THE EFFECTS OF CYANOBACTERIA AND THE CYANOBACTERIAL TOXIN MICROCYSTIN-LR ON Ca$^{2+}$ TRANSPORT AND Na$^{+}$/K$^{+}$-ATPase IN TILAPIA GILLS

N. R. BURY$^1$, G. FLIK$^{1,*}$, F. B. EDDY$^2$ AND G. A. CODD$^2$

$^1$Department of Experimental Zoology, University of Nijmegen, Toernooiveld, Nijmegen, The Netherlands and $^2$Department of Biological Sciences, University of Dundee, Dundee, DD1 4HN, Scotland

Accepted 4 March 1996

Summary

The effects of cytotoxic substances from cyanobacteria on ionic transport processes in tilapia (Oreochromis mossambicus) were examined. Inhibitory effects on ionic transport including whole-body Ca$^{2+}$ fluxes and P-type ATPases of the gill were found. The compounds tested were (1) purified microcystin-LR (MC-LR), a heptapeptide hepatotoxin produced by the cyanobacterium Microcystis aeruginosa, (2) extracts from M. aeruginosa strain PCC 7820, a strain producing MC-LR and other microcystin variants, and (3) extracts of M. aeruginosa CYA 43, a strain producing toxins including small quantities of MC-LR. Whole-body Ca$^{2+}$ influx was inhibited by a 24 h exposure to extracts of M. aeruginosa CYA 43 and 7820, but not by exposure to an equivalent amount (90 mg l$^{-1}$) of purified MC-LR. Shorter exposure times (4 h) were ineffective. Fish exposed to extracts from M. aeruginosa showed significant plasma hypocalcaemia. Both strains of M. aeruginosa inhibited Ca$^{2+}$ uptake by basolateral plasma membrane vesicles (BLMVs), endoplasmic reticulum (ER) and mitochondria, as well as BLMV K$^{+}$-dependent p-nitrophenol phosphatase (pNPPase) activity. The hydrophobic fractions of the cyanobacterial extracts were the most potent, inhibiting BLMV, ER and mitochondrial Ca$^{2+}$ uptake by up to 99%, but they were less inhibitory of BLMV K$^{+}$-dependent pNPPase activity. Purified MC-LR was without effect on these preparations. In conclusion, cytotoxic substances from cyanobacteria have the potential to disrupt normal physiological processes dependent upon Ca$^{2+}$ transport processes in tilapia gills.

Key words: Ca$^{2+}$-ATPase, plasma membranes, fish gills, Oreochromis mossambicus, microcystin, cyanobacterial toxins.

Introduction

In the gills of freshwater fish, Ca$^{2+}$ influx is facilitated by a Ca$^{2+}$ channel in the apical membrane of the chloride cells (Verbost et al. 1989); extrusion of Ca$^{2+}$ from the cell across the basolateral membrane is mediated by a high-affinity Ca$^{2+}$-ATPase and possibly by a Na$^{+}$/Ca$^{2+}$ exchanger (Flik et al. 1993; Verbost et al. 1994). The intracellular Ca$^{2+}$ concentration could be regulated by this extrusion process, by Ca$^{2+}$-binding proteins, by active Ca$^{2+}$ uptake into endoplasmic reticulum (ER) (Somlyo, 1985) or by sequestration by mitochondria via a uniporter (Carafoli, 1982; Gunter and Pfieffer, 1990; Gunter et al. 1994). Inhibition of the Ca$^{2+}$-ATPase of the basolateral membrane of chloride cells by Cd$^{2+}$ (Verbost et al. 1987a, 1988, 1989) causes hypocalcaemia (Giles, 1984), indicating the importance of this branchial Ca$^{2+}$ pump in calcium homeostasis.

