Pergamon S0031-9422(96)00091-X

Phytochemistry, Vol. 42, No. 3, pp. 599–605, 1996

0031-9422/96 $15.00 + 0.00

INHIBITION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE BY CURCUMIN

M. HASMEDA and G. M. POLYA*

School of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia

(*Received in revised form* 11 *December* 1995)

Key Word Index—*Curcuma longa*; Zingeberaceae; curcumin; protein kinase inhibitor.

Abstract—Curcumin [diferuloylmethane; 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], a major bioactive secondary metabolite found in the rhizomes of turmeric (*Curcuma longa*), is an inhibitor of Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) and of the catalytic subunit (cAK) of cyclic AMP-dependent protein kinase (IC_{50} values 15 and 4.8 μM, respectively). Curcumin inhibits plant Ca^{2+}-dependent protein kinase (CDPK) (IC_{50} 41 μM), but does not inhibit myosin light chain kinase or a high affinity 3′,5′-cyclic AMP-binding phosphatase. Curcumin inhibits cAK, PKC and CDPK in a fashion that is competitive with respect to both ATP and the synthetic peptide substrate employed. The IC_{50} values for inhibition of cAK by curcumin are very similar when measured with kemptide (LRRASLG) (in the presence or absence of ovalbumin) or with casein or histone III-S as substrates. However, the presence of bovine serum albumin (0.8 mg ml^{-1}) largely overcomes inhibition of cAK by curcumin.

INTRODUCTION

Plants elaborate a variety of defensive metabolites of which 1000s have so far been resolved and characterized [1, 2]. A variety of such plant defensive compounds are of medicinal importance, and high affinity biochemical sites for action have been established for such compounds [3, 4]. Nevertheless, the high affinity biochemical targets of most plant defensive metabolites have yet to be determined.

A variety of natural products, including plant defensive compounds, can interact with second messenger-regulated protein kinases [5–17]. Thus, a variety of flavonoids inhibit cyclic AMP-dependent protein kinase catalytic subunit (cAK) [10, 11], Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) [12] and myosin light chain kinase (MLCK) [10]. However, flavonoids in general are very poor inhibitors of plant Ca^{2+}-dependent protein kinase (CDPK) [10]. Other plant defensive compounds that inhibit cAK (as well as some other protein kinases) include certain xanthones [13], anthraquinones [14], condensed flavans [15] and gallic acid esters [16]. The potent inhibition of cAK by a large number of plant defensive compounds is consistent with the apparent absence of this enzyme from plants [18].

Some plant defensive compounds selectively inhibit protein kinases other than cAK. Thus, the benzophenanthridine alkaloid chelerythrine [17] is a relatively selective inhibitor of PKC. Curcumin (diferuloylmethane), a major bioactive secondary metabolite from rhizome of turmeric (*Curcuma longa*) and the other spice plants *C. aromatica* and *C. xanthoriza* (Zingerberaceae) [2, 19], is a competitive and selective inhibitor of phosphorylase *b* kinase [19]. Curcumin is a relatively poor inhibitor of cAK and PKC when histone H2B and histone H1, respectively, are used as substrates [19]. However, the present paper shows that curcumin is an effective competitive inhibitor of both cAK and of PKC when the target enzymes are assayed using synthetic peptide substrates.

*Author to whom correspondence should be addressed.

RESULTS

As part of a continuing survey of edible and medicinal plants for the occurrence of protein kinase inhibitors [20], we found that fresh turmeric root contains a large amount of cAK inhibitory activity, namely 1.1×10^6 cAK inhibition units g^{-1} fresh weight (where one inhibition unit is the amount of material causing 50% inhibition of cAK activity as measured in the standard assay conditions). Purification of the active component of fresh turmeric roots by a HPLC-based procedure resolved one major peak of methanol-soluble material having cAK inhibitory activity. This material has the same ES mass spectral-derived mass (368.2 Da) as calculated for curcumin (368.39 Da). The UV–visible spectra (200–500 nm) of this material and of commercially obtained curcumin are identical. The purity of the curcumin standard used (Fluka) was confirmed by HPLC, ES mass spectrometry and ^{1}H NMR.

When non-fresh, commercial, powdered turmeric was used as the starting material, three peaks of curcumin-related material were resolved by reversed phase HPLC involving elution of a C18 column with a linear gradient of increasing methanol concentration in H_2O (eluting at 93, 95 and 97% methanol, respectively). The mass spectrum showed average molecular masses of 368.2 Da for the material in all three peaks and we presume that these include storage-generated curcumin isomers.

