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RESEARCH PAPER

Modulation of protein kinase C by curcumin; inhibition and activation switched by calcium ions

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Background and purpose: Previous studies have identified the natural polyphenol curcumin as a protein kinase C (PKC) inhibitor. In contrast, we found significant stimulation of PKC activity following curcumin treatment. Thus, the mechanism of curcumin interaction with PKC was investigated.
Experimental approach: We employed phosphorylation assays in the presence of soluble or membrane-bound PKC substrates, followed by SDS–PAGE, autoradiography and phosphorylation intensity measurements.
Key results: Curcumin inhibited PKC in the absence of membranes whereas stimulation was observed in the presence of membranes. Further analysis indicated that curcumin decreased PKC activity by competition with Ca^{2+} stimulation of the kinase, resulting in inhibition of activity at lower Ca^{2+} concentrations and stimulation at higher Ca^{2+} concentrations. The role of the membrane is likely to be facilitation of Ca^{2+}-binding to the kinase, thus relieving the curcumin inhibition observed at limited Ca^{2+} concentrations. Curcumin was found to mildly stimulate the catalytic subunit of PKC, which does not require Ca^{2+} for activation. In addition, studies on Ca^{2+}-independent PKC isoforms as well as another curcumin target (the sarcoplasmic reticulum Ca^{2+}-ATPase) confirmed a correlation between Ca^{2+} concentration and the curcumin effects.
Conclusions and Implications: Curcumin competes with Ca^{2+} for the regulatory domain of PKC, resulting in a Ca^{2+}-dependent dual effect on the kinase. We propose that curcumin interacts with the Ca^{2+}-binding domains in target proteins. To our knowledge, this is the first study that defines an interaction domain for curcumin, and provides a rationale for the broad specificity of this polyphenol as a chemopreventive drug.
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Keywords: curcumin; PKC; Ca^{2+} binding; phosphorylation; polyphenols

Abbreviations: DAG, diacyl glycerol; ECL, enhanced chemiluminescence; PC, phosphatidyl choline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidyl serine; SDS, sodium dodecyl sulfate; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase

Introduction

Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent enzyme that phosphorylates serine and threonine residues in a wide variety of cellular proteins. PKC comprises a family of enzymes that significantly differ in structure, cofactor requirements and cellular compartmentalization. The PKC family has been divided into three groups: conventional isoforms (α, β_I, β_{II} and γ) that require Ca^{2+} and diacylglycerol (DAG) for activation; novel isoforms (δ, ε, η, θ and μ) that require only DAG and atypical isoforms (ζ, ι and λ) that require neither Ca^{2+} nor DAG (Newton, 2001). The involvement of individual PKC isoforms in different signal transduction pathways in health and disease has been well documented (Nishizuka, 2003; Zhang *et al*., 2004). Inhibitors of PKC are currently being used in clinical trials for various types of cancer (Cohen, 2002).

Scientific interest in medicinal plants has increased strongly during the last decade, and many efforts have been made to understand and explain the beneficial effects of many plant-derived chemicals. Curcumin (diferuloylmethane), the active constitute of *Curcuma longa*, is one of the best-studied natural compounds. Curcumin has been used as a spice and coloring agent for centuries but only recently have the anti-inflammatory, antiviral, anti-infectious and anti-carcinogenic effects of this molecule have been realized (for reviews see Surh, 2003; Joe *et al*., 2004; Singh and Khar, 2006). In addition, the pharmacological effects of curcumin have been recently substantiated by epidemiological studies (Ng *et al*., 2006). Curcumin has been shown to modulate the activity of seemingly unrelated proteins such as protein kinases (Liu *et al*., 1993; Hasmeda and Polya, 1996), membrane ATPases (Zheng and Ramirez,

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2000; Logan-Smith *et al*., 2001; Sumbilla *et al*., 2002; Mahmmoud, 2005) and transcription factors (Singh and Aggarwal, 1995; Choi *et al*., 2006). This broad specificity suggests that curcumin interacts with common domain(s) on target proteins. The molecular mechanism behind the physiological effects of curcumin is not well understood. In particular, whether curcumin intake provides a safe way to treat cystic fibrosis has been a matter of controversy (see Egan *et al*., 2002, 2004; Song *et al*., 2004; Mall and Kunzelmann, 2005; Grubb *et al*., 2006).

The positive interference of curcumin with the tumor-promoting effects of phorbol esters has presumably been attributed to a direct inhibitory effect of curcumin on the phorbol ester receptor, PKC (Lin *et al*., 1997). In the present study, we observed that addition of curcumin to PKC phosphorylation assays resulted in stimulation, not inhibition, of the PKC-catalyzed phosphorylation of a membrane-bound substrate. This is, however, in significant disagreement with previous reports documenting inhibition of PKC activity by curcumin *in vivo* (Liu *et al*., 1993) and *in vitro* using membrane-free systems (Reddy and Aggarwal, 1994). Hence, we investigated the effect of curcumin on PKC in a well-defined reconstituted membrane system and in isolated native plasma membranes. It is concluded that the effects of curcumin on PKC and possibly other curcumin targets may be switched from inhibition to stimulation by the concentration of Ca^{2+}.

