Inhibitory effect of curcumin on mammalian phospholipase D activity

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Abstract Curcumin, the major yellow pigment of turmeric (*Curcuma longa*), has strong anti-carcinogenic and anti-inflammatory activities. We examined the effects of curcumin on enzyme activities of the following phospholipases in a cell-free system: G protein-mediated phospholipase D (PLD), phosphatidylinositol-specific phospholipase C, and phospholipase A_2 from mouse macrophage-like cell line J774.1 cells, sphingomyelinase from bovine brain, and phosphatidylcholine-phospholipase C from *Bacillus cereus*. Curcumin inhibited several types of phospholipases, most effectively PLD among those tested. It also inhibited 12-*O*-tetradecanoylphorbol-13-acetate-induced PLD activation in intact J774.1 cells in a dose-dependent manner. These results suggest that the anti-inflammatory and anti-carcinogenic action of curcumin is partly due to the inhibition of PLD.

Key words: Curcumin; Phospholipase D

1. Introduction

Curcumin is the major pigment in turmeric, the powdered rhizome of *Curcuma longa*, which is widely used for the treatment of a variety of inflammatory conditions and other diseases [1]. Recent studies have shown that curcumin possesses anti-oxidant and anti-inflammatory activities [2–6]. Curcumin potently inhibits the initiation and promotion of chemical carcinogen-induced tumor formation in mice [7,8] and the proliferation of animal epithelial and muscle cells [9,10]. It also inhibits the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-mediated induction of c-Jun/AP-1 [11] and protein kinase C (PKC) activity [12] in mouse NIH/3T3 cells, induction of nitric oxide synthase in activated macrophages [13], TNF-α/TNF-R-mediated NF-κB activation in human myeloid cells [14], and EGF-induced activation of EGF-R phosphorylation [15], but it remains unclear what molecule(s) curcumin directly modulates in cells. Furthermore, curcumin effectively inhibits type 1 human immunodeficiency virus long terminal repeat-directed gene expression and the virus replication [16].

In response to various stimuli, the breakdown of phospholipids plays crucial roles in the early steps of the signaling pathways [17]. For example, phospholipase A_2 (PLA_2), phosphatidylinositol-specific phospholipase C (PI-PLC), and sphingomyelinase (SMase) are involved in various receptor-mediated signalings [17], and the breakdown of phosphatidylcholine (PC) by phospholipase C or D (PC-PLC, PLD) is suggested to be involved in the signalings in response to TNF-α and platelet-derived growth factor [18]. In the present study, we examined whether curcumin modulated the activities of these phospholipases and found that it inhibited the activities of various phospholipases, most effectively PLD activity, in a cell-free system and in intact cells.

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TNF, tumor necrosis factor; FCS, fetal calf serum (heat-inactivated); PC, phosphatidylcholine; PA, phosphatidic acid; PBut, phosphatidyl butanol; PC-PLC, PC-specific phospholipase C; PLD, phospholipase D; G proteins, guanine-nucleotide binding proteins; ARF, ADP-ribosylation factor; GTPγS, guanosine 5′-*O*-[γ-thio]triphosphate; DMSO, dimethylsulfoxide; PDTC, pyrrolidine dithiocarbamate

2. Materials and methods

2.1. Materials

TPA was purchased from Sigma Chemicals (St. Louis, MO, USA). Curcumin was purchased from Wako Pure Chemical (Tokyo, Japan) and stored as a 100 mM stock solution in dimethylsulfoxide (DMSO) at −30°C under light-shielding conditions. ADP-ribosylation factor (ARF) was prepared according to Tanigawa et al. [19].

2.2. Cell culture

Mouse macrophage-like J774.1 cells were maintained in Ham's F-12 medium supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) newborn calf serum (NCS, ICN Biomedicals, CA, USA) at 37°C in a water-saturated atmosphere of 5% CO_2 in air.

2.3. Cell-free assays of phospholipases

J774.1 membranes were prepared according to Hara-Kuge et al. [20], and protein concentrations were determined by the method of Bradford [21], using bovine serum albumin as the standard. Membranes were preincubated with or without curcumin at 37°C for 30 min, then the phospholipase reactions were started by addition of radioactive phospholipid substrates. Assays were performed at 37°C, and lipids were extracted according to Bligh and Dyer [22].