Eutrophication of water bodies has resulted in an increased frequency of cyanobacterial (blue-green algal) blooms that may produce neuro- and/or hepatotoxins (Codd et al. 1989; Carmichael, 1992). On senescence of a bloom, these toxins are released into the water and they may cause fish kills (e.g. Schwimmer and Schwimmer, 1968). The majority of kills have been linked with hypoxia caused by the high biological oxygen demand on senescence of cyanobacterial blooms and scums. However, oxygen levels were above 90% saturation when moribund brown trout were retrieved after the lysis of a bloom of the toxic blue-green alga Anabaena flos-aquae at Loch Leven, Scotland (Rodger et al. 1994). Histopathology revealed that these fish had severe liver damage similar to that of fish administered extracts of Microcystis spp. containing the hepatotoxic microcystin-LR (MC-LR) or purified MC-LR injected intraperitoneally (Phillips et al. 1985; Sugaya et al. 1990; Råbergh et al. 1991) or after gavage experiments with microcystin-producing cyanobacteria (Tencalla et al. 1994). However, immersion trials using concentrations of extracts of the hepatotoxic cyanobacterial cells similar to those found in eutrophic environments have produced sublethal effects in fish, e.g. increased plasma cortisol and glucose levels, while plasma Na$^{+}$ and Cl$^{-}$ concentrations decreased over a 4 h exposure.

*Author for correspondence.
period (Bury et al. 1996). Long-term exposure, for 63 days, was detrimental to fish growth and resulted in disturbances to ionic regulation (Bury et al. 1995).

In vitro, MC-LR specifically inhibits protein phosphatases 1 and 2A (Eriksson et al. 1990; Honkanen et al. 1990; MacKintosh et al. 1990; Matsuhashi et al. 1990). There is also one report that MC-LR inhibits Na⁺/K⁺-ATPase activity in carp gill microsomes (Gaete et al. 1994), and these observations imply that aspartic acid–phosphatase hydrolyase activity of P-type ATPases is also a target for MC-LR. In view of these results, the present study examines the effects of purified MC-LR and of extracts of cells from the cyanobacteria Microcystis aeruginosa 7820 (which produces a range of microcystins including MC-LR) or M. aeruginosa CYA 43 (which produces toxins containing very small quantities of MC-LR) on Ca²⁺ transport in tilapia, Oreochromis mossambicus. This investigation focuses on whole-body Ca²⁺ influxes, on Ca²⁺ transport in a gill basolateral membrane vesicle (BLMV) preparation and in endoplasmic reticulum (ER) and mitochondria and on Na⁺/K⁺-ATPase activity of the gill BLMVs.

Materials and methods

Fish holding conditions

Freshwater tilapia (Oreochromis mossambicus Peters) were obtained from laboratory stocks and were held in running Nijmegen tap water (in mmol l⁻¹, 0.7 Ca²⁺, 0.5 Na⁺, 0.06 K⁺, pH 7.8, 27 °C) under a light regime of 12 h:12 h light:dark. Fish were fed Trouvit fish pellets (Trouw and Co., Putten, The Netherlands) at a ration of 1.5 % body mass per day.

Cyanobacterial culture, toxin purification and fraction preparation

A method for culturing Microcystis aeruginosa PCC 7820 has been described previously (Bury et al. 1995). Chlorophyll a levels were measured according to MacKinney (1941). Cells were harvested in the early stationary phase of batch culture by continuous centrifugation in a Sharples centrifuge (Sharples Ltd, Surrey), the cell pellet was collected, freeze-dried and microcystin-LR (MC-LR; molecular mass 994 kDa) extracted by high-performance liquid chromatography (HPLC) (Lawton et al. 1994). M. aeruginosa CYA 43 was similarly cultured, but does not yield MC-LR at levels detectable by HPLC, (detection limit, 5 ng of microcystin on the HPLC column).

For in vitro Ca²⁺ transport and Na⁺/K⁺-ATPase assays, freeze-dried cyanobacterial cells were twice extracted in methanol (0.314 g per 50 ml methanol), centrifuged at 100g for 15 min (Sorval RC-5B), the supernatants were combined, dried under vacuum, and the extract was resuspended in 2 ml of methanol and stored at −20 °C. Samples were dried and resuspended in sucrose buffer containing 250 mmol l⁻¹ sucrose and 10 mmol l⁻¹ Hepes/Tris, pH 7.4, to give a final concentration of 150 mg dry mass ml⁻¹, equivalent to 0.5 mg ml⁻¹ MC-LR for M. aeruginosa PCC 7820 extracts. Additions of the cyanobacterial extracts will be referred to as milligrams of freeze-dried cells per milligram of membrane protein. Purified microcystin-LR was resuspended in sucrose buffer to a concentration of 2 μg ml⁻¹ MC-LR.

