Volume 264, number 2, 187–192 FEBS 08427 May 1990

Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants

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Received 23 March 1990

The cyclic heptapeptide, microcystin-LR, inhibits protein phosphatases 1 (PP1) and 2A (PP2A) with K_i values below 0.1 nM. Protein phosphatase 2B is inhibited 1000-fold less potently, while six other phosphatases and eight protein kinases tested are unaffected. These results are strikingly similar to those obtained with the tumour promoter okadaic acid. We establish that okadaic acid prevents the binding of microcystin-LR to PP2A, and that protein inhibitors 1 and 2 prevent the binding of microcystin-LR to PP1. We discuss the possibility that inhibition of PP1 and PP2A accounts for the extreme toxicity of microcystin-LR, and indicate its potential value in the detection and analysis of protein kinases and phosphatases.

Microcystin; Cyanobacteria; Toxin; Protein phosphatase; Protein kinase; Okadaic acid

1. INTRODUCTION

Members of the cyanobacterial genera, *Microcystis, Oscillatoria* and *Anabaena* produce cyclic peptides, termed microcystins, which are potent hepatotoxins [1,2]. These substances are responsible for the deaths of fish, birds, wild animals and agricultural livestock in many countries where freshwaters contain toxic cyanobacterial blooms, and adverse effects of the toxins on human health have been recognised [3,4]. Microcystins are cyclic heptapeptides with the basic structure cyclo(D-Ala-L-X-*erythro*-β-methyl-D-iso-Asp-L-Y-Adda-D-iso-Glu-*N*-methyldehydro-Ala), where L-X and L-Y represent variable L-amino acid residues and Adda is the β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid [5,6]. Microcystin-LR, in which the two variable amino acids are leucine and arginine (Fig. 1), is the most commonly found variant of the nine microcystins recognised to date.

Oral and intraperitoneal administration of the microcystins to mammals causes extensive loss of hepatic lobular and sinusoidal architecture with hepatocyte necrosis and hepatic haemorrhage. 1–2 μg of microcystin-LR constitutes a lethal intraperitoneal dose to mice, with most of the toxin accumulating in the liver, and death occurring in about 60 min from haemodynamic shock and heart failure [1,2,7–9]. At the cellular level, microcystin-LR causes rapid, characteristic deformation, e.g. hepatocyte plasma membrane bleb formation and loss of microvilli, with a major reorganisation of microfilaments as determined by electron microscopy and fluorescent staining of actin [10–12].

Recently, we learned from Dr R.E. Moore [13] that H. Fujiki and co-workers (National Cancer Center, Tokyo) have found that microcystin-LR is an inhibitor of protein phosphatases 1 and 2A (PP1, PP2A), two of the major phosphatases in eukaryotic cells that dephosphorylate serine and threonine residues [14,15]. Here, we demonstrate that microcystin-LR is a highly specific and potent inhibitor of PP1 and PP2A from phyla as diverse as mammals, protozoa and higher plants, and that it inhibits these enzymes in a remarkably similar manner to okadaic acid, a potent tumour promoter that is also the toxin responsible for diarrhetic shellfish poisoning (see [16] for a review). We present kinetic evidence which demonstrates that the binding of okadaic acid to PP2A prevents its interaction with microcystin-LR, and that microcystin is unable to bind to PP1 in the presence of inhibitor-1 or inhibitor-2, two thermostable proteins that inhibit PP1 specifically.