For assay of ARF/GTPγS-dependent PLD activity, J774.1 membranes (0.3 mg protein/ml) were incubated with [^{14}C]dipalmitoyl-PC (4.2 GBq/mmol, DuPont New England Nuclear, Boston, MA, USA) in the presence of 1% butanol [23], and formation of [^{14}C]phosphatidylbutanol ([^{14}C]Pbut) was measured [24]. For PI-PLC assay, J774.1 membranes (0.3 mg protein/ml) were incubated with [*myo*-inositol-^{3}H]PI (407 GBq/mmol, DuPont New England Nuclear), and the release of [^{3}H]inositol was measured [25]. For PLA_2 assay, J774.1 cell lysates (0.3 mg/ml) were incubated with 1-acyl-2-[1-^{14}C]arachidonyl-L-3-phosphatidylethanolamine (2.04 GBq/mmol, Amersham, London, UK), and release of [^{14}C]arachidonic acid was determined [26]. Since activities of acidic and neutral SMases in J774.1 membranes were very low, bovine brain cell lysates were used as an enzyme source for SMase assays. Neutral and acidic SMase activities in bovine brain cell lysates (0.2 mg/ml) were measured according to Schütze et al. [27] by using [*methyl*-^{14}C]choline-sphingomyelin (2.04 GBq/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO) as the substrate. Activity of PC-PLC from *Bacillus cereus* (Boehringer Mannheim, 0.4 U/ml) was assayed using [^{14}C]dipalmitoyl-PC as the substrate [28].

2.4. Effect of curcumin on TPA-induced PLD activity in J774.1 cells

J774.1 cells (1×10^{7} cells) were cultured in 6 ml of F-12 containing 1% FCS and [^{14}C]palmitate (1.92 GBq/mmol, Moravek Biochemicals Inc., CA) at 37°C for 1 day, then harvested by centrifugation ($500\times g$, 5 min). The prelabeled cells suspended in F12/0.1% NCS (approximately 1×10^{6} cells/ml) were incubated with or without curcumin at 37°C for 30 min. After addition of 1% (v/v) butanol to the cell sus-

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pension, the reaction was started by addition of 100 nM TPA, and [^{14}C]PBut formation was measured [24].

3. Results

3.1. Effect of curcumin on activities of various phospholipases in cell-free systems

A membrane fraction prepared from J774.1 cells was used as an enzyme source for assays of ARF/GTPγS-dependent PLD and PI-PLC, and a cytosol fraction prepared from J774.1 cells was used for assay of PLA_2. Cell lysates of bovine brain were used for assays of acidic and neutral SMases. Curcumin potently inhibited ARF/GTPγS-dependent PLD activity in a dose-dependent manner (Fig. 1A). The curcumin concentration required to inhibit the PLD activities by 50% (IC_{50}) was about 10 μM, and 50 μM curcumin inhibited the PLD activity by 80%. Although 10 μM curcumin did not affect other phospholipase activities, higher concentrations of curcumin substantially inhibited the cytosolic PLA_2 activity (IC_{50} = 50 μM) and bacterial PC-PLC activity (IC_{50} = 80 μM). Curcumin also inhibited PI-PLC activity in a dose-dependent manner up to 100 μM, but the maximum inhibition of PI-PLC by curcumin was only 50% (Fig. 1C). Curcumin up to 200 μM did not appreciably inhibit either acidic or neutral SMase activity (Fig. 1E). These results suggest that curcumin inhibited PLD more potently than other phospholipases.