Curcumin inhibits cAK, PKC and CDPK as measured in the standard assay conditions involving the synthetic substrates kemptide, epidermal growth factor receptor-derived synthetic peptide (EGFRP) and myosin light chain-based synthetic peptide (MLCP), respectively (Fig. 1). The IC_{50} values for curcumin are 4.8, 15 and 41 μM for cAK, PKC and CDPK, respectively. Curcumin inhibits cAK in a fashion that is competitive with respect to both ATP and the synthetic peptide employed (Fig. 2), the K_i value ($\pm$ standard deviation from four estimates) being 2.0 ± 1.1 μM. Similarly, curcumin competitively inhibits both PKC (Fig. 3) and CDPK (Fig. 4) with respect to both ATP and the synthetic peptide substrate used for each protein kinase, the K_i values being 15.0 ± 2.6 and 58 ± 35 μM for PKC and CDPK, respectively. The curcumin K_i values for cAK, PKC and CDPK are in agreement with the curcumin IC_{50} values for these enzymes.

It has been previously shown that curcumin is a very poor inhibitor of cAK and PKC when these enzymes are assayed with histone H2B and histone H1 as substrates, respectively (IC_{50} values *ca* 1 mM in both cases) [19]. Accordingly, we have examined the effect of protein substrate on such inhibition of cAK by curcumin. However, the IC_{50} values for inhibition of cAK by curcumin are very similar whether measured with 20 μM kemptide, 0.8 mg ml^{-1} dephosphorylated casein or 0.8 mg ml^{-1} histone III-S preparation as substrate (4.8, 2.3 and 6.0 μM, respectively) (Fig. 5). While inclusion of 0.8 mg ml^{-1} ovalbumin does not significantly change the IC_{50} for curcumin when cAK is assayed with kemptide as substrate, inclusion of 0.8 mg ml^{-1} bovineserum albumin (BSA) abolishes such inhibition (Fig. 5).

While curcumin is an effective inhibitor of cAK and PKC, little inhibition of MLCK is observed at 60 μM final concentration of curcumin. Since curcumin inhibits cAK, PKC and CDPK in a fashion that is competitive with respect to ATP (Figs 2–4), the possible effect of curcumin on a potato phosphatase that is competitively inhibited by the adenine nucleotide 3′-5′-cyclic AMP [21] was examined. However, 40 μM curcumin does not inhibit this high affinity cyclic nucleotide-binding phosphatase. Thus, curcumin exhibits specificity as well as potency as an inhibitor of cAK and PKC.

DISCUSSION

The present paper shows that curcumin is a relatively effective inhibitor of cAK when the enzyme is assayed with synthetic peptide substrate in the absence of proteins that can evidently interfere with curcumin binding such as certain histones [19] and BSA (Fig. 5). It is likely that these proteins either bind curcumin or occlude a curcumin-binding region on cAK. The same interpretation applies to lack of inhibition of PKC by low concentrations of curcumin in the presence of certain histones [19]. We have previously found that the