2. MATERIALS AND METHODS

2.1. *Materials*

The catalytic subunits of PP1 and PP2A [17] (Mr D. Schelling), PP2C [18] (Dr C. McGowan), inhibitor-1 (Miss L. MacDougall) and inhibitor-2 (Dr M. Hubbard) [19] were purified to homogeneity from rabbit skeletal muscle and provided by other members of the Protein Phosphorylation Group at Dundee. PP1 and PP2A in extracts of the protozoan *Paramecium tetraurelia* were separated by chromatography on heparin-Sepharose [20]. PP2B from bovine brain (calcineurin) was a gift from Dr Claude Klee, National Cancer In-

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00145793/90/$3.50

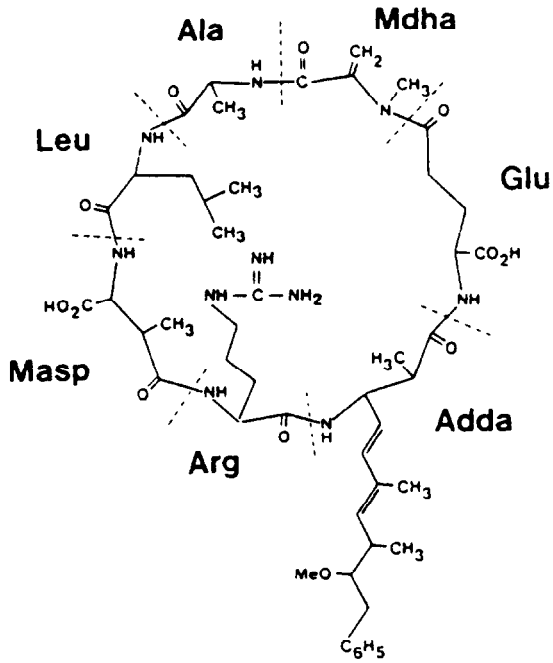

Fig. 1. Structure of microcystin-LR. Abbreviations: Masp, β-methylaspartic acid; Mdha, *N*-methyldehydroalanine; Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.

stitute, NIH, USA. Potato acid phosphatase and calf intestinal alkaline phosphatase were purchased from Boehringer. Okadaic acid was a gift from Dr Y. Tsukitani, Fujisawa Chemical Company, Japan.

2.2. *Isolation and analysis of microcystins*

Microcystin-LR was purified from *Microcystis aeruginosa* harvested from Rutland Water, England. The microcystin-LR was isolated and purified by high-performance liquid chromatography essentially as described [21,22]. The toxin was dissolved in 10% methanol to give a 0.4 mM solution and its concentration determined by amino acid analysis using a Water's PICOTAG system. Hydrolysis in 6 N HCl for 3 h at 150°C was essential to obtain complete peptide bond cleavage. Microcystin-LR was diluted in 50 mM Tris-HCl, pH 7.0 (4°C) containing 0.03% Brij 35 and retained its phosphatase inhibitory activity for at least a month at concentrations as low as 0.01 nM.

2.3. *Preparation of cell extracts*

Extracts were prepared from rat liver [23], *Brassica napus* (rape) seeds [24], and *Escherichia coli* [25] as described. Extracts were also prepared from nine strains of cyanobacteria (provided by Dr P. Rowell, Dept of Biological Sciences, University of Dundee) by sonication in 100 mM Hepes, pH 7.4, 0.1% (v/v) 2-mercaptoethanol, 2 mM EDTA, 1 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride, and freed from low-molecular-mass substances by gel filtration on Sephadex G50 equilibrated in the same buffer.

2.4. *Phosphatase assays*

PP1 and PP2A were assayed in the absence of divalent cations using 10 μM ^{32}P-labelled glycogen phosphorylase [17], PP2B in the presence of 1 mM $MnCl_2$ using 1 μM [^{32}P]phosphorylase kinase [26] and PP2C in the presence of 10 mM $MgCl_2$ using 6 μM [^{32}P]casein [18]. One unit of protein phosphatase was that amount which catalysed the dephosphorylation of 1 μmol of substrate in 1 min. PP1 and PP2A were also assayed with other substrates where specified. The bacteriophage λ protein phosphatase (orf 221) was assayed in the presence of 1 mM $MnCl_2$ and [^{32}P]casein, using extracts from phage λgt10-infected *E. coli* prepared just prior to cell lysis [25]. Alkaline and acid phosphatases were assayed at 37°C using 0.5 mM *para*-nitrophenyl phosphate at pH 8 and pH 5, respectively.