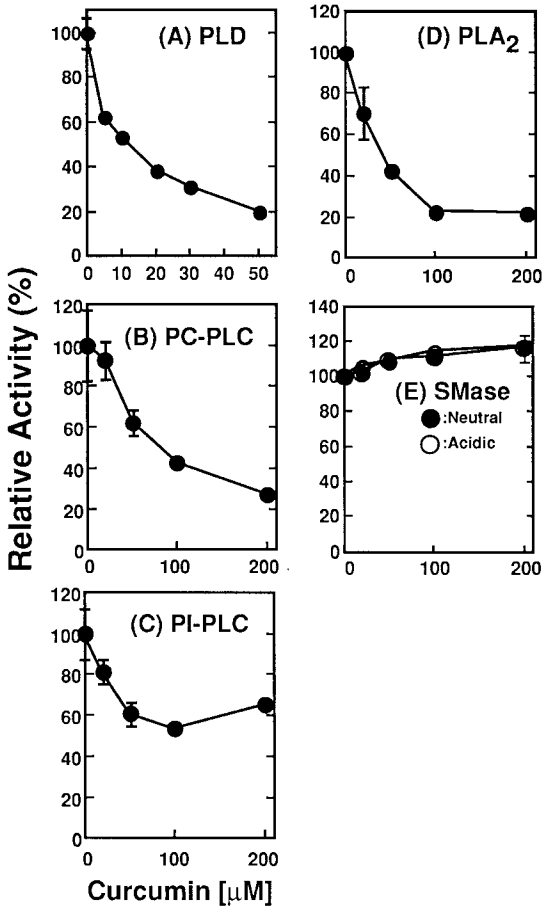

Fig. 1. Effect of curcumin on phospholipase activities. Sources of the indicated enzymes were preincubated with various concentrations of curcumin for 30 min. Then phospholipase assays were started by addition of phospholipid substrates to the enzyme sources, and the activities were determined as described in Section 2. The activities after treatment with curcumin are represented as percentages of the control activities. The data shown are mean values ± S.D. of three independent experiments. A: ARF/GTPγS-dependent PLD. B: PC-PLC. C: PI-PLC. D: PLA_2. E: Acidic and neutral SMases.

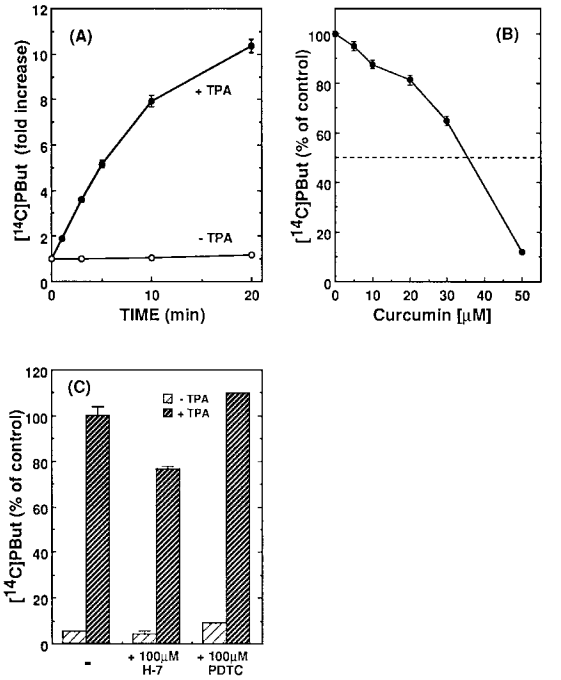

Fig. 2. TPA-induced PLD activation. A: [^{14}C]palmitate-labeled J774.1 cells were stimulated with 100 nM TPA (closed circle) in the presence of 1% butanol. The level of [^{14}C]PBut in the absence of TPA at 0 min was taken as 1. B: Dose-dependent effect of curcumin on PLD activity. [^{14}C]Palmitate-labeled J774.1 cells were incubated with various concentrations of curcumin at 37°C for 30 min, then stimulated with 100 nM TPA at 37°C for 20 min. The level of [^{14}C]PBut in the presence of TPA without curcumin was taken as 100%. C: [^{14}C]Palmitate-labeled J774.1 cells were incubated with or without 100 μM H-7 and 100 μM PDTC at 37°C for 30 min, then stimulated with 100 nM TPA at 37°C for 20 min. The level of [^{14}C]PBut in the presence of TPA without drugs was taken as 100%. The data shown are mean values ± S.D. of three independent experiments.