The methanol extracts from M. aeruginosa 7820 and CYA 43 were fractionated by a Pharmacia ‘Smart’ system using the eluents acetonitrile/0.5 % trifluoroacetic acid (TFA) and milliQ water/0.05 % TFA. A 65 % acetonitrile (v/v) gradient was built up over a 25 min period with a flow of 150 μl min⁻¹, during which fractions of 100 μl were collected and pooled into six sequential groups, fraction 1 being the most hydrophilic and fraction 6 being the most hydrophobic. Fractions were resuspended in sucrose buffer in a volume equivalent to the volume of methanol injected onto the column to allow comparisons of toxin levels in fractions and cell extracts (see also toxin application).

In vivo exposure

Purified MC-LR (1 mg) was dissolved in 2 ml of ethanol and diluted in 100 ml of distilled water. Freeze-dried M. aeruginosa 7820 (0.314 g yielded 1 mg of MC-LR) was extracted twice with 50 ml of 4 % (v/v) ethanol in water and centrifuged at 1000 g (Sorval RC-5B) and the supernatants combined. A similar quantity of M. aeruginosa CYA 43 was similarly extracted.

Tilapia (13–35.9 g) were starved for 48 h and then six fish were held for 24 h in 41 vessels containing Nijmegen tap water at 27 °C, to which 90 μg l⁻¹ MC-LR, freeze-dried extract of M. aeruginosa 7820 (86.4 μg l⁻¹ MC-LR, 27 mg dry mass l⁻¹) or M. aeruginosa CYA 43 (27 mg dry mass l⁻¹) was added. MC-LR levels were determined by HPLC. Fish exposed to 0.025 % (v/v) ethanol in water acted as controls.

Exposure was for 4 and 24 h. Whole-body Ca²⁺ influx was measured by the uptake of ⁴⁵Ca²⁺ (0.11 MBq l⁻¹, Dupont) over 4 h. Fish were killed after this time by an overdose of the anaesthetic 2-phenoxynethanol, blood was removed via the caudal vessel and the plasma was collected by centrifugation (1 min at 13000 g).

Carcasses were digested for 48 h in 50 ml of H₂O₂ at 60 °C, 1 ml of digest was removed and 4 ml of Aqualumar added. ⁴⁵Ca²⁺ was measured in a Pharmacia Wallac 1410 liquid scintillation counter (all subsequent measurements of ⁴⁵Ca²⁺ levels are made with the same counter) and calculated on the basis of carcass total radioactivity (disints min⁻¹) and water specific activity (disints min⁻¹ ml⁻¹). Plasma osmolality was measured with a Vogel osmometer using distilled water and a 300 mosmol kg⁻¹ standard as references. Plasma total Ca²⁺ concentrations were measured using a calcium kit (Sigma Diagnostics), Na⁺ concentrations were measured by atomic absorption (Pye-Unicam SP9 atomic absorption spectrophotometer) and Cl⁻ concentrations were determined with a chloride meter (Jenway PCLM3 chloride meter).

Gill membranes and permeabilised cell preparations

Methods for basolateral membrane vesicle preparations are described by Flik et al. (1985). Briefly, gills were excised from tilapia (250 g), washed in buffer containing 250 mmol l⁻¹...
Cyanobacterial toxins and Ca$^{2+}$ transport in tilapia gill

The red blood cells and cellular debris were removed from the homogenate by centrifugation at 550 g for 10 min. Membranes were collected by centrifugation at 30,000 g (Sorval RC-5B) and were resuspended in buffer using a Douncer-type homogenisation device (100 strokes). The resulting suspension was differentially centrifuged; 1000 g for 10 min, 10,000 g for 10 min and 30,000 g for 30 min, and the final pellet was resuspended in buffer containing 150 mmol l$^{-1}$ NaCl, 0.8 mmol l$^{-1}$ MgCl$_2$ and 20 mmol l$^{-1}$ Hepes/Tris, pH 7.4, for the Na$^+$/K$^+$-ATPase assay or 150 mmol l$^{-1}$ KCl, 0.8 mmol l$^{-1}$ MgCl$_2$ and 20 mmol l$^{-1}$ Hepes/Tris, pH 7.4, for Ca$^{2+}$ transport measurements. The vesicles were resuspended by 10 passages through a 23 gauge needle, giving 19.2–30% inside-out (IOV) and 29–44% right-side-out vesicles (ROV) (Flik et al. 1985; Verbost et al. 1994). The amount of membrane protein present was determined using a commercial kit (Bio-Rad) with bovine serum albumin (BSA) as a standard and was adjusted to 1.5 mg ml$^{-1}$. To obtain maximum Na$^+$/K$^+$-ATPase activity, vesicles were permeabilised with saponin (0.2 mg ml$^{-1}$protein) to ensure optimal substrate accessibility.

