported to increase after 72 h (2). These results indicate that the increase in phosphorylated PKC is due to the activation, but not expression of PKC by TS. This conclusion was supported by our previous report (8): i.e., TS-induced superoxide generation by NADPH oxidase, which is activated through PKC activation, in VSMC was observed 12 h after the addition of TS, and then apoptosis was induced. As TS appeared to be taken up immediately into VSMC without being hydrolyzed (8), it was itself responsible for the

Fig. 2. Effect of TS on auto-phosphorylation of PKC α in VSMC. (A) The concentration-dependent auto-phosphorylation of PKC α by treatment with TS for 30 min. (B) Time-dependent change of PKC α phosphorylation by treatment with 10 μM TS. Phosphorylated PKC α (p-PKC) was estimated by Western blot analysis with an anti-phosphoryl PKC α antibody. The degree of phosphorylation was estimated by calculating the area of a positive band corresponding to PKC α on X-ray film. Values are means $\pm$ SD ($n=4$). * $p \leq 0.01$, ** $p \leq 0.05$.

Fig. 3. Effects of TS and PMA on the activity of isolated PKC in vitro. The concentrations of TS (○) and PMA (●) are indicated on the upper and lower X-axes, respectively. PKC activity was estimated by phosphorylation of the substrate peptide MARCKS by ELISA with an anti-phosphoserine antibody. Values are means $\pm$ SD ($n=3$). * $p \leq 0.01$, ** $p \leq 0.05$.

activation of PKC. Our results that TS has the ability to activate PKC in the VSMC are different from those of Neuzil and his co-workers (12–15). The discrepancy of the TS effect on PKC may be due to a difference in experimental conditions, including the cell lines as reported by Gopalakrishna et al. (11). The effect of TS is unlikely

to be caused by its detergent-like function, because TS makes bilayer membranes in itself (28).

To confirm that TS activated PKC directly, we compared the effect of TS with that of the PKC activator PMA on the activity of PKC isolated from rat brain (α , β and γ isoenzymes) in a non-cellular system by ELISA of phosphorylation of the substrate peptide MARCKS. TS increased PKC activity concentration-dependently like PMA, although the effective concentrations of TS were significantly different from those of PMA (Fig. 3). The K_m value for TS estimated from a Lineweaver-Burk plot of the reciprocal of PKC activity against the reciprocal of TS concentration was approximately 160-fold that for PMA: i.e., the K_m values for TS and PMA were 1.22 μM and 7.66 nM, respectively. These results suggest that TS activates PKC, like PMA, by direct interaction with it.

To determine why TS, the chemical structure of which is different from that of PMA, activates PKC, and to obtain information about the large difference in the effective concentrations of TS and PMA, we performed theoretical computations to obtain steric structural information about TS. TS has been suggested to bind to the phorbol 12,13-dibutyrate-binding site of PKC, because TS prevented the binding of phorbol ester with PKC isolated from rat brain (α , β and γ isoenzymes) (11). From a previous report (16) on the crystalline structure of the PKC δ activator-binding domain in the complex with phorbol ester, it appears that a carbonyl oxygen at position C3 (3 C=O) and a β -hydroxyl hydrogen at position C4 (4 βOH) of the phorbol ester bound with Gly253, and a hydroxyl group at its C20 position (20 OH) also interacted with Thr242 and Leu251. Thus, if TS binds to the activator-binding domain of PKC, hydrophilic groups in the succinyl moiety of TS might interact with PKC like those of phorbol ester: i.e., a conformation of TS, especially its succinyl moiety, might correspond to that of the phorbol ester binding with PKC. Next, we compared all the possible conformations of TS with the active conformation of PMA using a computational approach.

In this study, we used a model compound of the phorbol ester 4 β -OH-phorbol 12 β ,13 α -diacetate (β PAA, chemical structure shown in Fig. 1) to simplify computation of the active conformation of PMA, because the long hydrocarbon chain of PMA is not required for direct interaction with the PKC molecule (16). Furthermore, as it was difficult to obtain a single active conformation of phorbol ester because of various conformations of β PAA, we examined the molecular superimpositions of β PAA on its analogous PKC activators, ingenol and mezerein (29, 30), which have rigid conformations, and 4 α -OH-phorbol 12 β ,13 α -diacetate (α PAA), which is an inactive sterical isomer of β PAA. By molecular superimposition of β PAA, ingenol and mezerein, we obtained an active conformer with a common 3D-structure of azulenon moiety of the three compounds from 700 conformations of β PAA (Fig. 4). In the conformations of β PAA, the active conformer was the most stable, and the Boltzmann distribution implying its conformational population was determined as

Table 1. Sterical data for the conformation of βPAA and TECS.