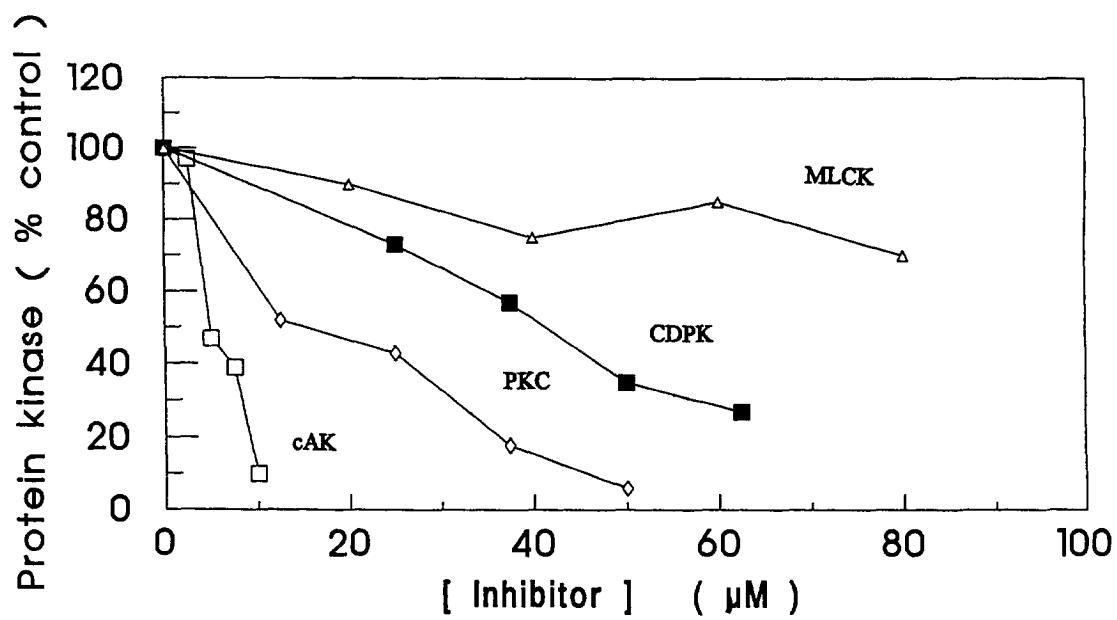

Fig. 1. Inhibition of cAK, PKC and CDPK by curcumin. PKC, cAK and CDPK were measured in standard conditions (see Experimental) with 20 μM EGFRP, 20 μM kemptide or 20 μM MLCP as substrate, respectively. Protein kinase activity is expressed as % of control (no added curcumin). □, cAK; ◇, PKC; ■, CDPK; △, MLCK.