The preparation of permeabilised gill cells was based on the methods of Verbost et al. (1994). Gills were excised and the epithelial scrapings incubated in lysis medium containing 9 parts of 0.17 mmol l$^{-1}$ NH$_4$Cl and 1 part of 0.17 mmol l$^{-1}$ Tris/HCl, pH 7.4, for 20 min. The cells and red cell material were resuspended at the beginning and end of lysis by passage through a 10 ml pipette, large cell clusters were removed by passage through a 100 µm nylon mesh. Cells were collected, and the lysed red cells removed by centrifugation at 150 g for 5 min. The resulting pellet was resuspended in buffer containing 150 mmol l$^{-1}$ NaCl, 0.8 mmol l$^{-1}$ MgCl$_2$ and 20 mmol l$^{-1}$ Hepes/Tris, pH 7.4, and incubated with 0.3 mg ml$^{-1}$ saponin at 37 °C for 5 min. The cells were centrifuged at 5 min at 150 g and the pellet washed twice and from which KCl was omitted. Toxin-treated vesicles (10 µl) were mixed with 500 µl of either medium A or medium E and incubated for 15 min at 37 °C. The K$^+$-dependent ouabain-sensitive pNPPase activity was defined as the difference in activity measured between medium A and medium E at 420 nm.

Toxin application

The BLMVs and permeabilised cell preparations were exposed to purified MC-LR at concentrations in the range 1.56–333 µg mg$^{-1}$ membrane protein (equivalent to 1.57–335 nmol mg$^{-1}$ membrane protein) and for experiments with methanol extracts of cyanobacteria in the range 0.12–25 mg of freeze-dried material per milligram membrane protein. For experiments with the six fractions obtained from the methanol extracts, a volume of 83.3 µl of cyanobacterial extract fraction per milligram membrane protein was applied, which was equivalent to 12.5 mg of freeze-dried material per milligram membrane protein. For permeabilised cell preparations, thapsigargin (10 µmol l$^{-1}$) was used to inhibit endoplasmic reticulum Ca$^{2+}$ transport and Ruthenium Red (1 µmol l$^{-1}$) to inhibit mitochondrial Ca$^{2+}$ transport. All preparations were incubated for 2 h on ice prior to assays.

K$^+$-dependent pNPPase activity

K$^+$-dependent p-nitrophenol phosphatase (pNPPase) activity, which reflects the dephosphorylation step (i.e. the phosphatase activity) of the Na$^+$/K$^+$-ATPase, was determined. Medium A contained 100 mmol l$^{-1}$ KCl, 75 mmol l$^{-1}$ MgCl$_2$, 300 mmol l$^{-1}$ imidazole, 10 mmol l$^{-1}$ trans-1,2-diaminocyclohexane-N,N',N'-tetraacetic acid (CDTA) and 5 mmol l$^{-1}$ p-nitrophenol phosphate (pNPP), pH 7.4, whilst medium E consisted of medium A to which 1 mmol l$^{-1}$ ouabain was added and from which KCl was omitted. Toxin-treated vesicles (10 µl) were mixed with 500 µl of either medium A or medium E and incubated for 15 min at 37 °C. The K$^+$-dependent ouabain-sensitive pNPPase activity was defined as the difference in activity measured between medium A and medium E at 420 nm.