	The most stable conformation of βPAA	The most stable conformation of TECS	TECS superimposed on the active conformer of βPAA
Steric energy (kcal/mol)	239.63	9.24	11.72
Boltzmann distribution (%)	99.59	4.90	0.077
Average energy (kcal/mol)	239.65	10.23	—
Number of surveyed conformations	700	1,850	1

Boltzmann distribution and average energies were calculated at 25°C.

Fig. 4. Molecular superimposition of TECS on βPAA. The light gray, dark gray and white in each structure represent carbon, hydrogen and oxygen atoms, respectively. Corresponding atoms in TECS to those in the active conformation of βPAA are indicated by circles, and are linked by dotted lines.

99.59% (Table 1). The active conformer of βPAA was the same as the crystal structure of a phorbol ester binding with the PKCδ activator-binding domain (16). Accordingly, the phorbol ester is suggested to take the active conformation at all times. However, an inactive isomer αPAA showed a quite different conformation (data not shown) because of the inversed configuration of 4OH in αPAA.

Similarly, to simplify the calculation of TS conformations, we used 2,5,7,8-tetramethyl-2-ethyl-chroman-6-yl succinate (TECS, chemical structure shown in Fig. 1) as a model compound for TS (Fig. 1). From a comprehensive search of possible conformations of TECS, 1,850 conformations were found, but the chroman ring moiety took only two conformations. The Boltzmann distribution of the most stable conformer of TECS was only 4.90% (Table 1). Accordingly, it is indicated that the structure of the succinyl moiety is highly flexible.

Then we examined the molecular superimpositions of the conformations of TECS with the determined active conformer of βPAA. From the calculation, we found that the 156th conformer from the most stable conformation of TECS superimposed on the active conformer of βPAA (Fig. 4); namely, a carbonyl oxygen at position C2' (2'C=O), an oxygen at position 1' (1'O) and a hydroxyl group at position C6' (6' OH) in succinyl moiety of TECS correspond to 3 C=O, 4 βOH and 20 OH of βPAA, respectively. In this result, the 1' O corresponding to 4 βOH of PMA would not form a hydrogen bond with the carbonyl oxygen of Gly253 in PKC. The 4 βOH has been suggested to be a key moiety of phorbol ester

action, because 4 αOH phorbol ester has no ability to activate PKC (31). However, it was reported that 4-deoxy phorbol ester showed activity even though the affinity to PKC was diminished (16). Furthermore, the sterical structure of αPAA obtained by theoretical computation was significantly different from that of βPAA. Thus, it is suggested that 4 βOH is important for the sterical structure of phorbol ester, but not essential for activation of PKC. On the other hand, the chroman ring moiety of TECS corresponds to βPAA bulky rings, which are inserted lengthwise into a narrow groove of PKC, like the phorbol ester (16). The differential energy between the superimposed and most stable conformations of TECS was 2.48 kcal/mol, and the value of the Boltzmann distribution was 0.077% (Table 1). Thus, it is indicated that approximately 1/1,000 of the total TS molecules should take the conformation superimposed on the active conformer of PMA. Namely, 1/1,000 of the TS molecules surrounding PKC should bind to the phorbol ester-binding domain. This result could explain why the Km for TS was extremely higher than that for PMA, and is also consistent with the reported result that high concentrations of TS (10⁻⁵ molar order of magnitude) prevented the binding of low concentrations of phorbol ester (10⁻⁹ molar order of magnitude) with PKC (11). The experimentally obtained ratio of TS to PMA required for PKC activation was not completely consistent with that theoretically determined. This inconsistency would be due to the theoretical values, which were computed as values in a vacuum. Furthermore, the high structural flexibility of TS shown by the theoretical computation suggests that TS interacts with various proteins such as PKC, and shows versatile effects.

In conclusion, we found that TS activates PKC directly due to binding with PKC by taking an active conformation like that of the phorbol ester.

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