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Curcumin is a non-competitive and selective inhibitor of phosphorylase kinase

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Abstract

Recently, we reported that curcumin (diferuloylmethane) inhibits the growth of several different kinds of tumor cells. In order to investigate the mechanism of this inhibition, we examined the effects of curcumin on different protein kinases: highly purified protein kinase A (PkA), protein kinase C (PkC), protamine kinase (cPK), phosphorylase kinase (PhK), autophosphorylation-activated protein kinase (AK) and pp60$^{c\text{-}src}$ tyrosine kinase. While all kinases tested were inhibited by curcumin, only PhK was completely inhibited at relatively lower concentrations. At around 0.1 mM curcumin, PhK, pp60$^{c\text{-}src}$, PkC, PkA, AK, and cPK were inhibited by 98%, 40%, 15%, 10%, 1%, and 0.5%, respectively. Lineweaver–Burk plot analysis indicated that curcumin is a non-competitive inhibitor of PhK with a K_i of 0.075 mM. Overall, our results indicate that curcumin is a potent and selective inhibitor of phosphorylase kinase, a key regulatory enzyme involved in the metabolism of glycogen. This has important implications for the anti-proliferative effects of curcumin.

Key words: Curcumin; Phosphorylase kinase; pp60$^{c\text{-}src}$; Inhibitor; Protein kinase; Phosphorylation

1. Introduction

Curcumin (diferuloylmethane) is a major active component of the food flavor, turmeric (Curcuma longa). The anti-carcinogenic properties of curcumin in animals has been demonstrated by its inhibition of both tumor initiation induced by benz[α]pyrene and 7,12 dimethylbenz[α]anthracene [1–4] and tumor promotion induced by phorbol esters [5,6]. Besides its anti-carcinogenic effects, curcumin exhibits anti-inflammatory properties in vivo [7–9]. The pharmacological safety of curcumin is demonstrated by its consumption for centuries of up to 100 mg/day by people in certain countries [7].

In vitro, curcumin inhibits neutrophil activation, suppresses mitogen-induced proliferation of blood mononuclear cells, inhibits mixed lymphocyte reaction and inhibits proliferation of smooth muscle cells [10,11]. Curcumin is also a potent scavenger of reactive oxygen species, protects hemoglobin from nitrite-induced oxidation to methemoglobin, and inhibits lipid peroxidation [12–14]. Some of these activities are also responsible for its ability to protect DNA from free radical-induced damage and to protect hepatocytes against various toxins [14,15].

The mechanism underlying these diverse effects of curcumin is not fully understood. Among the possibilities, it has been shown that the phorbol ester-induced transcriptional factor c-*jun*/AP-1, are suppressed by curcumin [16]. Recently, curcumin has been shown to be highly effective in inhibiting type 1 human immunodeficiency virus long terminal repeat-directed gene expression and virus replication [17]. We have recently reported that curcumin inhibits the growth of a wide variety of tumor cells (Gupta and Aggarwal, submitted). In an attempt to investigate the mechanism of action of curcumin, we examined its effect on the activities of various protein kinases. The results presented in this report indicate that, among the kinases tested, curcumin is a selective and non-competitive inhibitor of phosphorylase kinase.

2. Materials and methods

Cytosolic protamine kinase and autophosphorylation-activated protein kinase were a generous gift from Dr. Z. Damuni, Department of Biological Sciences, Unviersity of South Carolina, Columbia, SC. These protein kinase preparations were judged to be homogeneous based on SDS-PAGE and gel permeation chromatography. Phosphorylase kinase (PhK) (170 U/mg), phosphorylase *b*, catalytic subunit of protein kinase A (41 U/mg), histone H-1 and H-2B, protamine sulfate (salmine), myelin basic protein, curcumin, polyglutamic acid-tyrosine (4:1) and phosphatidyl-L-serine were from Sigma Chemical Co. Protein

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Abbreviations: PkA, protein kinase A; PkC, protein kinase C; cPK, protamine kinase; PhK, phosphorylase kinase; AK, autophosphorylation-activated protein kinase; pp60$^{c\text{-}src}$, cellular tyrosine kinase with a molecular weight of 60 kDa; TCA, trichloroacetic acid.