Ca$^{2+}$ transport

Basolateral membrane vesicles

Toxin-treated vesicles (12.5 µl) were added to 50 µl of assay medium; 150 mmol l$^{-1}$ KCl, 1 mmol l$^{-1}$ ‘free’ Ca$^{2+}$, 0.8 mmol l$^{-1}$ ‘free’ Mg$^{2+}$, buffered with 0.5 mmol l$^{-1}$ EGTA, 0.5 mmol l$^{-1}$ N-(2-hydroxyethyl)-ethylenediamine-N,N',N'-tetraacetic acid (HEEDTA), 0.5 mmol l$^{-1}$ NTA, with or without 3 mmol l$^{-1}$ ATP and containing 45Ca$^{2+}$. Both solutions were

![Fig. 1. Whole-body Ca$^{2+}$ influx (µmol h$^{-1}$ 100 g$^{-1}$) in tilapia after 24 h of exposure to purified microcystin-LR (MC-LR, 90 µg l$^{-1}$) or extracts of Microcystis aeruginosa 7820 (7820, 86 µg l$^{-1}$, 27 mg dry mass l$^{-1}$) or M. aeruginosa CYA 43 (CYA 43, 27 mg dry mass l$^{-1}$). Values are means ± s.e.m., N=6, apart from controls where N=5. Asterisks indicate a significant difference from control values (P<0.05).](https://example.com/image)
pre-warmed to 37 °C and subsequent incubations performed at this temperature for 1 min, to determine initial velocities. The transport was stopped by the addition of 1 ml of ice-cold stop buffer containing 150 mmol l\(^{-1}\) KCl, 1 mmol l\(^{-1}\) EGTA and 10 mmol l\(^{-1}\) Hepes/Tris, pH 7.4. Vesicles were collected by rapid filtration using Schleicher and Schuell ME25 mixed cellulose filters, pore size 0.45 mm, and were rinsed twice with ice-cold stop buffer. Filters were placed in scintillation vials and dissolved in 4 ml of Aqualuma for 30 min before activity was counted.

Permeabilised gill cells

Permeabilised gill cell preparations were mixed thoroughly and 10 µl of the suspension was added to 50 µl of assay medium, both having been pre-warmed to 28 °C. Assay medium contained the same concentration of ligands and KCl as above, but with 5 mmol l\(^{-1}\) ATP. *Free* [Mg\(^{2+}\)] was set at 0.8 mmol l\(^{-1}\); for endoplasmic reticulum Ca\(^{2+}\) transport measurements, ‘free’ [Ca\(^{2+}\)] was set at 0.1 µmol l\(^{-1}\), and for mitochondrial Ca\(^{2+}\) transport measurements, ‘free’ [Ca\(^{2+}\)] was 1 µmol l\(^{-1}\) (Verbost et al. 1987b). Cells were incubated at 28 °C for 2 min and transport was stopped by the addition of 1 ml of stop buffer, as above. Cells were collected on Schleicher and Schuell (GF92 diameter 25 mm) glass filters, and rinsed and counted as above.

**Table 1. Plasma osmolality and Ca\(^{2+}\), Na\(^{+}\) and Cl\(^{-}\) concentrations in tilapia exposed for 24 h to purified microcystin (MC-LR, 90.8 µg l\(^{-1}\) MC-LR) or extracts of Microcystis aeruginosa 7820 (7820, 86 µg l\(^{-1}\) MC-LR, 27 mg l\(^{-1}\) of freeze-dried material) or M. aeruginosa CYA 43 (CYA 43, 27 mg l\(^{-1}\) of freeze-dried material)**

<table>
<thead>
<tr>
<th>Osmolality</th>
<th>Control</th>
<th>MC-LR 7820</th>
<th>CYA 43</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mosmol kg(^{-1}))</td>
<td>301±1.9</td>
<td>294±5</td>
<td>306±1.9</td>
</tr>
<tr>
<td>[Ca(^{2+})] (mmol l(^{-1}))</td>
<td>2.71±0.15</td>
<td>2.68±0.05</td>
<td>2.39±0.26</td>
</tr>
<tr>
<td>[Na(^{+})] (mmol l(^{-1}))</td>
<td>153±4.6</td>
<td>161±4</td>
<td>169±26*</td>
</tr>
<tr>
<td>[Cl(^{-})] (mmol l(^{-1}))</td>
<td>129.5±2.7</td>
<td>125±2.8</td>
<td>139±1.4*</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m., N=6, apart from controls where N=5. Asterisks indicate significant differences (P<0.05) compared with control values at 24 h.