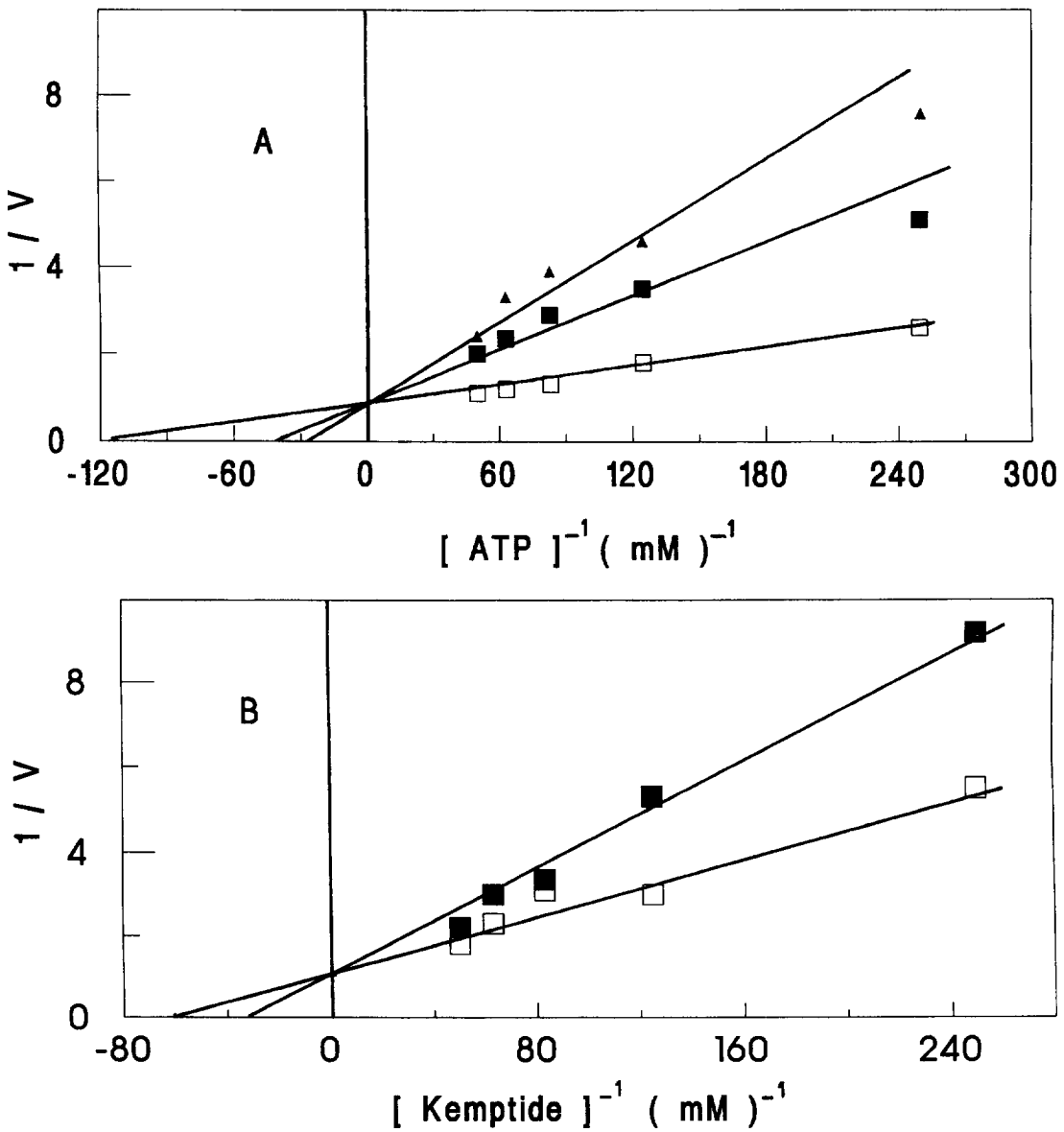

Fig. 2. Competitive inhibition of cAK by curcumin. Protein kinase was assayed in duplicate in standard assay conditions in the presence or absence of various concentrations of curcumin, and either ATP concentration (A) or peptide substrate concentration (B) was varied. □, No added curcumin; ■, 2 μM curcumin; ▲, 4 μM curcumin. The data as a whole in (A) and (B) are interpreted as indicating competitive inhibition of protein kinase with respect to ATP and the peptide substrate, respectively.

histone III-S preparation decreases flavonoid inhibition of cAK and inhibition of plant CDPK by certain gallic acid esters [16], but it does not significantly affect inhibition of plant CDPK by the xanthones γ-mangostin and mangostin [13].

A range of plant-derived flavonoids [10, 11], xanthones [13], anthraquinones [14], condensed flavans [15], gossypol [11], a variety of synthetic azaacridines [22], isoquinoline derivatives [8, 23], phenanthrene derivatives [24] and the microbial indole carbazole staurosporine and related compounds [8, 25] inhibit cAK, the common structural feature of these inhibitors being a planar, polycyclic ring structure substituted with particular polar residues [8, 10, 11, 13–15, 22–25]. However, the most potent plant-derived cAK inhibitors found so far are esters of gallic acid (3,4,5-trihydroxybenzoic acid) in which particular gallic acid residues are separated by about six carbon or oxygen atoms [16]. Curcumin resembles these gallic acid ester inhibitors in having two phenolic groups separated by a seven atom spacer. It is notable that certain non-phenolic plant defensive compounds, namely particular acidic triterpenoids and the acidic carotenoid crocetin, also inhibit cAK [26]. These compounds resemble curcumin in having polar residues separated by a spacer region [26].

Curcumin is a major component in the spice preparation turmeric used in curry and is relatively non-toxic