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kinase C (PkC) (1,200 U/mg) was from Calbiochem Corp. [γ-^{32}P]ATP was obtained from ICN Biomedicals Inc. pp60$^{c\text{-}src}$ (40 U/mg) was a generous gift from Dr. Raymond Budde of the M.D. Anderson Cancer Center, Houston, TX.

2.1. Protein kinase assays

Protein kinase A (PkA), PkC, and cytosolic protamine kinase (cPK) were assayed as described previously [18] with some modifications. Briefly, the assays were performed in 0.05 ml mixtures containing 25 mM Tris-HCl, pH 7.3, 10% glycerol, 1 mM benzamidine, 14 mM β-mercaptoethanol, 0.2 mM phenylmethyl sulfonyl fluoride, 100 μg/ml leupeptin, 4 μM microcystin, 2 μg/ml aprotinin, protein kinase, 50 μg histone H-1 (PkC) or histone H-2B or 100 μg protamine sulfate, 10 mM $MgCl_2$, and 0.2 mM [γ-^{32}P]ATP (200–500 cpm/pmol). The reaction was initiated by adding $MgCl_2$ and ATP. After 10 min incubation at 37°C, the reaction was terminated by the addition of 1 ml 10% trichloroacetic acid (TCA). Protein in the TCA-terminated mixtures was spun into pellets by centrifugation for 2 min in a Beckman centrifuge at 15,000 × g. The pellets were washed five times with TCA. 1 ml scintillant was added and the radioactivity was counted in a Packard liquid scintillation counter. Control tubes were treated in an identical manner except that protein kinase was excluded from the mixture.

PkC was assayed as described above except that the incubation mixture also included 0.5 mM $CaCl_2$ and 40 μg/ml phosphatidyl-L-serine.

Phk was assayed as described previously [19], with some modifications. The assay mixture contained 25 mM Tris-HCl, pH 7.3, 10% glycerol, 1 mM benzamidine, 14 mM β-mercaptoethanol, 0.2 mM phenylmethyl sulfonyl fluoride, 100 μg/ml leupeptin, 4 μM microcystin LR, 2 μg/ml aprotinin, protein kinase, 50 μg phosphorylase b and 0.5 mM $CaCl_2$. Following incubation for 10 min at 37°C, the reaction was terminated with 1 ml of 10% TCA and treated as described above.

The autophosphorylation-activated protein kinase was purified as a single polypeptide of apparent $M_r \approx 36{,}000$ [20]. Based on substrate specificities and chromatographic properties this enzyme was differentiated from all other known protein kinases. When incubated with ATP, AK rapidly undergoes autophosphorylation ($t_{1/2} \approx 0.5$–1 min) which leads to a 10-fold enhancement in its activity. Both, the autophosphorylation and activation were reversed by protein phosphatase 2A. It has also been reported recently that this new kinase phosphorylates and inactivates protein phosphatase 2A and could thus play an important role in the regulation of cellular processes by extracellular factors. In order to determine the activity of this kinase, it was first pre-activated and then assayed with myelin basic protein as substrate [20].

The activity of pp60$^{c\text{-}src}$ was determined as described previously with some modifications [21]. The incubation mixture (0.05 ml) contained 25 mM Tris-HCl, pH 7.3, 10% glycerol, 1 mM benzamidine, 14 mM β-mercaptoethanol, 0.2 mM phenylmethyl sulfphonyl fluoride, 100 μg/ml leupeptin, 2 μg/ml aprotinin, protein kinase, 50 μg polyglutamic acid- tyrosine (4:1), 10 mM $MgCl_2$ and 0.2 mM [γ-^{32}P]ATP (200–500 cpm/pmol). Following incubation at 37°C for 10 min the mixture was blotted onto filter paper and immediately immersed in 10% TCA. The paper was then washed with 10% TCA before counting radioactivity in the presence of scintillant.