**Statistics and calculations**

All results were compared with control values using a Student’s t-test on the Minitab computer package. *Free* Ca\(^{2+}\) and Mg\(^{2+}\) concentrations were calculated using a matrix computer program developed by Schoenmakers et al. (1992).

**Results**

In vivo experiments

Whole-body Ca\(^{2+}\) influx rates were inhibited by 40% after 24 h of exposure to extracts of *M. aeruginosa* 7820 and by 31% with *M. aeruginosa* CYA 43 extracts (Fig. 1). Table 1 shows that plasma Ca\(^{2+}\) concentrations were significantly lower in fish exposed for 24 h to *M. aeruginosa* CYA 43, while plasma Na\(^{+}\) and Cl\(^{-}\) levels showed a small but significant increase in fish exposed to *M. aeruginosa* 7820. Purified microcystin-LR (MC-LR) had no effect on plasma electrolyte levels or whole-body Ca\(^{2+}\) influx when compared with controls at 24 h (Table 1; Fig. 1). None of the treatments had a significant effect on whole-body Ca\(^{2+}\) influx or body ion concentrations after 4 h of exposure.

**Table 2. Percentage inhibition of K\(^{+}\)-dependent pNPPase activity in gill basolateral membrane vesicles (BLMVs) exposed to fractions 1–6 from methanol extracts of Microcystis aeruginosa 7820 or CYA 43**

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition by 7820 (%)</td>
<td>9±17</td>
<td>8±14</td>
<td>5±10</td>
<td>7±17</td>
<td>35±14</td>
<td>12±13</td>
</tr>
<tr>
<td>Inhibition by CYA 43 (%)</td>
<td>12±15</td>
<td>5±14</td>
<td>0±17</td>
<td>7±13</td>
<td>37±14</td>
<td>27±13</td>
</tr>
</tbody>
</table>

See Materials and methods for details of fraction preparation. Values are percentage of control values ± s.e.m., N=5.
Cyanobacterial toxins and Ca\textsuperscript{2+} transport in tilapia gill

K\textsuperscript{+}-dependent pNPPase activity

BLMV K\textsuperscript{+}-dependent pNPPase activity was not affected by purified MC-LR, but was inhibited by increasing doses of methanol extracts of both *M. aeruginosa* 7820 and CYA 43 (Fig. 2). Fraction 5 of the cyanobacterial extracts appeared to produce the greatest reduction in K\textsuperscript{+}-dependent pNPPase activity (Table 2), but this was not significant when compared with the effects of other fractions.

Ca\textsuperscript{2+} transport

Basolateral membrane vesicles

Purified MC-LR had no effect on Ca\textsuperscript{2+} uptake in BLMVs, although methanol extracts of both *M. aeruginosa* 7820 and CYA 43 progressively inhibited Ca\textsuperscript{2+} uptake with increasing doses (Fig. 3). *M. aeruginosa* 7820 and CYA 43 extract fractions 4, 5 and 6 significantly inhibited Ca\textsuperscript{2+} uptake in BLMVs compared with controls (Table 3).

Permeabilised gill cells

Ca\textsuperscript{2+} uptake specific to the endoplasmic reticulum (ER) of the permeabilised cells was verified using the inhibitor thapsigargin at 0.1 \textmu mol\textsuperscript{1} \textsuperscript{-1} Ca\textsuperscript{2+}, which produced an 82% inhibition. Ca\textsuperscript{2+} uptake specific to mitochondria of the permeabilised cells was verified using Ruthenium Red at 1 \textmu mol\textsuperscript{1} \textsuperscript{-1} Ca\textsuperscript{2+} as an inhibitor, which resulted in 80% inhibition. Purified MC-LR had no effect on ER or mitochondrial uptake of Ca\textsuperscript{2+} (Figs 4, 5). The methanol extracts from both strains of *M. aeruginosa* inhibited Ca\textsuperscript{2+} uptake by ER (Fig. 4) and mitochondria (Fig. 5), most noticeably at higher concentrations. *M. aeruginosa* 7820 and CYA 43 extract fractions 4, 5 and 6 all inhibited Ca\textsuperscript{2+} transport in both systems compared with controls, with fraction 5 being the most potent (Table 4).