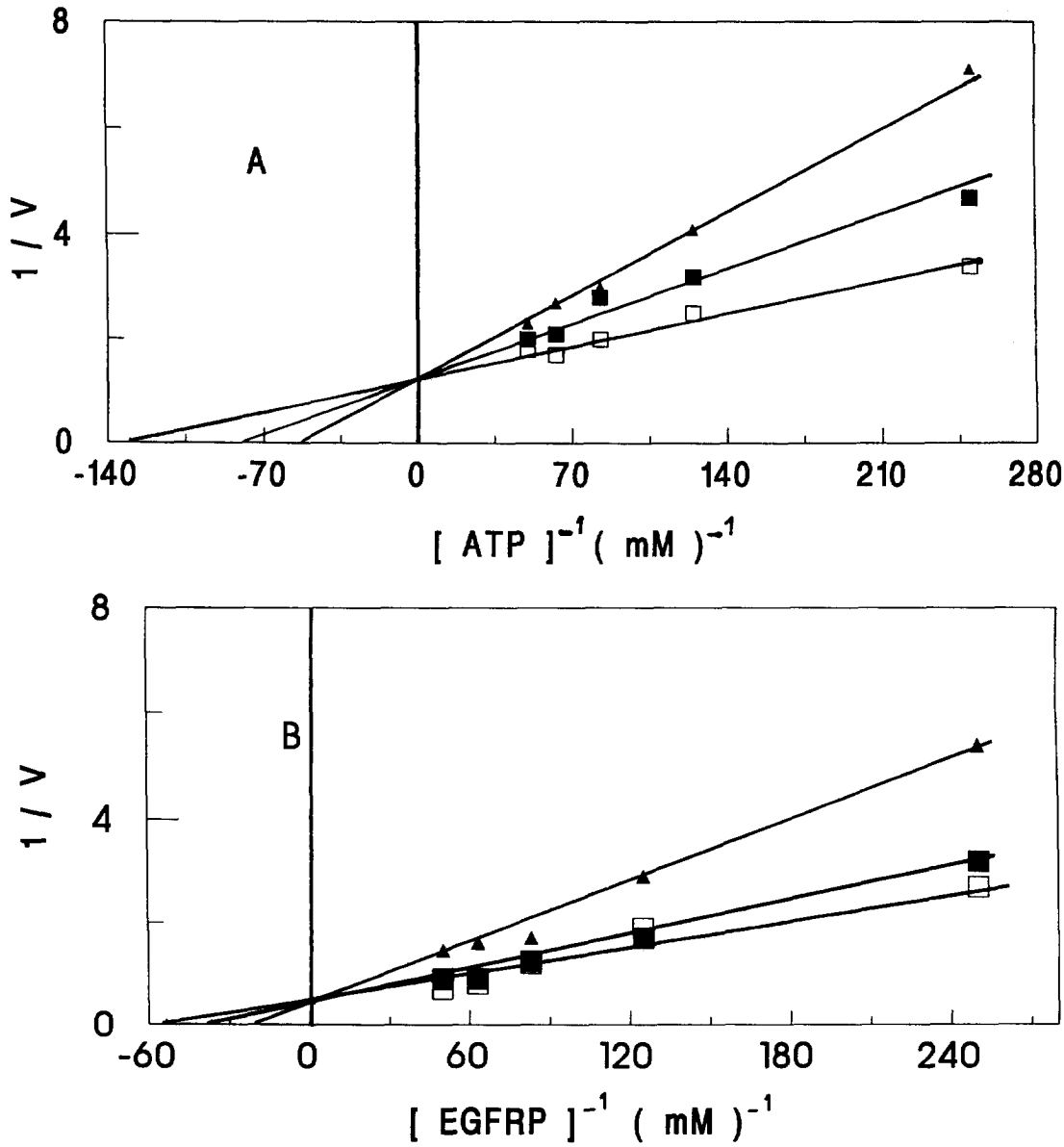

Fig. 3. Competitive inhibition of PKC by curcumin. □, No added curcumin; ■, 10 μM curcumin; ▲, 20 μM curcumin. Other details as for Fig. 2.

[27]. We have found that commercial powdered turmeric, but not fresh *C. longa* rhizome, contains several compounds with the same average M_r as curcumin and which we infer are curcumin isomers that are evidently produced during rhizome industrial processing and storage and which can be separated by HPLC. The structures of these components have not been determined.

Curcumin is an antioxidant [28, 29] and has anti-inflammatory [30], cytoprotective [31] and cytotoxic [31] effects. Curcumin is anti-carcinogenic, inhibiting tumour induction by particular carcinogens [32, 33] and inhibiting tumour promotion by phorbol esters [34, 35]. It is notable that other inhibitors of both cAK and PKC, namely the isoquinoline derivative H-7 [8, 9] and the flavonoid quercetin [11, 12], can inhibit tumour promotion by the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate [8, 36, 37]. Such inhibition of phorbol ester-induced tumour promotion by H-7, quercetin and curcumin has been related to inhibition of PKC by these compounds [8, 37]. The present paper shows that, as measured in the absence of interfering proteins, curcumin is a relatively potent inhibitor of both cAK and PKC. Curcumin is a further type of plant defensive compound that is an inhibitor of particular eukaryote signal regulated protein kinases. Inhibition of protein kinase-mediated signalling in the target organisms may contribute to the plant defensive effectiveness of curcumin and of other plant defensive secondary metabolites that are also protein kinase inhibitors [16].