One unit of protein kinase activity was defined as the amount of enzyme that incorporated 1 nmol of phosphoryl groups into substrate/min. To ensure linearity the extent of incorporation of phosphoryl groups was limited to < 1 nmol.

3. Results

Curcumin, or diferuloylmethane (Fig. 1), constitutes as much as 5% of the food flavor, turmeric (*Curcuma longa*). We examined the effect of different concentrations of curcumin on the activity of six different protein kinases. The result of this experiment (Fig. 2A) indicates that curcumin inhibited all the kinases examined but to different extents. At around 0.1 mM curcumin, PhK, pp60$^{c\text{-}src}$, PkC, PkA, AK, and cPK were inhibited by

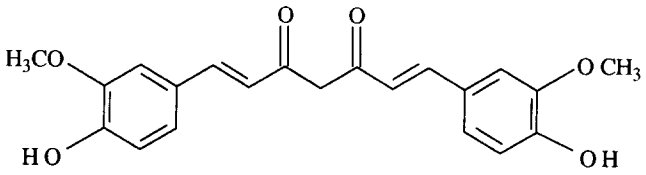

Fig. 1. Structure of curcumin (M_r 368).

98%, 40%, 15%, 10%, 1%, and 0.5%, respectively; however, higher concentrations of curcumin inhibited 98%, 95%, 46%, 49%, 17% and 2% of the activity, respectively. The inhibitory effect was dose-dependent. Because among all the six kinases PhK could be inhibited completely by a relatively lesser concentration of curcumin, this observation was further investigated in detail. The inhibitory effects of curcumin at lower doses on PhK is shown in Fig. 2B. Results indicate that the effect of curcumin could be noted at as low as 5 μM and it plateaued at around 0.3 mM. A reduced activity of PhK paralleled with the decrease in the phosphorylation of phosphorylase b as analyzed by SDS-PAGE (data not shown). The inhibitory effects of curcumin could be noted at ~5 μM, and no phosphorylated product was observed at 1.36 mM. The inhibition kinetics of PhK by curcumin was then analyzed by a Lineweaver–Burk plot (Fig. 3A) which indicated that curcumin is a non-competitive inhibitor of PhK. When the slope of each curve from Fig. 3A was re-plotted against different concentrations of curcumin a K_i of 0.075 mM was obtained (Fig. 3B).

4. Discussion

We report here that curcumin is an inhibitor of several different protein kinases. Among the kinases examined, only PhK was completely inhibited at relatively low concentrations of curcumin. Previously it was reported that curcumin could inhibit cellular PkC [22]. Our results indicate that inhibition of PkC by curcumin is only 40–50%. Why curcumin inhibits PkC, AK, PkA, and cPK only partially, whereas PhK is inhibited completely is not clear. Skeletal muscle PhK is a hexadecamer composed of four α, β, γ, and δ subunits [19]. The γ subunit is catalytic and shows significant homology to other protein kinases. The α, β, and δ subunits of PhK are regulatory, with δ being an integral calmodulin subunit. At least 90% of the enzyme mass is involved in its regulation. Therefore, it is possible that curcumin is maximally effective against PhK due to its size and multiple regulatory sites. Conformational change in the β subunit has been shown to eliminate the catalytic activity of the γ subunit completely [23]. Therefore it is possible that curcumin interacts with the β-subunit, resulting in a change in its conformation. Both the α and β subunits have been

shown to undergo phosphorylation in response to PkA. Besides kinase activity, PhK also displays ATPase activity [24]. Whether this activity and the autophosphorylation activity of PhK are also affected by curcumin is not clear at present.