3.2. Curcumin inhibited TPA-induced PLD activation in intact cells

To examine whether curcumin effectively inhibits PLD activity in intact cells, J774.1 cells were labeled with [^{14}C]palmitate, then stimulated with 100 nM TPA in the presence of 1% butanol. As shown in Fig. 2A, significant elevation of [^{14}C]PBut level was detectable within 1 min after stimulation, and the accumulation of [^{14}C]PBut reached a maximum level at 20 min. When [^{14}C]palmitate-labeled J774.1 cells were incubated with various concentrations of curcumin for 30 min before stimulation with 100 nM TPA in the presence of 1% butanol, the TPA-induced [^{14}C]PBut accumulation was prevented by the curcumin treatment in a dose-dependent manner (Fig. 2B). The IC_{50} value of curcumin to inhibit the TPA-induced PLD activation was 35 μM, and almost complete inhibition was observed at 50 μM curcumin.

Since it is known that TPA activates PKC and induces the stimulation of oxidative metabolism, that some PLDs are activated by PKC and hydrogen peroxide, and that curcumin

inhibits PKC catalytic activity and acts as an anti-oxidant, it is possible that curcumin may inhibit TPA-induced PLD activation as a consequence of its inhibitory effect on activation of PKC and/or oxidant production by TPA. To test this possibility, we examined the effects of a PKC inhibitor and an anti-oxidant on the TPA-induced PLD activation in J774.1 cells. Pretreatment of cells with H-7, a potent inhibitor of PKC due to competition with ATP for binding to the catalytic domain, even at 100 μM, caused only slight inhibition of the TPA-induced PLD activation (Fig. 2C). Pyrrolidine dithiocarbamate (PDTC), an antioxidant, at 100 μM did not affect the production of [^{14}C]PBut following TPA stimulation (Fig. 2C). These results suggest that the TPA-induced PLD activation does not require PKC catalytic activity or oxidative processes and that the inhibition of PLD activity by curcumin is not a consequence of PKC inhibition or anti-oxidation.

4. Discussion

Curcumin is a pharmacologically safe compound with anti-inflammatory, anti-carcinogenic and free radical scavenger properties [1–6]. In this study, we investigated the effects of curcumin on the activities of various phospholipases that might be involved in signaling pathways.

We found that curcumin potently inhibited ARF/GTPγS-dependent PLD activity in membrane fractions of J774.1 cells and TPA-induced PLD activity in intact J774.1 cells even at low concentrations (10–30 μM). Several PLDs are activated by PKC and small G proteins, such as ARF, Rho, and CDC42. It has recently been shown that TPA induces PLD activation via modulation of PKC which is independent of PKC catalytic activity [29,30]. Our results showed that curcumin inhibited both ARF/GTPγS-dependent and TPA/PKC-induced PLD activities, suggesting that the inhibition of PLD activity by curcumin is due to a direct interaction of curcumin with PLD but not to inhibition, if any, of ARF or PKC by curcumin.

Curcumin moderately inhibited bacterial PC-PLC (IC_{50} = 80 μM). Since no cell-free assay system for mammalian PC-PLC has been established, we did not address the question of whether curcumin inhibited mammalian PC-PLC. But the moderate inhibition of bacterial PC-PLC by curcumin suggests that curcumin might also inhibit the putative PC-PLC of mammalian cells. Curcumin also inhibited PI-PLC activity in a dose-dependent manner up to 100 μM, but higher concentrations caused no further increase of inhibition. This plateau level of about 50% inhibition might reflect the presence of some PI-PLC isoforms that are inhibited by curcumin and others that are not, since PI-PLC is known to possess multiple isozyme forms with different properties and different subcellular localizations, including three well-defined groups, PI-PLC β, γ, and δ [31]. Curcumin did not appreciably inhibit SMase activity even at 200 μM.

Pretreatment of cells with 40–60 μM curcumin inhibited NF-κB activation by TNF-α in human leukemia cells [14] and by LPS in J774.1 cells (our unpublished data). Stimulation with TNF-α or LPS induces diacylglycerol (DAG) production from PC, leading to NF-κB activation [28,32]. *ras*-dependent transformation of NIH/3T3 cells is also associated with elevation of the DAG level via action of PC hydrolysis [33]. Our findings that curcumin strongly inhibited PLD activity in intact cells as well as in a cell-free system raise the possibility that the anti-inflammatory/anti-carcinogenic effects of curcumin may be, at least partly, due to the inhibition of PLD activity.

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