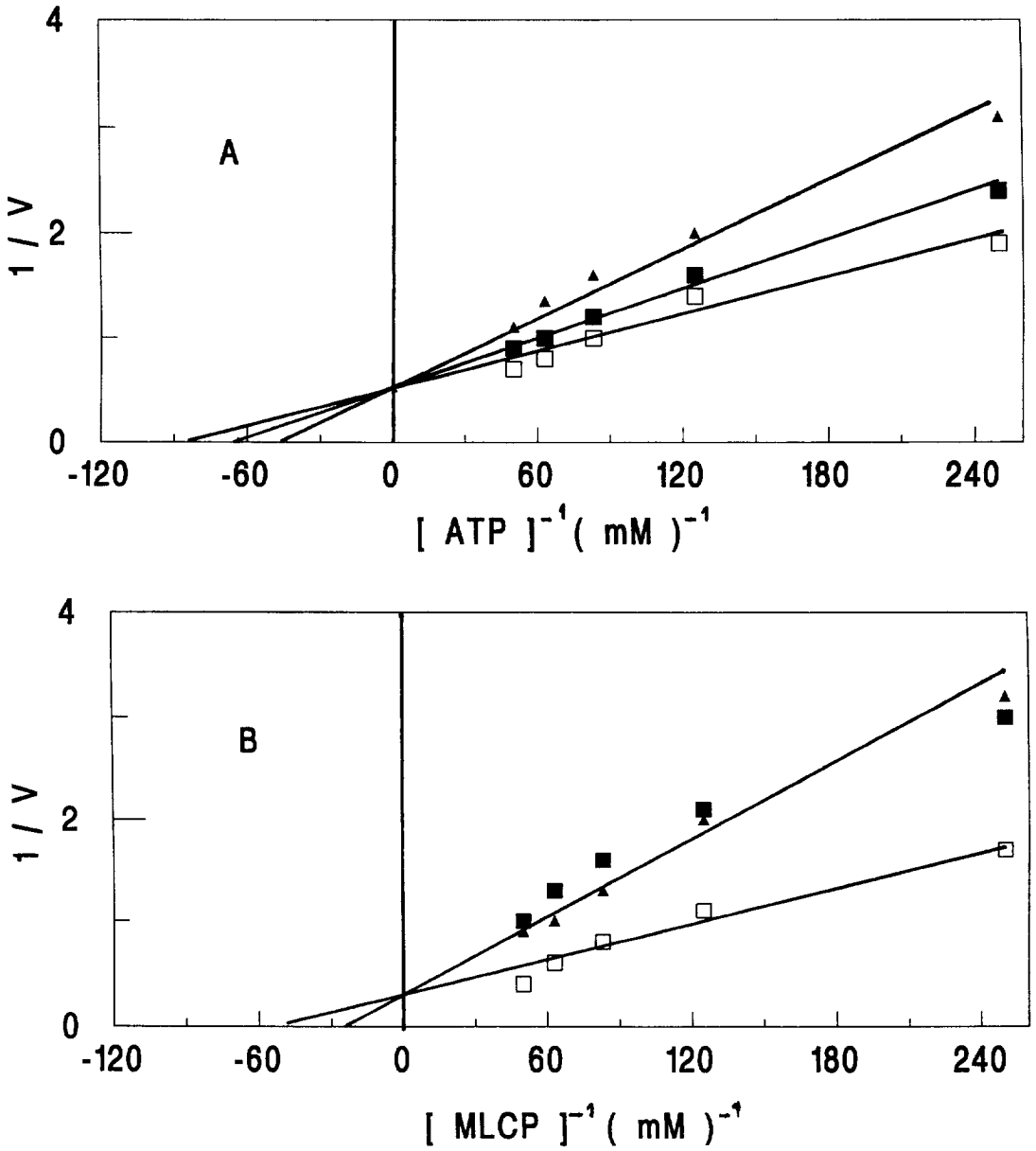

Fig. 4. Competitive inhibition of CDPK by curcumin. □, No added curcumin; ■, 30 μM curcumin; ▲, 50 μM curcumin. Other details as for Fig. 2.

EXPERIMENTAL

Materials. [γ-^{32}P]ATP (specific activity 4000 Ci mmol^{-1}) was obtained from Bresatec (Adelaide). Kemptide (LRRASLG), EGFRP (VRKRTLRRL-NH_2) and MLCP (KKRAARATSNVFA-NH_2) were obtained from Auspep (Melbourne). Curcumin was obtained from Fluka. All other compounds used were obtained from Sigma.

Isolation of curcumin from turmeric. Turmeric rhizome (10 g) was frozen in liquid N_2, ground in a pestle and mortar and then extracted in MeOH. After filtration and concn by rotevaporation, the MeOH extract was subjected to reversed phase HPLC on a C18 column (Vydak; 4.6 mm × 25 cm; 5 μm) eluted with a linear gradient of increasing MeOH concn (50–100% MeOH in 50 min; flow rate 1 ml min^{-1}). A single peak of material was obtained. UV–VIS spectra were obtained using a Carey IE UV/VIS spectrophotometer. ESMS of the isolated curcumin was conducted by introducing curcumin (20 μg in 10 μl MeOH–H_2O–HOAc, 99:99:2) at 3 μl min^{-1} into a VG B10-Q electrospray mass spectrometer (VG Biotech, Cheshire, U.K.). The first quadrupole of the mass spectrometer was repeatedly scanned from m/z 300–1000 (10 sec scan^{-1}).

Protein kinase and phosphatase isolation and assay. Rat brain PKC (sp. act. 0.6 μmol min^{-1} mg^{-1} protein with 3.5 μM EGFRP as substrate) chicken gizzard MLCK (sp. act. 0.05 μmol min^{-1} mg^{-1} protein with 20 μM MLCP as substrate), wheat embryo CDPK (sp.