Protein tyrosine phosphatase activity was assayed in NP40 lysates of actively growing rat fibroblasts using ^{32}P-labelled poly (Glu,Tyr) (4:1) phosphorylated by a protein tyrosine kinase purified from MDCK cells. These experiments were performed by Dr E. Ulug and Dr S. Courtneidge, EMBL, Heidelberg.

2.5. *Isolation and assay of protein kinases*

The catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle (Dr C. Smythe), phosphorylase kinase (Mr D. Schelling), casein kinases 1 and 2 (Dr A. Lavoinne and Miss S. Nakielny) and glycogen synthase kinase 3 (Dr C. Smythe) from rabbit skeletal muscle, the multifunctional calmodulin-dependent protein kinase from rat brain (Dr D.G. Hardie) and the AMP-activated protein kinase from rat liver (Mr John Weekes) were provided by other colleagues in the Protein Phosphorylation Group at Dundee. Protein kinase C from rat brain was a gift from Dr Alastair Aitken, National Institute for Medical Research, London. Each enzyme was assayed using glycogen synthase (0.5 mg/ml, 4 μM) as substrate by standard procedures [27]. Phosphorylation was limited to <0.15 mol phosphate incorporated per mol glycogen synthase subunit, to ensure that initial rate conditions were met.

3. RESULTS

The dephosphorylation of glycogen phosphorylase by the purified catalytic subunits of PP1 and PP2A from rabbit skeletal muscle was potently inhibited by microcystin-LR, 50% inhibition (IC_{50}) of either enzyme occurring at ~0.1 nM microcystin-LR, when assays were performed at phosphatase concentrations of 0.2 mU/ml (Fig. 2). This compares with IC_{50} values of 10 nM (PP1) and 0.1 nM (PP2A) for inhibition by okadaic acid under the same conditions [28]. Similar IC_{50} values for PP1 and PP2A were obtained using [^{32}P]phosphorylase kinase, while with [^{32}P]casein, a specific substrate for PP2A [29], the IC_{50} was 0.2 nM (data not shown).

The IC_{50} for microcystin-LR is similar to the concentrations of PP1 and PP2A in the assays, demonstrating that the toxin-phosphatase interactions are extremely strong. Consistent with essentially stoichiometric binding, the IC_{50} values for inhibition of PP1 and PP2A depended on the phosphatase concentration in the assays (Fig. 3), the effect being most pronounced for PP2A (Fig. 3). This would be expected, since the molar concentration of PP2A in the assays is 10-fold higher than PP1 (the specific activity of PP2A towards glycogen phosphorylase being ~10-fold lower than PP1 [30]). Extrapolation to zero phosphatase concentrations of the data in Fig. 3 (and other experiments) indicates that the K_i for PP1 is about 0.06 nM, while for PP2A it is even lower, probably below 0.01 nM.

The amino acid sequences of PP1 and PP2A show 50% identity in the catalytic domain (reviewed in [14,15]). The Ca^{2+}/calmodulin-regulated protein phosphatase (PP2B) is a member of the same gene family, but despite showing 40% identity to PP1 or PP2A in the catalytic domain (reviewed in [15]), it is far less sen-

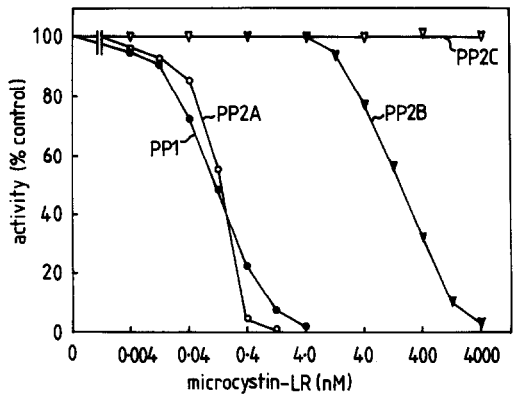

Fig. 2. Inhibition of different protein phosphatases by microcystin-LR. The catalytic subunits of PP1 (●) and PP2A (○) were assayed at 0.21 and 0.23 mU/ml, respectively, using ^{32}P-labelled glycogen phosphorylase as substrate, PP2B (▼) at 0.04 mU/ml using [^{32}P]phosphorylase kinase and PP2C (▽) at 0.02 mU/ml using [^{32}P]casein.