Materials and methods

PKC phosphorylation

PKC phosphorylation was performed essentially as described previously (Mahmmoud *et al*., 2000). The assay medium (25 μl) contained 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), pH 7.5, 10 mM $MgCl_2$, 300 μM Ca^{2+} (giving a free Ca^{2+} concentration of $\sim$260 μM, unless indicated otherwise), 40 μg membrane vesicles (see below) containing 10 mol% phosphatidyl serine (PS) (roughly 4 μg per reaction), 2 μM phorbol 12-myristate 13-acetate (PMA), 100 μM Tris-ATP (containing 0.03–0.06 μCi [^{32}P]-ATP), 4 μg histone H1, 0.15 μg PKC (known to contain the Ca^{2+}-dependent isoforms), and different curcumin concentrations, as described in figure legends. In other experiments, 4 μg of purified membranes containing Na,K-ATPase (purified from the rectal gland of the spiny dog fish, *Squalus acanthias*) was added as a kinase substrate (Mahmmoud *et al*., 2000). After short pre-incubation (3 min at 24°C) of the kinase, activators, and the substrate, phosphorylation was started with the addition of ATP (both labelled and unlabelled ATP were thoroughly mixed before addition), allowed to proceed for 20 min at 24°C and terminated with sodium dodecyl sulfate (SDS) sample buffer (Laemmli, 1970). The PKC-catalyzed phosphate incorporation into histone and the catalytic subunit of Na,K-ATPase was found to increase linearly, reaching a maximum at 20 min (Mahmmoud *et al*., 2000). In some experiments (Figures 4 and 5), different Ca^{2+} concentrations were used and phosphorylation was performed under conditions otherwise identical to those stated above. The free Ca^{2+} concentration was calculated using WinmaxC (http://www.stanford.edu /~cpatton/winmaxc2.html). In general, all experiments were repeated at least twice and gave similar results each time.

To measure the effect of curcumin on ATP activation of PKC, phosphorylation reactions were performed in the presence of the same amount of [^{32}P]-ATP ($\sim$0.04 μCi) but at increasing amounts of unlabelled ATP (from 20 to 300 μM). Because of the dilution of [^{32}P]-ATP, the radioactivity associated with the phosphorylated bands will decrease upon increasing the cold ATP concentration. The relative stimulation of phosphorylation was calculated by multiplying the obtained area intensities of the phosphorylated bands by the ratio $[ATP_{unabelled}]/[ATP_{labelled}]$.

Gel electrophoresis

Phosphorylated proteins in sample buffer were loaded on Tricine-based polyacrylamide gels (3% stacking gel, 9% intermediate gel and 16% resolving gel). Phosphorylated proteins (1–2 μg) were loaded in each lane and electrophoresis was run overnight at 200 V and 15 mA. After electrophoresis, gels were stained with Coomassie blue, destained, dried, and analyzed with autoradiography on high-performance films overnight at −80°C. Scanning and intensity determination of the autoradiograms was performed using ImageQuant TL image analysis software (Amersham Biosciences, Buckinghamshire, UK), as previously applied (Cornelius *et al*., 2005). In other experiments, the Coomassie-stained bands corresponding to the α-subunit were excised from the gel and the radioactivity associated with them was measured in a scintillation counter. The data were comparable to that obtained by area intensity measurements. Data were analyzed by unpaired *t*-test, and *P*-values <0.01 were considered significant.

Liposome preparation

Reconstitution of membrane vesicles was performed as described previously (Szoka and Papahadjopoulos, 1980). Lipids, dissolved in chloroform, were mixed and subjected to rotary evaporation under a stream of nitrogen to remove the organic solvent and form a homogenous lipid film. The dried lipid film was put in a vacuum tube for at least 1 h to remove traces of remaining organic solvent. Membrane vesicles were formed by hydration of the lipid film in a buffer containing 20 mM Tris, pH 7.0 and 100 mM sucrose. The mixture was sonicated four times (each for 20 s), subjected to two freezing/thawing cycles, and kept on ice until use in phosphorylation experiments. The lipid and cholesterol concentration was expressed in mole percentage (mol%), which is the mole fraction of the lipid (or cholesterol) as a percentage of the total concentration of lipids present in the system.

Ca^{2+}-ATPase measurements

Sarcoplasmic reticulum (SR) membrane fragments were prepared as described previously (Meissner *et al*., 1973). Ca^{2+}-ATPase activity was determined by measuring the Ca^{2+}-dependent inorganic phosphate liberation from ATP,

after incubation at 24°C in the presence of different curcumin concentrations, at either 0.1 or 1.0 mM Ca^{2+}. The reaction mixture contained 20 mM histidine, pH 7.03, 100 mM KCl, 3 mM Tris-ATP, 3 mM $MgCl_2$ and 10 μg purified SR membrane fragments. Control samples contained ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) instead of Ca^{2+}. Inorganic phosphate was determined using a colorimetric method, as described previously (Baginski *et al*., 1967).

Statistical analysis

Mean values are shown and differences between means were assessed with the unpaired *t*-test. The number of experiments and the *P*-values obtained are shown in the legends to the figures, as appropriate. All procedures were taken from Graph Pad Prism.

Materials

Highly purified curcumin (Cat-C7727) and histone H1 (Cat-4524) were purchased from Sigma–Aldrich (St Louis, MO, USA). The enhanced chemiluminescence (ECL) reagents and [^{32}P]-ATP (Cat-AA0068, specific activity 3000 Ci. mmol^{-1}) were from Amersham Biosciences. Rat brain PKC holoenzyme, purified catalytic subunits of PKC, as well as recombinant PKC isoforms and substrate peptides were from CalBiochem (San Diego, CA, USA). Lipids were from Avanti polar lipids. All other chemicals were of the highest analytical grade available.