Discussion

The toxins from cyanobacteria have been reported to impede Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity of carp gill microsomes (Gaete *et al.* 1994), and so we focused our studies on the ATPases that mediate Ca\textsuperscript{2+} transport in the gill of tilapia. Our results show that, in vitro, MC-LR did not inhibit Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity, measured as K\textsuperscript{+}-dependent pNPPase (Fig. 2), or the ATP-driven Ca\textsuperscript{2+} transport of the gill basolateral plasma membrane (Fig. 3); in addition, there was no inhibition of SERCA-type ATPases of the endoplasmic reticulum (ER, Fig. 4) or of the Ca\textsuperscript{2+} uniporter of the mitochondria (Fig. 5). In contrast, extracts, particularly in the hydrophobic fractions, from the 7820 and CYA 43 strains of *M. aeruginosa* inhibited all the systems investigated. Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity appeared to be less sensitive than the Ca\textsuperscript{2+}-dependent transporters to the toxic substances within the cyanobacteria.

The cyanobacterial toxin microcystin-LR (MC-LR) inhibits protein phosphatases 1 and 2A (PP1 and PP2A) (Eriksson *et al.* 1990; Honkanen *et al.* 1990; MacKintosh *et al.* 1990; Matsushima *et al.* 1990). If MC-LR also inhibits Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity of carp gill microsomes (Gaete *et al.* 1994), this would imply either that the inhibitory action of MC-LR is wider, i.e. it would also inhibit P-type ATPase activity (where the phosphatases show an aspartic acid–phosphate hydrolase activity) and/or that the fish ATPase activity is aberrant in having a PP1/PP2A-type phosphatase activity as part of its Na\textsuperscript{+}/K\textsuperscript{+}-ATPase cycle. However, in the present study, MC-LR did not inhibit any Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity in tilapia gill (Fig. 3), nor has MC-LR inhibition been found in carp gill.

![Fig. 3. Initial rates of ATP-driven Ca\textsuperscript{2+} uptake (nmol min\textsuperscript{-1} mg\textsuperscript{-1}) measured at 1 \textmu mol\textsuperscript{1} \textsuperscript{-1} Ca\textsuperscript{2+} in BLMV preparations of controls (C) and following exposure to varying concentrations of MC-LR or to methanol extracts of *Microcystis aeruginosa* 7820 or *M. aeruginosa* CYA 43 (see legend to Fig. 2 for details). Values are means ± S.E.M., N=5. Asterisks indicate a significant difference from control values (P<0.05).](image)

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition by 7820 (%)</td>
<td>12±11</td>
<td>5±10</td>
<td>19±16</td>
<td>32±5*</td>
<td>88±5*</td>
<td>77±9*</td>
</tr>
<tr>
<td>Inhibition by CYA 43 (%)</td>
<td>26±12</td>
<td>31±12</td>
<td>12±9</td>
<td>40±12*</td>
<td>88±5*</td>
<td>74±9*</td>
</tr>
</tbody>
</table>

Table 3. Percentage inhibition of Ca\textsuperscript{2+} transport in gill BLMVs exposed to fractions 1–6 from methanol extracts of *Microcystis aeruginosa* 7820 or CYA 43

See Materials and methods for details of fraction preparation. Values are percentage of control values ± S.E.M. Asterisks indicate significant differences (P<0.05) compared with control values before percentage transformation, N=5.
dog kidney or human erythrocytes (N. R. Bury and G. Flik, personal observations). Moreover, other Ca\(^{2+}\) -ATPases in the plasma membranes and ER proved to be unaffected by the toxin, in line with the specificity of the toxin to PP1 and PP2A. We have no doubt of the potency of our MC-LR preparations, as acute hepatotoxicity had been ascertained by intraperitoneal injection in mice (Falconer *et al.* 1981). Moreover, the preparation appeared as a single homogeneous peak on HPLC with the same retention time as that previously published for MC-LR (Lawton *et al.* 1994).