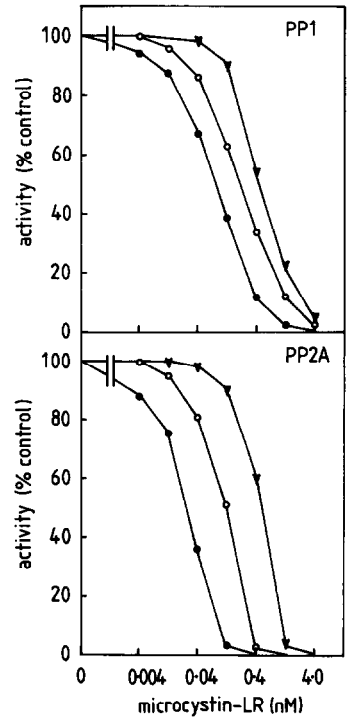

Fig. 3. Inhibition of the purified catalytic subunits of PP1 and PP2A from rabbit skeletal muscle by microcystin-LR. PP1 was assayed with glycogen phosphorylase at 0.05 mU/ml (●), 0.41 mU/ml (○) and 2.3 mU/ml (▼). PP2A was assayed with the same substrate at 0.05 mU/ml (●), 0.25 mU/ml (○) and 0.75 mU/ml (▼).

sitive to okadaic acid (IC_{50} ~5 μM) [31]. Fig. 2 shows that microcystin-LR inhibits PP2B, but with an IC_{50} value (0.2 μM) at least 1000-fold higher than that observed with PP1 or PP2A.

PP2A activity in the ciliated protozoan *Paramecium tetraurelia* is unique among eukaryotic cells so far examined in being unaffected by okadaic acid [20], while a protein phosphatase encoded in the genome of bacteriophage λ (orf221) that is structurally related to PP1/PP2A is also unaffected by this tumour promoter [25]. Neither of these phosphatases was affected by microcystin-LR at concentrations as high as 4 μM (data not shown). In contrast, PP1 activity in *Paramecium*, which is inhibited by okadaic acid [20], was inhibited by microcystin-LR similarly to mammalian PP1 (IC_{50} = 0.13 nM).

The specificity of microcystin-LR was tested by examining its ability to inhibit phosphatases that are structurally unrelated to PP1/PP2A. At a concentration of 4 μM, microcystin-LR (like okadaic acid) had no effect on the activity of protein phosphatase 2C (Fig. 2), while at 1 μM it had no effect on potato acid phosphatase, calf intestinal alkaline phosphatase, and protein tyrosine phosphatase activity towards ^{32}P-labelled poly (Glu,Tyr) in extracts of rat fibroblasts. Similarly, microcystin-LR (4 μM) was without effect on the following eight protein kinases; cyclic AMP-dependent protein kinase, the multifunctional calmodulin-dependent protein kinase, phosphorylase kinase, protein kinase C, the AMP-activated protein kinase, casein kinase 1, casein kinase 2 and glycogen synthase kinase 3.

In view of the striking similarities in the inhibition by microcystin-LR and okadaic acid, it was of interest to determine whether these toxins bind to the same site on PP1/PP2A. However, because both toxins are 'tight-binding inhibitors', standard Michaelis-Menten kinetics cannot be used to solve this problem. The inhibition of PP1 by microcystin-LR was therefore examined in the presence of a large molar excess of PP2A which had first been inactivated by incubation with NaF and PP_i (see legend to Fig.4). This caused an increase in the IC_{50} for microcystin-LR (Fig. 4), indicating that PP2A, although inactive, could still bind this toxin. However, the increase in IC_{50} was abolished by preincubating inactive PP2A with okadaic acid (Fig. 4), using a concentration in the assay (1 nM) sufficient to form a complex with PP2A, but too low to inactivate PP1 [28]. These experiments demonstrated that the binding of okadaic acid to PP2A prevented the subsequent binding of microcystin-LR.