Results

Effect of curcumin on PKC in a membrane environment

Intact cells (Liu *et al*., 1993), as well as mixtures containing purified proteins (Reddy and Aggarwal, 1994) were used previously to show inhibition of PKC activity by curcumin. In the present study, we discovered that curcumin treatment activated PKC phosphorylation of a membrane-bound substrate (see below). We therefore investigated the effect of curcumin on PKC phosphorylation of histone in the absence or presence of PS-containing liposomes. As seen in Figure 1, curcumin inhibited PKC activity in the presence of PS alone (Figure 1a) but significantly increased it in the presence of PS in vesicles containing 10 mol% PS with phosphatidyl choline (PC) (60 mol%) and cholesterol (30 mol%) (Figure 1b), showing that the membrane is indeed a cofactor that determines the functional effects of curcumin on PKC activity. The C2 domain of classical PKCs binds to membranes through Ca^{2+} bridging to PS. In the absence of membranes, limited availability of PS in the lipid micelle might decrease PKC activity at a given Ca^{2+} concentration. To confirm that the inhibition produced by curcumin in the absence of membranes is not due to the limited availability of PS in this system, we increased the concentration of PS in the mixture by threefold (from 4 to 12 μg per phosphorylation reaction), which showed similar inhibition to that observed in Figure 1a (data not shown), indicating that the inhibitory effect of curcumin is not due to diluted PS in the

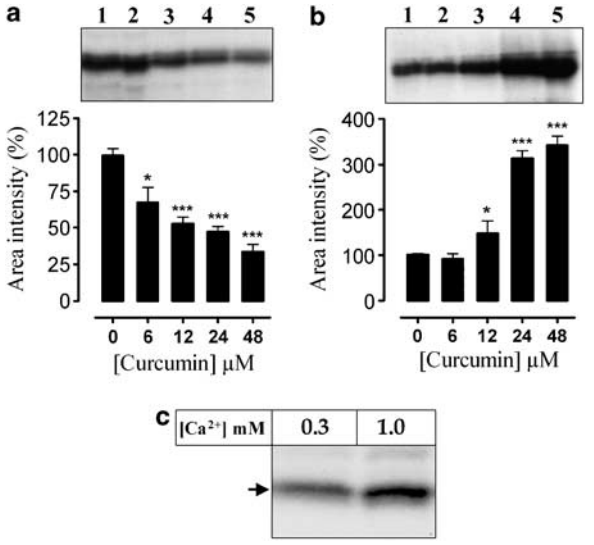

Figure 1 Curcumin effects on PKC are membrane-dependent. (**a**) Upper panel. A representative autoradiogram showing PKC phosphorylation of histone in the absence of phospholipid vesicles but in the presence of 4 μg PS and a range of concentrations of curcumin. Phosphorylation was performed as described in Materials and methods. Phosphorylated proteins in sample buffer were loaded onto SDS-gels and electrophoresis run overnight. The dry gels analyzed by autoradiography at −80°C. The experiment was performed twice (each in duplicate) and gave similar results each time. Lower panel: a representative histogram of the area intensity of the phosphorylated bands, expressed as decreases in PKC activity relative to that without curcumin (100%). Data are mean ± s.e.m. of double determinations. The Ca^{2+} concentration was ~260 μM. Data were analyzed using unpaired *t*-test, *$P<0.01$; ***$P<0.0001$. (**b**) Upper panel: a representative autoradiogram showing PKC phosphorylation of histone in the presence of 40 μg PC–Chol–PS (60/30/10 mol%, respectively) phospholipid vesicles, otherwise the experiment was identical to that in panel a. The experiment was performed twice (each in duplicate) and gave similar results each time. Lower panel: a representative histogram of the area intensity of the phosphorylated bands, showing the changes in PKC activity. Data are mean ± s.e.m. of double determinations. (**c**) PKC phosphorylation of histone was measured as described in panel a but in the presence of 24 μM curcumin, 4 μg PS, and either 0.3 or 1 mM Ca^{2+}, as indicated.

micelle system. Interestingly, when the Ca^{2+} concentration was increased threefold (in the absence of membrane vesicles), the inhibition of histone phosphorylation was partially reversed (Figure 1c), indicating the involvement of Ca^{2+} in the activation process (see below). To further investigate the role of the membrane in the curcumin-mediated activation of PKC, we considered the possibility that curcumin affects PKC activity by modulating its interaction with the PC rather than the membrane. To test this possibility, we performed phosphorylation experiments in which the PC–Chol membrane vesicles were treated with a solubilizing concentration of the non-ionic detergent $C_{12}E_8$ (1 mM) before the phosphorylation incubation. This showed no stimulation of PKC activity by curcumin in the presence of the soluble lipid alone (data not shown).

It was also of interest to investigate the effect of curcumin on PKC phosphorylation of a membrane-bound substrate. It is well documented that the catalytic (α) subunit of Na,K-ATPase is phosphorylated by PKC at its N-terminal domain (Feschenko and Sweadner, 1997; Mahmmoud *et al*., 2000;

Mahmmoud and Cornelius, 2002). Thus, we performed PKC phosphorylation assays in the presence of increasing concentrations of curcumin, using membrane-bound Na,K-ATPase as a PKC target and the phosphorylation intensity as a function of curcumin concentration was investigated by autoradiography. The intensity of the Coomassie-stained α-subunits in the gel were determined to ensure that equal amount of protein was loaded in each lane. As seen in Figure 2, curcumin also stimulated PKC-catalyzed phosphorylation of the Na,K-ATPase α-subunit (stimulation reached ~80% at 48 μM curcumin). Interestingly, PKC autophosphorylation was also stimulated by curcumin treatment.