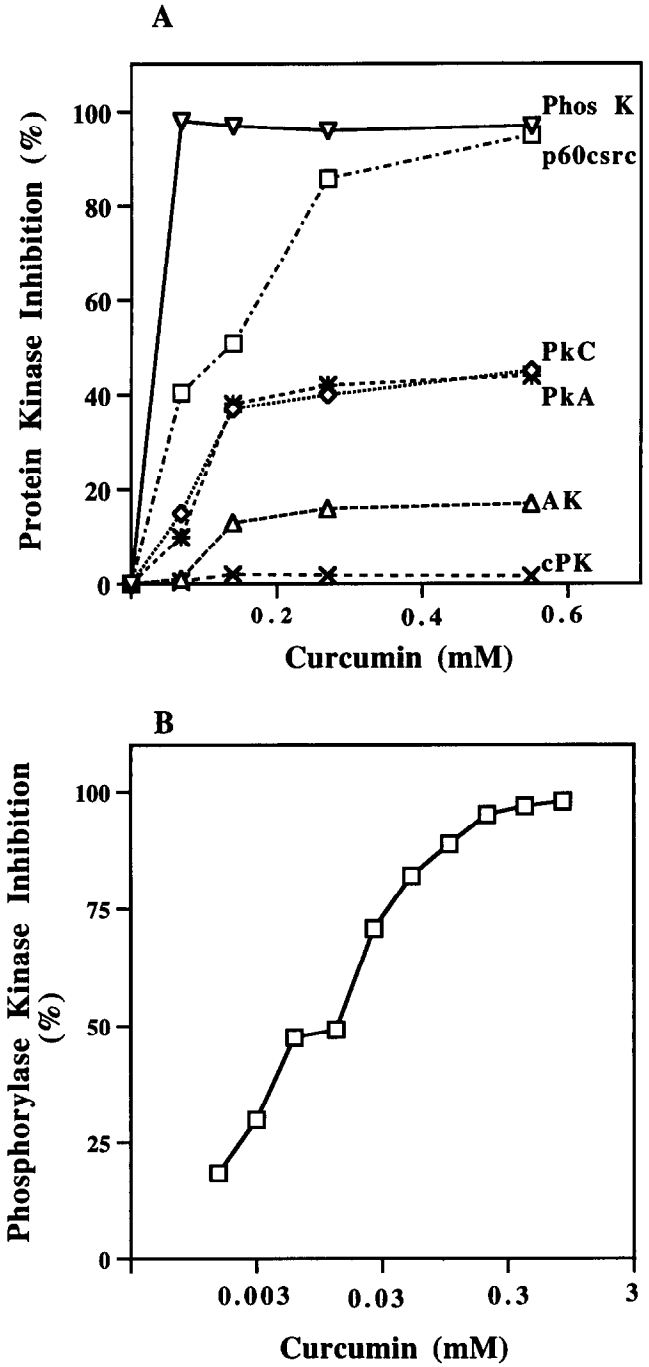

Fig. 2. (A) Effect of curcumin on the activities of various protein kinases. Preparations of PhK (▽, 134 U/ml), PkC (◇, 6.8 U/ml), PkA (*, 5 U/ml), cPK (×, 500 U/ml), AK (△, 500 U/ml) and pp60$^{c\text{-}src}$ tyrosine kinase (□, 8 U/ml) were assayed with phosphorylase *b*, histone H-1, histone H-2B, protamine sulfate, myelin basic protein and polyglutamic acid-tyrosine as substrates, respectively, as described in section 2 in the presence of the indicated concentrations of curcumin. Controls were treated in an identical manner except that dimethylsulfoxide was substituted for curcumin. (B) Dose–response of phosphorylase kinase with curcumin. Phosphorylase kinase (134 U/ml) was assayed with phosphorylase *b* as described in section 2 in the presence of the indicated concentrations of curcumin.