We next tested the effect of inhibitor proteins 1 and 2, which inhibit PP1 specifically (reviewed in [14]), on the subsequent binding of microcystin-LR to PP1. PP2A was assayed in the presence of excess PP1 using a substrate ([^{32}P]casein) that is selective for PP2A [29]. The presence of PP1 caused a 3-fold increase in the IC_{50} for inhibition of PP2A by microcystin-LR (Fig. 5), which was abolished by preincubating PP1 with either inhibitor-1 or inhibitor-2 (Fig. 5). These experiments demonstrated that the binding of inhibitors 1 or 2 to PP1 prevented the binding of microcystin-LR.

The free catalytic subunits of PP1 and PP2A do not normally exist in vivo, but are complexed to regulatory

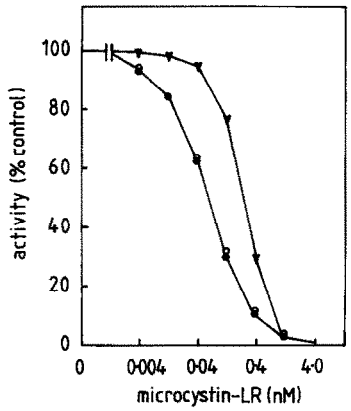

Fig. 4. Okadaic acid prevents the interaction of microcystin-LR with PP2A. The purified catalytic subunit of rabbit skeletal muscle PP2A (0.6 ml, 60 mU/ml) was completely inactivated by incubation for 1 h on ice in 50 mM Tris-HCl, pH 7.0 (4°C), 0.1 mM EGTA, 0.1% 2-mercaptoethanol (solution A) containing 100 mM NaF, 5 mM sodium pyrophosphate and 1 mg/ml bovine serum albumin, and then passed through a 25 × 1.2 cm column of Sephadex G-50 Superfine equilibrated in solution A to remove fluoride and pyrophosphate. The inhibition of the catalytic subunit of PP1 (0.03 mU/ml) by microcystin-LR was then examined using ^{32}P-labelled glycogen phosphorylase as substrate, in the presence (▼) and absence (●) of the G50 eluate (diluted a further six-fold in the assay). The open circles show the effect of preincubating the G50 eluate with 6 nM okadaic acid (diluted to 1 nM okadaic acid in the assay, a concentration that does not inhibit PP1 [28]). Similar results were obtained in three separate experiments.