Oxidative modification has been considered as a putative regulatory mechanism for modulation of PKC activity (Gopalakrishna and Jaken, 2000). As curcumin is an antioxidant (Joe *et al.*, 2004), it is possible that the effects of curcumin might be related to modulation of the redox state of PKC. If curcumin exerts its effects merely through preventing oxidative modification of PKC, then addition of reducing agents should abolish (or at least reduce) the curcumin effects. As seen in Figure 3, addition of 1 mM cysteine to the phosphorylation mixture did not abolish the stimulatory effect of curcumin on PKC phosphorylation of the Na,K-ATPase. Indeed, addition of cysteine slightly enhanced the stimulatory effect of curcumin (Figure 3).

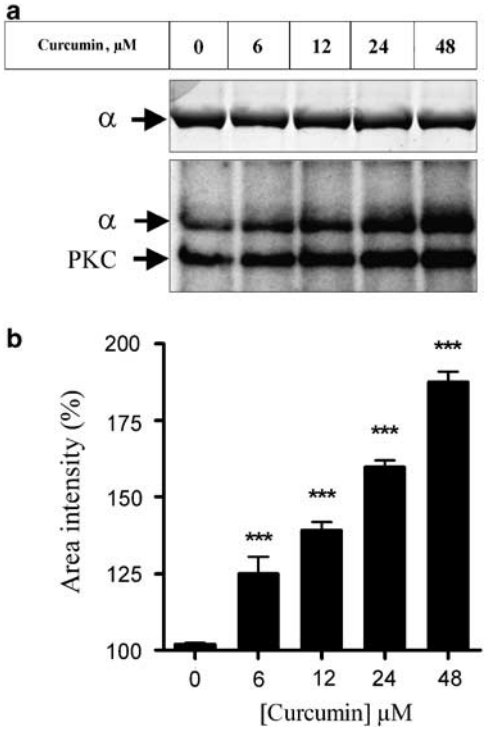

Figure 2 Curcumin activates PKC phosphorylation of Na,K-ATPase in native plasma membranes. (**a**) A representative autoradiogram showing stimulation of PKC phosphorylation of the Na,K-ATPase α-subunit by curcumin. Phosphorylated proteins in sample buffer were loaded onto SDS-gels and electrophoresis was run overnight. Dry gels were subjected to autoradiography analysis and the phosphorylation levels were estimated by measuring the intensity of the bands. The upper panel depicts the Coomassie-stained α-subunit bands, which have the same intensity, indicating loading of equal amounts of the protein in each lane. Shown is a representative of three independent experiments. (**b**) A representative histogram showing mean ± s.e.m. ($n=2$) of the changes in PKC phosphorylation intensity, expressed as a percentage of control (without curcumin but containing only the same volume of dimethyl sulphoxide (DMSO)). Data were analyzed using unpaired *t*-test; ***$P<0.0001$.

Curcumin competes with Ca^{2+} for the Ca^{2+}-binding domain of PKC

The result obtained in Figure 1c as well as the requirement for membranes to show stimulation of PKC activity by curcumin suggested that Ca^{2+} is involved in the activation process (see Discussion). We therefore investigated the effect of Ca^{2+} on phosphorylation of histone by PKC, in the absence or in the presence of curcumin. As seen in Figure 4, curcumin increased the EC_{50} for Ca^{2+} activation by almost threefold (from 28 ± 4.4 μM for control to 92 ± 8.5 μM for the curcumin-treated mixtures). This is consistent with a threefold decrease in the affinity for Ca^{2+} activation. On the other hand, the inhibitory effect of curcumin was found to diminish at higher Ca^{2+} concentrations, consistent with competition.

In addition to the experiments described in Figure 4, we measured the curcumin dependence of PKC activity at fixed Ca^{2+} concentrations. Thus, we repeated the phosphorylation assays described in Figure 2, but in the presence of 25, 50 or 250 μM Ca^{2+}. A representative result is depicted in

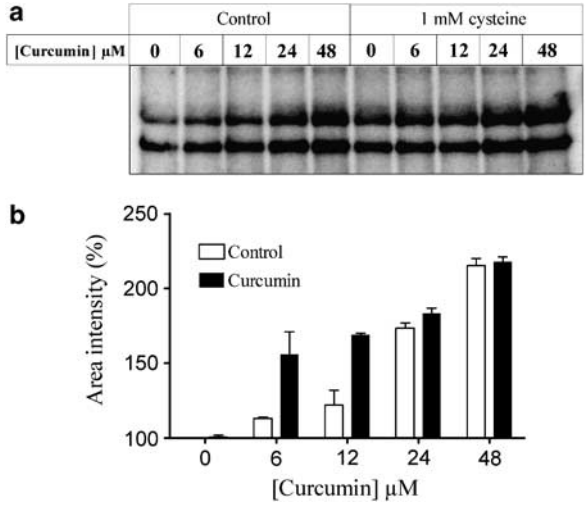

Figure 3 Cysteine does not abolish the stimulatory effect of curcumin on PKC. (**a**) Autoradiogram showing stimulation of PKC phosphorylation of Na,K-ATPase by curcumin, in the absence or in the presence of 1 mM cysteine, as indicated. Phosphorylated proteins in sample buffer were loaded onto SDS-gels and electrophoresis was run overnight. Dry gels were subjected to autoradiography analysis and phosphorylation levels were estimated by measuring the intensity of the bands. Shown is a representative of two independent experiments. (**b**) A representative histogram showing the changes in PKC phosphorylation intensity as a percentage of control (containing only the same volume of DMSO); there was additive stimulation of PKC activity by cysteine at low curcumin concentrations. Data are mean ± s.e.m. ($n=2$).