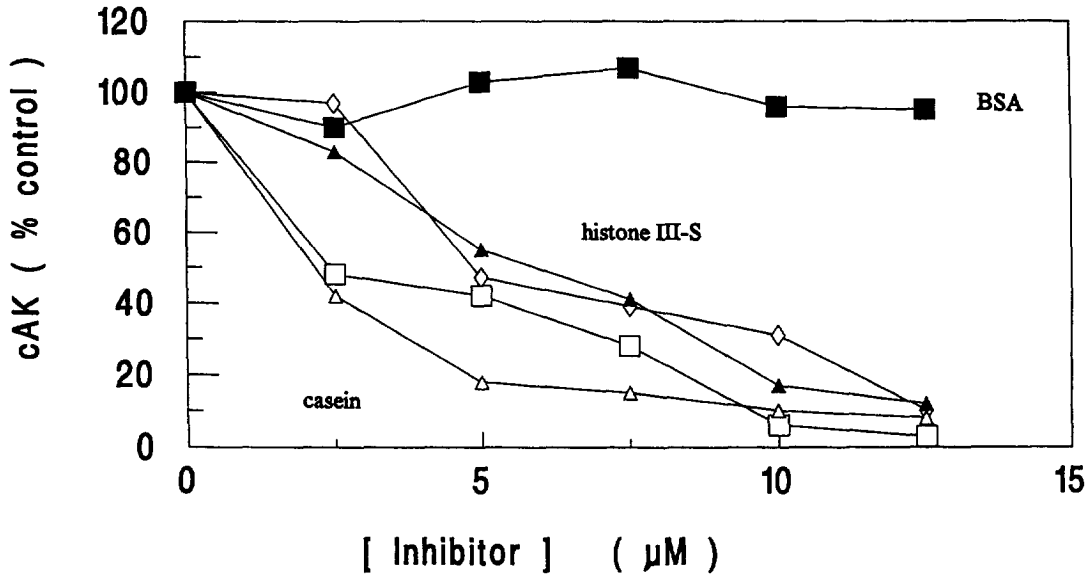

Fig. 5. Inhibition of cAK by curcumin. Protein kinase was assayed as described in the Experimental with the indicated substrates and with the further additions indicated in parentheses: ◇, 20 μM kemptide; □, 20 μM kemptide (plus 0.8 mg ml^{-1} ovalbumin); ■, 20 μM kemptide (plus 0.8 mg ml^{-1} BSA); △, 0.8 mg ml^{-1} dephosphorylated casein; ▲, 0.8 mg ml^{-1} histone III-S. Protein kinase activity is presented as % of control activity (no added curcumin).

act. 0.01 μmol min^{-1} mg^{-1} protein with 1 mg ml^{-1} histone type III-S as substrate) and rat liver cAK (sp. act. 0.3 μmol min^{-1} mg^{-1} protein with 20 μM kemptide as substrate) were extensively purified and assayed radiochemically in standard assay conditions at pH 8 and at 30° as described previously [15]. MLCK was assayed without the inclusion of BSA.

Inhibitor IC_{50} values (concns for 50% inhibition of particular protein kinases in standard assay conditions) were determined from interpolation of plots of protein kinase activity versus inhibitor concn. Control protein kinase activity (no added inhibitor) was determined ($\times 6$) and that assayed with inhibitor included was determined $\times 2$. All assay results were corrected by subtraction of blank values from assays conducted in absence of added protein kinase. Standard deviations associated with control protein kinase assays were *ca* 10% of mean values. Inhibitor compounds were routinely dissolved in MeOH and added to protein kinase assays to give the following final MeOH concns: 17% (v/v) (MLCK and PKC assays) and 20% (v/v) (cAK and CDPK assays). Control protein kinase assays (without added inhibitor) were conducted with inclusion of appropriate concn of MeOH.

Potato tuber phosphatase was purified to homogeneity [21] and assayed in a reaction medium containing 0.1 M NaOAc (pH 5), 4 mM $MgCl_2$ and 1 mM *p*-nitrophenylphosphate as described previously [21].