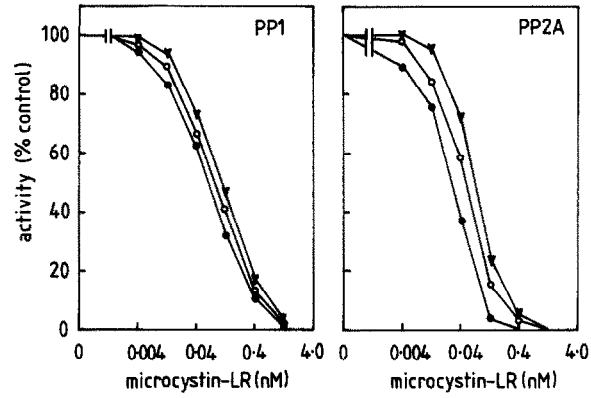

Fig. 6. The active high-molecular-mass forms of PP1 and PP2A in mammalian and plant extracts are inhibited by microcystin-LR with similar potency to the catalytic subunits. Rat livers and *B. napus* seeds were frozen in liquid nitrogen, powdered and extracts prepared as described [23,24]. The extracts were then assayed at a 600-fold dilution (liver extracts, ▼) and 225-fold dilution (*B. napus* extracts, ○) using glycogen phosphorylase as substrate. PP1 activity was measured in the presence of 1 nM okadaic acid to inhibit PP2A, and PP2A after preincubation with 0.25 μM inhibitor-2 to inactivate PP1 [28]. The concentrations in the assays were 0.08 mU/ml (liver PP1), 0.07 mU/ml (liver PP2A), 0.065 mU/ml (*B. napus* PP1) and 0.053 mU/ml (*B. napus* PP2A). The IC_{50} values are similar to those obtained with the purified catalytic subunits of PP1 and PP2A (both 0.06 mU/ml) from rabbit skeletal muscle (●). Similar results were obtained in two experiments using different liver and *B. napus* extracts.

and/or targetting subunits (reviewed in [14,15]). Fig. 6 demonstrates that the active forms of PP1 and PP2A in dilute extracts of rat liver or *Brassica napus* (rape) seeds are inhibited by microcystin-LR with similar potency to the free catalytic subunits. PP1 and PP2A in other plant extracts (carrot cells, wheat leaf and pea leaf) were inhibited by microcystin-LR in a similar manner (data not shown). The slightly higher IC_{50} values for the native enzymes (Fig. 6) are probably explained by their higher molar concentrations in the assays, the native enzymes having lower specific activities than the catalytic subunits [14].

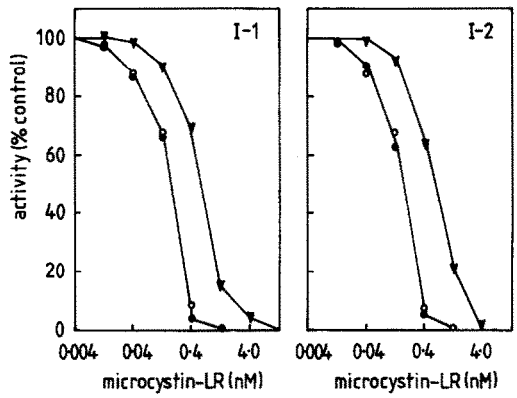

Fig. 5. Inhibitor-1 (I-1) and inhibitor-2 (I-2) prevent the interaction of microcystin-LR with PP1. Inhibition of the catalytic subunit of rabbit skeletal muscle PP2A (0.14 mU/ml) was examined using [^{32}P]casein as substrate in the presence (▼) and absence (●) of the PP1 catalytic subunit (2.9 mU/ml). The open circles show the effect of preincubating PP1 with either I-1 or I-2 (final concentration 0.25 μM in assay). Casein is a selective substrate for PP2A, PP1 only accounting for 12% of the casein phosphatase activity in the absence of I-1 or I-2, and for none for the activity in the presence of these inhibitors. Similar results were obtained in three separate experiments.

4. DISCUSSION

The results presented in this paper establish that microcystin-LR is a highly specific inhibitor of PP1 and PP2A and that this toxin inhibits these enzymes in a remarkably similar manner to the C_{44} polyketal fatty acid okadaic acid ([30], reviewed in [16]). Thus, both inhibitors inhibit PP2A more potently than PP1, inhibit PP2B at least 1000-fold less strongly, do not inhibit the structurally related protein phosphatase of bacteriophage λ or the type 2A-like protein phosphatases of *Paramecium tetraurelia*, and do not inhibit any other protein phosphatase or protein kinase that has been tested. Like okadaic acid, microcystin-LR inhibits plant and mammalian PP1 and PP2A in an identical manner (Fig. 6, [24]), an observation explained by the extreme structural conservation of these phosphatase catalytic subunits (R.W. MacKintosh, personal communication).