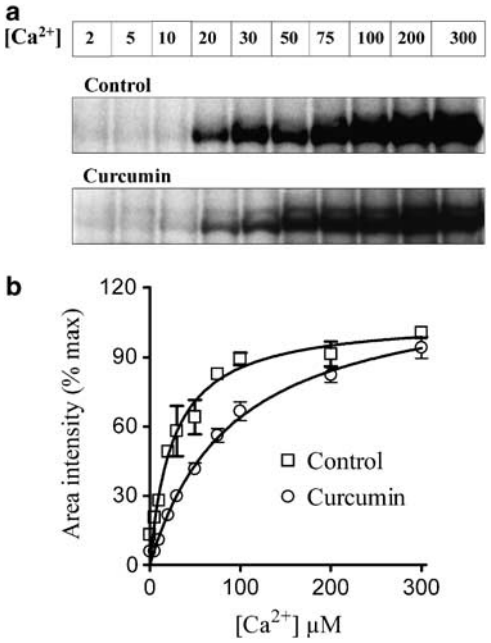

Figure 4 Curcumin competes with Ca^{2+} for the regulatory domain of PKC. (**a**) A representative autoradiogram showing PKC phosphorylation of histone measured in the presence of 4 μg PS and the Ca^{2+} concentrations shown in the figure. Phosphorylation was performed in the presence of either DMSO (control) or 20 μM curcumin, as indicated. Phosphorylated proteins in sample buffer were loaded onto SDS-gels and the dry gels were analyzed by autoradiography. A representative result from one of the three independent experiments is shown. (**b**) A graph depicting the increase in PKC activity as a function of Ca^{2+} concentration for control and curcumin-treated mixtures. Data are mean$\pm$s.e.m. ($n=3$) and expressed as percentage of control (phosphorylation performed in the presence of 0.1 mM EGTA). Analysis of the data using a hyperbolic function gave EC_{50} for Ca^{2+} activation of 28.0$\pm$4.4 μM, for the control conditions and 92$\pm$8.5 μM for the curcumin-treated mixtures. Data were analyzed by *t*-test. Mean values were significantly different, $P=0.0002$.

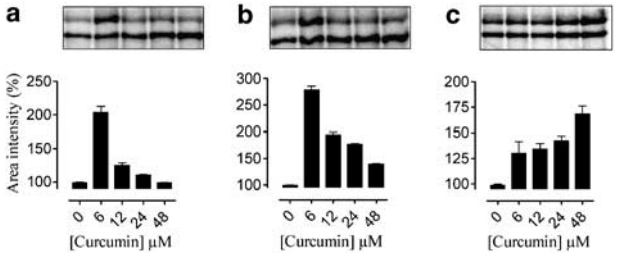

Figure 5 Direct demonstration of the biphasic effect of curcumin on PKC. A representative autoradiograms showing the effect of curcumin on PKC phosphorylation of the catalytic subunit of Na,K-ATPase, measured in the presence of 25 μM Ca^{2+} (**a**), 50 μM Ca^{2+} (**b**) and 250 μM Ca^{2+} (**c**). The experiments performed as described in the legend to Figure 2. The area intensities of the phosphorylated bands were determined and plotted in histograms (lower panels), demonstrating the interplay between curcumin and Ca^{2+} in modulating the PKC-mediated phosphorylation.

Figure 5, demonstrating that curcumin has a biphasic effect at a suboptimal Ca^{2+} concentration (25 μM), where low curcumin concentrations stimulated kinase activity, and increasing the curcumin concentration to 12 μM abolished the initial stimulation produced by 6 μM curcumin (Figure 5a). At 50 μM Ca^{2+}, the inhibition by the higher curcumin concentrations was only moderate (Figure 5b) compared to that observed at 25 μM Ca^{2+} (Figure 5a). On the other hand, when the concentration of Ca^{2+} was in excess of that of curcumin (250 μM), only activation is observed (Figure 5c), demonstrating unambiguously that Ca^{2+} modulates the curcumin effect on PKC. An interesting observation is that, in the presence of membrane vesicles, no inhibition of control PKC activity could be seen. Inhibition by curcumin of PKC in the presence of membranes will possibly require higher concentrations of curcumin, than those used here.

The light absorbance spectrum of a given molecule significantly changes following the addition of ions that specifically interact with the molecule (Atwood *et al*., 1998). Curcumin was previously shown to directly bind the divalent cations copper and iron, as estimated from the changes in absorbance of light upon mixing curcumin with these ions (Baum and Ng, 2004). In this regard, the possibility arises that curcumin might simply act as a Ca^{2+} chelator (without interacting with PKC), which would decrease the free Ca^{2+} concentration in the medium leading to inhibition of PKC activity. Therefore, we tested this possibility by measuring the absorption maxima of curcumin solution in the presence of Ca^{2+}, as reported previously (Baum and Ng, 2004). Applying different conditions of varying buffer, curcumin and Ca^{2+} concentrations, we were unable to demonstrate any changes in the absorption spectrum of curcumin following the addition of Ca^{2+} (data not shown).