Tilapia exposed for 24 h to extracts from either strain of *M. aeruginosa*, 7820 or CYA 43, showed inhibited Ca\(^{2+}\) influx (Fig. 1), which resulted in hypocalcaemia in fish exposed to *M. aeruginosa* CYA 43 (Table 1). The small but significant increase in plasma Na\(^+\) and Cl\(^{-}\) concentrations in fish exposed to *M. aeruginosa* 7820 (Table 1) was unexpected, and there is no immediate explanation. A possible explanation for the lack of *in vivo* effects at 4 h is that the toxin has yet to exert an inhibitory effect at the basolateral membrane on Ca\(^{2+}\) transporting mechanisms (see Flik *et al.* 1985, 1993).

Toxicity tests with invertebrates and bacteria have verified the toxicity of the microcystins (Penaloza *et al.* 1990; Demott *et al.* 1991; Kiviranta *et al.* 1991), but have also illustrated the presence of additional cytotoxic substances, as yet unidentified, within cyanobacteria (Nizan *et al.* 1986; Jungmann *et al.* 1991; Jungmann, 1992; Jungmann and Benndorf, 1994; Campbell *et al.* 1994). Penaloza *et al.* (1990) found that cyanobacterial fractions toxic to zooplankton contained factors with a molecular mass similar to that of MC-LR, but toxicity was lost upon boiling, while microcystins are heat-stable to 160 °C (e.g. Jungmann and Benndorf, 1994). Cyanobacteria have been screened for compounds with therapeutic potential and are a rich source of novel bioactive substances; for example, antineoplastic compounds (Patterson 2000).

Table 4. Percentage inhibition of Ca\(^{2+}\) uptake by gill endoplasmic reticulum (ER) and mitochondria in permeabilised cell preparations exposed to fractions 1–6 from methanol extracts of *Microcystis aeruginosa* 7820 or CYA 43

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>7820</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition of ER Ca(^{2+}) transport (%)</td>
<td>0±23</td>
<td>0±18</td>
<td>10±12</td>
<td>33±12</td>
<td>88±7*</td>
<td>75±9*</td>
</tr>
<tr>
<td>CYA 43</td>
<td>8±21</td>
<td>10±21</td>
<td>1±11</td>
<td>35±12*</td>
<td>90±6*</td>
<td>50±19</td>
</tr>
<tr>
<td>Inhibition of mitochondrial Ca(^{2+}) transport (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7820</td>
<td>20±13</td>
<td>3±16</td>
<td>12±18</td>
<td>57±6</td>
<td>99±1*</td>
<td>96±2*</td>
</tr>
<tr>
<td>CYA 43</td>
<td>11±21</td>
<td>0±22</td>
<td>8±18</td>
<td>59±7</td>
<td>99±1*</td>
<td>97±2*</td>
</tr>
</tbody>
</table>

See Materials and methods for details of fraction preparation.
Values are percentage of control values ± S.E.M.
Asterisks indicate significant differences (P<0.05) compared with control values before percentage transformation, N=5.
et al. 1991), and lipophilic cyclic peptides (laxaphycins) that are antifungal and cytotoxic (Frankmolle et al. 1992). There is no account of these substances inhibiting ATPases, but fungi have been shown to possess citreoviridin, a polyene neurotoxin which inhibits mitochondrial ATPase (Sayood et al. 1989), and tentoxin, a cyclic tetrapeptide which inhibits ATPase activity in cyanobacteria (Ohta et al. 1993).

In conclusion, we have shown that strains of the cyanobacterium *Microcystis aeruginosa* produce compound(s) that inhibit Ca²⁺ uptake and gill K⁺-dependent pNPPase activity in tilapia. We also present evidence that these inhibitory effects are not due to the protein phosphatase inhibitor MC-LR. However, further work is required to determine the exact structure and inhibitory mode of action of the compound(s) which affect gill Ca²⁺ transport and may determine the exact structure and inhibitory mode of action of inhibitor MC-LR. However, further work is required to determine the exact structure and inhibitory mode of action of the compound(s) which affect gill Ca²⁺ transport and may inhibit physiological processes dependent upon Ca²⁺, thus contributing to the fish death that often accompanies senescence of a cyanobacterial bloom.

N.B. is funded by the NERC (GT/92/140/A). We also thank the European Science Foundation Ecotoxicology Short-Term Visiting Fellowship programme (SVF/94/37) for funding this study, F. A. T. Spanings for the husbandry of the tilapia and K. A. Beattie for help with the cyanobacterial cell culture.

### References