Our kinetic studies (Fig. 4) indicate that prior binding of okadaic acid to PP2A prevents it from interacting with microcystin-LR, suggesting that, despite their

quite different chemical compositions, these toxins may well bind to the same site(s) on PP1/PP2A. Intriguingly, the thermostable proteins inhibitor-1 and inhibitor-2, which inhibit PP1 specifically, also prevent the binding of microcystin-LR (Fig. 4). Thus all known substances that are potent inhibitors of PP1/PP2A may exert their effects by interacting at the same site on these enzymes.

Microcystin-LR is lethal to mice when 1-2 μg (1-2 nmol) are injected intraperitoneally, about 75% of the injected toxin accumulating in the liver. Since the intracellular concentrations of PP1 and PP2A in mammalian tissues are both about 1 μM, an adult mouse liver weighing about a gram would contain ~0.5 nmol of each enzyme. These observations are consistent with the possibility that phosphatase inhibition may underlie the extreme toxicity of microcystin-LR. PP1 and PP2A are involved in dephosphorylating regulatory proteins that control many cellular functions (reviewed in [14,15]), and it is not surprising that their complete inactivation would rapidly be lethal. It is even conceivable that inhibition of PP1 and PP2A might explain, in part, why reduced plant and animal species diversity is a common feature of waters containing toxic cyanobacterial blooms.

The presence of high levels of microcystin-LR in *Microcystis aeruginosa* raises the question of the biological function of this toxin which is present at high levels in hepatotoxic cyanobacteria. We have examined nine different strains of marine and freshwater cyanobacteria for enzymatic activities that resemble PP1 and PP2A, but failed to find any with significant activity towards glycogen phosphorylase (a specific substrate for PP1 and PP2A in eukaryotic cells). This is consistent with the absence of these enzymes in other prokaryotes, such as *E. coli* [25], and suggests that cyanobacteria do not contain PP1- and PP2A-like activities that have evolved resistance to microcystins. Since most of these strains of cyanobacteria do not synthesise toxins related to microcystins, it is unlikely that PP1 and PP2A-like activities were present, but masked by the presence of related cyclic heptapeptides.

Okadaic acid is becoming an extremely useful substance for the study of protein kinases, protein phosphatases and cellular processes that are controlled by phosphorylation. At a concentration of 1 nM it can be used as a specific inhibitor of PP2A in dilute tissue extracts, while above 1 μM it inhibits both PP1 and PP2A. This allows the quantitation in cell extracts of other protein phosphatases with similar substrate specificities, such as PP2C [28]. Furthermore, the routine inclusion of okadaic acid in protein kinase assays is to be recommended, since it can aid the detection of enzymes whose activities were previously masked by the presence of high levels of contaminating protein phosphatase activity [31]. Although microcystin-LR cannot be used to discriminate between PP1 and PP2A, it should be even more valuable than okadaic acid for the other purposes mentioned above, because it inhibits PP1 at a much lower concentration and is easier to isolate than okadaic acid. We have established that PP2C activity can be quantitated in dilute mammalian extracts if 10 nM microcystin is included in the assays (C. MacKintosh, unpublished experiments).

Acknowledgements: This work was supported by grants from the Medical Research Council and Royal Society (P.C.), the National Environmental Reseach Council (G.A.C.) and the Deutsche Forschungsgemeinschaft Grant SFB 323 (S.K. and Professor Joachim Schultz). We thank Dr Hugh Nimmo for valuable discussions about the kinetic aspects of this project, colleagues in the Protein Phosphorylation Group for the provision of purified proteins, Miss Linda Lawton for assistance in microcystin-LR purification and useful discussions, and Drs Emin Ulug and Sara Courtneidge for performing the protein tyrosine phosphatase assays.

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NOTE ADDED IN PROOF

After submission we noticed a paper by Runnegar, M.T.C., Andrews, J., Gerdes, R.G. and Falconer, I.R. [(1987) Toxicon 25, 1235–1239], who observed a time- and dose-dependent activation (phosphorylation) of phosphorylase in hepatocytes in response to a microcystin from *M. aeruginosa*. This is consistent with its action as a protein phosphatase inhibitor.