To substantiate further a role for Ca^{2+} in modulation of the effect of curcumin on PKC, we investigated the effect of curcumin on the phosphorylation of Na,K-ATPase by the catalytic subunit of PKC. The latter is obtained by proteolytic cleavage in the hinge region between the two subunits (Kishimoto *et al*., 1989). The catalytic subunit of PKC is constitutively active and does not require Ca^{2+} or phospholipids for activation. Results in Figure 6 show that curcumin also stimulated the catalytic subunit of PKC. This stimulation was not influenced by the addition of 0.5 mM Ca^{2+} (data not shown).

Curcumin mildly enhances the interaction of PKC with ATP

To account for the mild stimulation of the catalytic subunit of PKC by curcumin, we investigated the possibility that curcumin would alter the apparent affinity of PKC for ATP. Thus, PKC phosphorylation assays for control and curcumin were performed, in the presence of increasing concentrations of ATP, and the phosphorylation intensity was calculated as described in Materials and methods. As depicted in Figure 7, a mild stimulation of PKC phosphorylation was observed following curcumin treatment.

Effect of curcumin on other PKC isoforms

The data above imply that curcumin inhibits the Ca^{2+}-dependent PKC isoforms by interfering with Ca^{2+} binding.

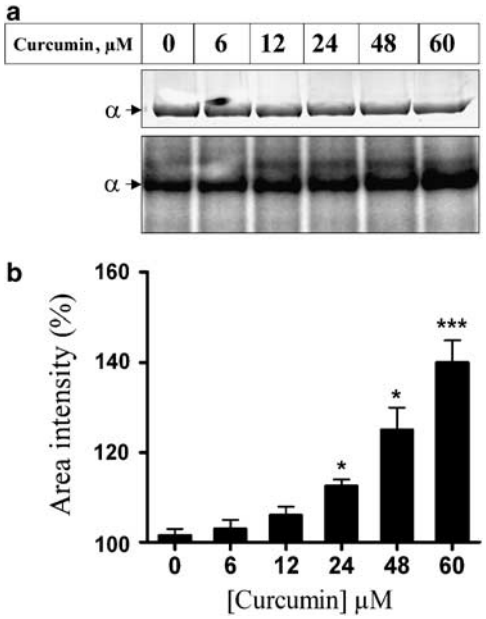

Figure 6 Curcumin stimulates the catalytic subunit of PKC. (**a**) A representative autoradiogram showing stimulation of the catalytic subunit of PKC by curcumin. PKC phosphorylation was performed as described in Materials and methods, in the presence of 4 μg purified membrane-bound Na,K-ATPase and the curcumin concentrations are shown. The upper panel depicts the Coomassie-stained α-subunit bands, having the same intensity at different curcumin concentrations. A representative result from one of two independent experiments, each performed in duplicate, is shown. (**b**) A histogram depicting the PKC phosphorylation intensity as a function of different curcumin concentrations, showing the stimulation by curcumin of the catalytic activity of PKC. Data are mean $\pm$ s.e.m. ($n=2$), and expressed as percentage of control (containing DMSO). Data were analyzed using unpaired *t*-test, $^{*}P<0.01$ and $^{***}P<0.0001$.

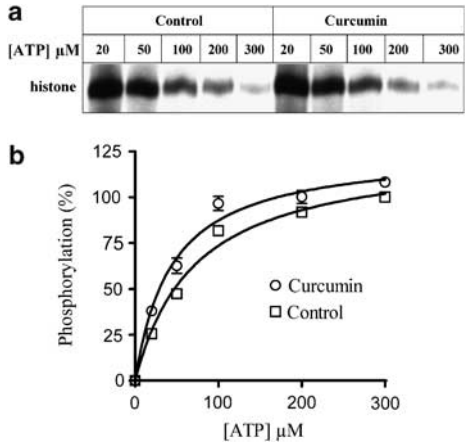

Figure 7 Curcumin increased PKC sensitivity to ATP. (**a**) A representative autoradiogram shows stimulation of PKC by ATP, measured in the absence or in the presence of 20 μM curcumin, as indicated. The phosphorylation reactions also contained 40 μg PC–Chol–PS lipid vesicles (60/30/10 mol%, respectively) and 260 μM Ca^{2+}. The concentration of cold ATP increased from 20 to 300 μM whereas the concentration of ^{32}P-ATP kept constant. The obtained area intensities were then multiplied by the fractional amount of unlabelled ATP, producing the relative amount of phosphorylation. Data shown are from one of two independent autoradiograms, each performed in duplicate. (**b**) Phosphorylation as a function of ATP concentration. Data are mean $\pm$ s.e.m. ($n=2$), and expressed as a percentage of control (containing only the same volume of DMSO).

We studied the effect of curcumin on two other PKCs: a novel and an atypical isoform, PKC_{θ} and PKC_{ζ}, respectively. If curcumin inhibits PKC by interfering with Ca^{2+} binding to the regulatory subunit, then it should not inhibit Ca^{2+}-independent PKC isoforms. As expected, curcumin only mildly stimulated the activity of the θ and ζ isoforms of PKC (Figure 8).