REFERENCES

1. Harborne, J. B. (1988) *Introduction to Ecological Biochemistry*, 3rd Edn. Academic Press, New York.
2. Harborne, J. B. and Baxter, H. (1993) *Phytochemical Dictionary. A Handbook of Bioactive Compounds from Plants*. Taylor and Francis, London.
3. Nogrady, T. (1985) *Medicinal Chemistry. A Biochemical Approach*. Oxford University Press, Oxford.
4. Rang, H. P. and Dale, M. M. (1991) *Pharmacology*, 2nd Edn. Churchill Livingston, London.
5. Gescher, A. and Dale, I. L. (1989) *Anti-cancer Drug Design* **4**, 93.
6. Powis, G. (1991) *Trends Pharm. Sci.* **12**, 188.
7. Powis, G. and Kozikowski, A. (1991) *Clin. Biochem.* **24**, 385.
8. Hidaka, H. and Kobayashi, R. (1992) *Annu. Rev. Pharmacol. Toxicol.* **32**, 377.
9. Hidaka, H. and Ishikawa, T. (1992) *Cell Calcium* **13**, 485.
10. Jinsart, W., Ternai, B. and Polya, G. M. (1991) *Biol. Chem. Hoppe-Seyler* **372**, 819.
11. Jinsart, W., Ternai, B. and Polya, G. M. (1992) *Biol. Chem. Hoppe-Seyler* **373**, 205.
12. Ferriola, P. C., Cody, V. and Middleton, E. (1989) *Biochem. Pharmacol.* **38**, 1617.
13. Jinsart, W., Ternai, B., Buddhasukh, D. and Polya, G. M. (1992) *Phytochemistry* **31**, 3713.
14. Jinsart, W., Tenai, B. and Polya, G. M. (1992) *Biol. Chem. Hoppe-Seyler* **373**, 903.
15. Polya, G. M. and Foo, L. Y. (1994) *Phytochemistry* **35**, 1399.
16. Polya, G. M., Wang, B. H. and Foo, L. Y. (1995) *Phytochemistry* **38**, 307.
17. Herbert, J. M., Augereau, J. M., Gleye, J. and Maffrand, J. P. (1990) *Biochem. Biophys. Res. Commun.* **172**, 993.
18. Polya, G. M., Chung, R. and Menting, J. (1991) *Plant Sci.* **79**, 37.

19. Reddy, S. and Aggarwal, B. B. (1994) *FEBS Letters* **341**, 19.
20. Larkin, M., Brazier, J., Ternai, B. and Polya, G. M. (1993) *Planta Med.* **59**, 525.
21. Polya, G. M. and Wettenhall, R. E. H. (1992) *Biochim. Biophys. Acta* **1159**, 179.
22. Chen, Q., Deady, L. W. and Polya, G. M. (1994) *Biol. Chem. Hoppe-Seyler* **375**, 223.
23. Hidaka, H., Inagaki, M., Kawamuto, S. and Sasaki, Y. (1984) *Biochemistry* **23**, 5036.
24. Wang, B. H., Ternai, B. and Polya, G. M. (1994) *Biol. Chem. Hoppe-Seyler* **375**, 527.
25. Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A. and Kareko, M. (1987) *Biochem. Biophys. Res. Commun.* **142**, 436.
26. Wang, B. H. and Polya, G. M. (1996) *Phytochemistry* **41**, 55.
27. Ammon, H. P. and Wahl, M. A. (1991) *Planta Med.* **57**, 1.
28. Sharma, O. P. (1976) *Biochem. Pharmacol.* **25**, 1811.
29. Toda, S., Miyase, T., Arich, H., Tarizawa, H. and Takino, Y. (1985) *Chem. Pharm. Bull. (Tokyo)* **33**, 1725.
30. Rao, T. S., Basu, N. and Siddiqui, H. H. (1982) *Indian J. Med. Res.* **75**, 574.
31. Donatus, I. A., Sardjoko and Vermeuler, N. P. E. (1990) *Biochem. Pharmacol.* **39**, 1869.
32. Huang, M-T., Wang, Z. Y., Georgiadis, C. A., Laskin, J. B. and Conney, A. H. (1992) *Carcinogenesis* **13**, 2183.
33. Azuine, M. A. and Bhide, S. V. (1992) *Int. J. Cancer* **51**, 412.
34. Huang, M-T., Smart, R. C., Wong, C-Q. and Conney, A. H. (1988) *Cancer Res.* **48**, 5941.
35. Conney, A. H., Lysz, T., Ferraro, T., Abidi, T. F., Marchard, P. S., Laskin, J. D. and Huang, M-T. (1991) *Adv. Enz. Regul.* **31**, 385.
36. Kato, R., Nakadate, T., Yamamoto, S. and Sugimura, T. (1983) *Carcinogenesis* **4**, 1301.
37. Liu, J-Y, Lin, S-J. and Lin J-K. (1993) *Carcinogenesis* **14**, 857.