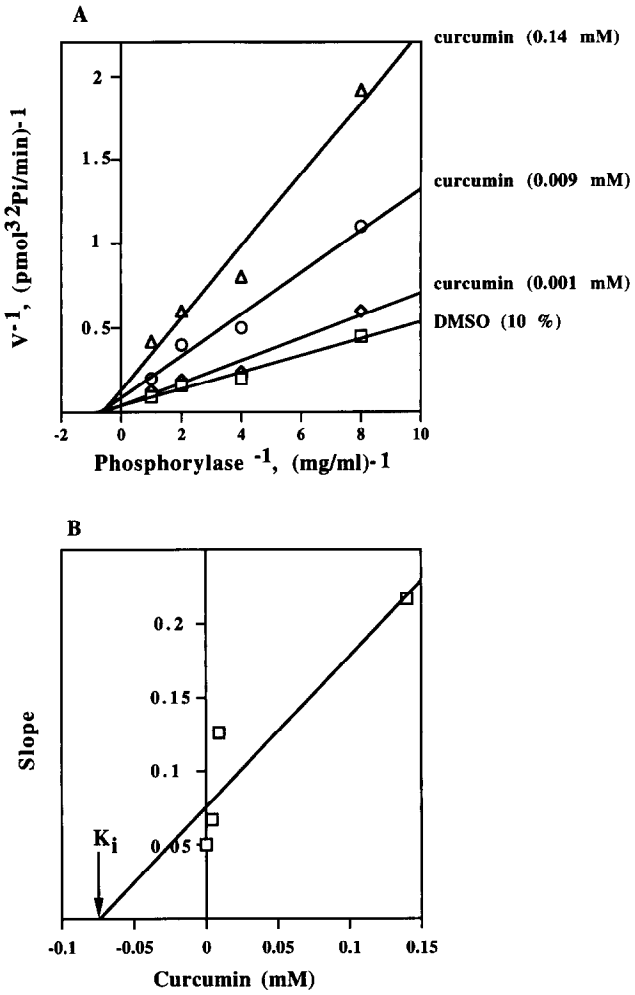

Fig. 3. (A) Lineweaver–Burk plot analysis of the inhibition of phosphorylase kinase by curcumin. The activities of phosphorylase kinase (0.03 U) were determined as described in section 2. The incubations contained various concentrations of curcumin as indicated. With each set of curcumin concentrations, the concentration of phosphorylase *b* was varied. The other two substrates, viz. Mg^{2+} and ATP, were present at saturating levels (2 mM and 0.2 mM, respectively). The rates of each reaction were calculated as pmol of ^{32}P incorporated into phosphorylase *b* per min. The reciprocal values of these velocities are plotted against reciprocal concentrations of phosphorylase *b* as a Lineweaver–Burk plot. (B) The slopes of the lines derived from the double reciprocal plot are plotted against the relevant concentrations of curcumin in order to derive the K_i value for curcumin.

Near complete inhibition of pp60$^{c\text{-}src}$ tyrosine kinase was also observed but at much higher concentrations of curcumin than that required for the total inhibition of phosphorylase kinase. This result could significantly reflect the ability of this compound to inhibit tyrosine phosphorylation of proteins and thus form a major part of its mechanism of action. Whether curcumin can inhibit the activities of receptor tyrosine kinases as well remains to be determined.

Recently, curcumin has been shown to down-modu-

late the expression of the c-*jun*/AP1 gene and also inhibit the replication of human immunodeficiency virus [16,17]. Since both of these activities have been shown to be kinase-dependent [25–27], it is possible that the effects of curcumin in these systems is also due to its effects on protein kinases. Because curcumin has been shown to inhibit tumor initiation and its promotion by carcinogens [1–6], it is possible that these effects are also mediated through the inhibition of protein kinases.

We have shown recently that curcumin could inhibit the growth of a wide variety of tumor cells, whereas normal cells were found to be relatively resistant (Gupta and Aggarwal, submitted). Whether the suppressive effects of curcumin on tumor cells is due to the inhibition of protein kinases described here is not certain. Since PhK is a key regulatory enzyme involved in glycogen metabolism it is possible that the inhibition of this enzyme may block the autocrine action of growth factors needed for cellular proliferation. Kinase inhibitors have been shown to display anti-proliferative activity in vitro and anti-tumor activity in vivo [28,29]. Because of its well-established pharmacological safety in mice and humans [7] and its in vitro anti-proliferative activity against tumor cells, curcumin may also display in vivo anti-tumor activity.

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