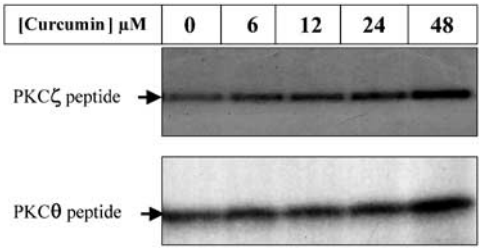

Figure 8 Curcumin stimulates some Ca^{2+}-independent PKC isoforms. Autoradiogram showing phosphorylation of specific substrate peptides of θ (novel) and ζ (atypical) isoforms of PKC, as indicated. The kinase was added to PKC phopshorylation mixture without Ca^{2+} together with substrate peptides and the reaction was performed as described in Materials and methods and in the legend to Figure 2.

Effect of curcumin on other Ca^{2+}-dependent enzyme

In order to support the conclusion that curcumin interferes with Ca^{2+} binding to target proteins, we needed to study the effect of curcumin on one of its targets, which is modulated by Ca^{2+} – the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA). Moderate curcumin concentrations ($>6\,\mu$M) were previously shown to inhibit SERCA in intact SR vesicles (Bilmen *et al.*, 2001; Logan-Smith *et al.*, 2001; Sumbilla *et al.*, 2002). In the absence of Ca^{2+} ionophores, Ca^{2+}-ATPase activity will depend on the rate of Ca^{2+} accumulation in the SR. In order to avoid using Ca^{2+} ionophores together with curcumin, we used broken SR membranes to measure the direct effect of curcumin on ATP hydrolysis by SERCA. As can be seen in Figure 9, a 10-fold increase in Ca^{2+} concentration not only increased the IC_{50} for curcumin inhibition, but also resulted in less inhibition of the Ca^{2+}-ATPase activity.

Discussion

Membranes and Ca^{2+} are cofactors that determine the functional effects of curcumin

By using well-defined phosphorylation assays and directly measuring phosphate incorporation into PKC substrates resolved on SDS-gels, we have provided evidence for the

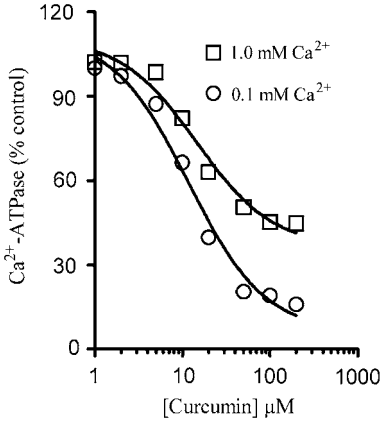

Figure 9 Increasing [Ca^{2+}] reduces curcumin inhibition of SERCA. Ca^{2+}-ATPase activity was measured as described in Materials and methods, in the presence of either 0.1 or 1 mM $CaCl_2$, as indicated. A sigmoid dose–response function was fitted to the data points, giving IC_{50} values for curcumin of $11 \pm 1.1\ \mu M$ at 0.1 mM Ca^{2+} and of $15 \pm 1.2\ \mu M$ at 1 mM Ca^{2+}. Data were analyzed by *t*-test. Mean values were significantly different, $P = 0.0019$.

interaction of curcumin with the Ca^{2+}-binding domain of PKC. Curcumin inhibited PKC activity at low Ca^{2+} concentrations, whereas activation was observed at saturating Ca^{2+} concentrations (Figure 1c). The curcumin interaction with PKC was also found to depend on the presence of membranes, where the addition of PS-containing membrane vesicles resulted in strong activation of PKC by curcumin (Figures 1b and 2). The Ca^{2+} and membrane effects might be explained in light of previous investigations on the role of these cofactors in PKC activation. Keranen and Newton (1997) reported a dual role for Ca^{2+} in PKC activation, where the concentration of Ca^{2+} promoting half-maximum membrane binding was found to be 40-fold higher than the apparent K_m for Ca^{2+} for PKC activation. In a series of studies (Bazzi and Nelsestuen, 1990, 1991), it was shown that PKC binds little Ca^{2+} in the absence of cell membranes, although the phosphorylation mixture contained $200\ \mu M$ Ca^{2+}. In the presence of membranes, the amount of Ca^{2+} bound to PKC increased by at least eightfold, and it was proposed that Ca^{2+}-binds with high affinity to PKC at the membrane interface. Thus, the most likely explanation for the membrane-dependent stimulation of kinase activity (Figure 1b) is a significant increase of Ca^{2+} binding to PKC observed in the presence of membranes. In the absence of membranes, it is to be expected that the Ca^{2+} concentration has to be high enough to overcome the inhibition produced by curcumin competition for the Ca^{2+}-binding domain (Figure 1c). Membrane-dependent activation might also explain the previously reported inhibition of PKC by curcumin measured in the presence of membrane-free mixtures (Reddy and Aggarwal, 1994). Measuring the effect of curcumin *in vivo* is complex because of the large number of cellular components that are modified by this molecule. However, measuring the curcumin effect on a single target permits obtaining definite information that can be extrapolated to understand the physiological effects of the molecule. In light of this, the previously reported inhibition of PKC in NIH 3T3 cells after curcumin treatment (Liu *et al.*, 1993) is probably not due to direct interaction of curcumin with the kinase. Such inhibition of PKC activity can be ascribed to a variety of reasons such as, for example, inhibition of Ca^{2+} influx into the cells following curcumin treatment (Dyer *et al.*, 2002), preventing PKC association with the plasma membrane and its subsequent activation.

Curcumin interacts directly with PKC and might have two binding sites on the kinase

The data in Figure 3 show that curcumin has direct effects on PKC distinct from being an antioxidant. In a previous report (Liu *et al.*, 1993), the inhibitory effect of curcumin was diminished after preincubation with thiol compounds, suggesting that the inhibition of PKC was indirect, by affecting the redox state of PKC. We believe that curcumin directly interacts with the kinase without affecting its redox state. Consistent with this, the effect of curcumin on membrane-bound Na,K-ATPase was found to be fully reversible (unpublished observations).

The stimulation of PKC by curcumin is likely to be due to a direct interaction of curcumin with the kinase, not the substrate. This was indicated from phosphorylation experiments in which different concentrations of histone were used, showing the same activation pattern observed in Figure 1b (data not shown). Interactions between PKC and its substrates have been shown to be important in PKC activation, as may be inferred from the differential effect of substrate on PKC autophosphorylation (Feschenko and Sweadner, 1994). In this regard, the activation by curcumin of PKC reported here was observed when using soluble substrate (Figure 1), intact membrane-bound substrate (Figure 2) or substrate peptides (Figure 8), suggesting that the modulation of kinase activity by curcumin is not related to kinase substrate interaction.

Cofactor binding to the regulatory domain of PKC induces long-range conformational changes that modulate major interdomain interactions. Curcumin seems to interact with the regulatory domain and disrupts the communication with the catalytic domain, which is normally facilitated by Ca^{2+} binding. The experiments shown here suggest that curcumin competes with Ca^{2+} for the regulatory domain of the kinase, producing inhibition at low Ca^{2+} concentrations. On the other hand, when the curcumin effect is abolished by a high Ca^{2+} concentration (or by addition of membranes, which in turn increases the Ca^{2+} affinity of the kinase), stimulation of kinase activity is observed. Activation of PKC *in vitro* requires the interaction of the enzyme with Mg^{2+}ATP, protein substrate, Ca^{2+}, anionic phospholipids and DAG (phorbol esters). The number of these cofactors and substrates, as well as their mutual interactions, precludes simple kinetic analysis of the enzyme mechanism. Inspite of the direct correlation between Ca^{2+} and curcumin stimulation of PKC, we cannot rule out the possibility that curcumin affects the interaction of PKC with the membrane, itself a Ca^{2+}-dependent process.

Curcumin treatment resulted in mild stimulation of the catalytic subunit of conventional PKC isoforms (Figure 6) as well as one novel and one atypical PKC isoform (Figure 8), which do not have Ca^{2+}-binding domains. Thus, the

stimulation by curcumin of the catalytic subunit of PKC might be accounted for by an increase in the apparent affinity for ATP. This result also showed that curcumin does not abrogate ATP binding to the kinase. Curcumin was found to abolish ATP binding to the SERCA), measured at 20 μM ATP (Bilmen *et al*., 2001). Although the stimulation of PKC isoforms ζ and θ is mild (Figure 8), we propose that curcumin binds at two sites on PKC: a major inhibitory site that interferes with Ca^{2+} binding and a stimulatory site located in the kinase domain.

Effect of curcumin on other targets suggests a common mechanism of action

Data from the literature support the conclusion that curcumin interferes with interactions of Ca^{2+} with target proteins. The molecular mechanism of curcumin action on SERCA and Na,K-ATPase has previously been studied in detail. Curcumin significantly reduces catalytic phosphorylation of SERCA (Bilmen *et al*., 2001; Sumbilla *et al*., 2002), thus inhibiting Ca^{2+}-ATPase transport activity (see also Figure 9). Ca^{2+}-binding at the intracellular side of the plasma membrane (and subsequent occlusion of two Ca^{2+} ions) stimulates transient phosphorylation of the Ca^{2+}-ATPase, giving a plausible explanation of why curcumin drastically reduces phosphorylation of SERCA. Interestingly, curcumin has the opposite effect on the closely related Na,K-ATPase (Mahmmoud, 2005), it increases enzyme phosphorylation but significantly reduces dephosphorylation that is dependent on K^+ binding at the extracellular side. In this regard, it has previously been shown that Ca^{2+} can bind at the K^+ binding site on the extracellular side of the Na,K-ATPase α-subunit (Shainskaya *et al*., 1998). Finally, curcumin was found to inhibit the inositol 1,4,5-trisphosphate receptor (Dyer *et al*., 2002).

Curcumin treatment has well-documented beneficial effects on many cell types (Surh, 2003; Joe *et al*., 2004; Singh and Khar, 2006) but how curcumin exerts its effects is largely unknown. Curcumin interference with Ca^{2+} binding to target proteins would provide an explanation to the diverse actions of this polyphenol. Thus, the chemopreventive action of curcumin can better be explained (at least in part) by considering the effects it could have on a global signalling molecule such as Ca^{2+}.

In summary, we have shown that curcumin competes with Ca^{2+} for the regulatory domain of PKC. This effect is dependent on a direct interaction between the kinase and the polyphenol. This is, to our knowledge, the first study that defines an interaction domain for curcumin. Further analyses of the interaction site of curcumin on some of its targets are in progress to gain more information about the minimum site requirements for interactions with curcumin.

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Conflict of interest

The author state no conflict of interest.

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