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FATTY ACIDS FROM THE CYANOBACTERIUM MICROCYSTIS AERUGINOSA WITH POTENT INHIBITORY EFFECTS ON FISH GILL Na⁺/K⁺-ATPase ACTIVITY

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Accepted 15 October 1997; published on WWW 9 December 1997

Summary

Fatty acids from two strains of the cyanobacterium Microcystis aeruginosa, PCC 7820 (a strain that produces the hepatotoxin microcystin-LR, MC-LR) and CYA 43 (a strain that produces only small quantities of MC-LR), were extracted, partially characterised and tested for their inhibitory effect on the K⁺-dependent p-nitrophenol phosphatase (pNPPase) activity of tilapia (Oreochromis mossambicus) gill basolateral membrane. Thin-layer chromatography of the lipids from dichloromethane:methanol extracts of M. aeruginosa PCC 7820 and CYA 43, using diethylether:isopropanol:formic acid (100:4.5:2.5) as solvent, yielded five inhibitory products from M. aeruginosa 7820 and six from M. aeruginosa CYA 43. None of these products could be related to MC-LR. The inhibitory behaviour of the products mimics that of a slow, tight-binding inhibitor. The inhibitory activity is removed by incubation of extracts with fatty-acid-free bovine serum albumin (FAF-BSA). However, FAF-BSA only partially reverses the inhibition of K⁺-dependent pNPPase on fish gills pre-exposed to the extracted products. We conclude that M. aeruginosa strains PCC 7820 and CYA 43 produce fatty acids with potent inhibitory effects on K⁺-dependent pNPPase. The release of these products following lysis of cyanobacterial blooms may help to explain fish kills through a disturbance of gill functioning.

Key words: Microcystis aeruginosa, Na⁺/K⁺-ATPases, lipids, fish, Oreochromis mossambicus, inhibition, tilapia.

Introduction

Eutrophication of fresh waters has led to an increased incidence of cyanobacterial blooms that may produce neuro-and hepatotoxins (Codd et al. 1989). Upon senescence of these blooms, the toxins that are released into the water may cause fish to die (e.g. Schwimmer and Schwimmer, 1968; Eriksson et al. 1986; Penalosa et al. 1990; Rodger et al. 1994).

The majority of such fish kills have been attributed to hypoxic water conditions resulting from the high oxygen demand caused by bloom respiration at night and/or by bloom senescence. However, dissolved oxygen levels were 90% of normal values in Loch Leven, Scotland, when moribund brown trout (Salmo trutta) were found after lysis of an Anabaena flos-aquae bloom (Rodger et al. 1994). These authors found histopathological evidence both for gill damage and for severe liver damage in the brown trout, which was similar to that observed in fish treated with microcystins (Phillips et al. 1985; Råbergh et al. 1991; Tencalla et al. 1994). However, immersion trials using concentrations of aqueous extracts of the hepatotoxic cyanobacterial cells similar to those found in eutrophic environments did not cause deaths (Bury et al. 1995). Consequently, the exact cause of death, i.e. the biochemical mechanism underlying death following exposure to cyanobacterial blooms, has yet to be established.

Recent research has advanced a number of explanations for the fish kills. First, fish may ingest the toxins or toxic cyanobacteria, which may then result in liver malfunction (Tencalla et al. 1994). Second, fish exposed to extracts from cyanobacteria exhibit a stress response (Bury et al. 1995, 1996a), which may be detrimental to their health. Third, toxic compounds present in cyanobacteria affect fish gill ion transport by inhibiting ATPase activities in the plasma membranes of the branchial epithelium (Gaete et al. 1994; Bury et al. 1996b; Zambrano and Canelo, 1996).

The toxic compounds present in the cyanobacteria that inhibit fish gill ATPase activities are associated with hydrophobic fractions of methanol extracts of M. aeruginosa, but are unrelated to the hepatotoxin microcystin-LR (MC-LR; Bury et al. 1996b). These results contrast with those of Gaete et al. (1994) and Zambrano and Canelo (1996), who attributed an inhibitory action on carp gill microsomal ATPase activity to MC-LR. However, we were unable to reproduce these results using ultrapure MC-LR on carp or tilapia gill ATPases (Bury et al. 1996b). In view of these results, the aim of this
study was to characterise the compounds other than MC-LR found in *M. aeruginosa* that inhibit gill ATPase activity. K⁺-dependent pNPPase activity in a basolateral plasma membrane (BLM) preparation of branchial epithelium of tilapia (*Oreochromis mossambicus*) was tested for its sensitivity to extracts from two strains of the cyanobacteria *Microcystis aeruginosa* PCC 7820 (which produces a range of microcystins), to *M. aeruginosa* CYA 43 (which produces toxins, but only very small quantities of microcystins) and to subfractions of these extracts.

**Materials and methods**

**Fish-holding conditions**

Tilapia (*Oreochromis mossambicus* Peters) with an approximate mass of 250 g were obtained from laboratory freshwater stocks and were held in running Nijmegen tap water under a light régime of 12h:12h 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**

The method used for culturing *Microcystis aeruginosa* PCC 7820 and CYA 43 has been described previously (Bury et al. 1995). Cells were harvested in the early stationary phase of batch culture by continuous centrifugation in a Sharples centrifuge (Sharples Ltd, Surrey); the pelleted cells were collected and freeze-dried. The freeze-dried material was extracted twice in methanol (50 ml methanol per 0.15 g), centrifuged at 100 g for 10 min (Sorval RC-5B) and the supernatants combined. The methanol was then evaporated by heating in a waterbath at 35 °C. Dried extract was resuspended in 1 ml of methanol to a final concentration of extract equivalent to 150 mg dry mass ml⁻¹ and stored at -20 °C. Samples of this stock were dried and resuspended in sucrose buffer (250 mmol l⁻¹ sucrose, 10 mmol l⁻¹ Heps/Tris, pH 7.4) for the K⁺-dependent pNPPase assay.

**Gill membrane preparation**

Methods for basolateral membrane isolation were described by Bury et al. (1995). Briefly, gills were excised from tilapia, washed in buffer containing 250 mmol l⁻¹ sucrose, 5 mmol l⁻¹ NaCl, 5 mmol l⁻¹ Hepes/Tris, pH 7.4, aprotonin (0.01 trypsin inhibitor unit (TIU) ml⁻¹) and kept on ice. All subsequent procedures were performed at 1-4 °C. The epithelium was scraped off with a glass microscope slide and homogenised in the washing buffer for 2 min using a Polytron Ultra-Turrax homogeniser fitted with an Ultra-Turrax dispersing tool S25 and set at 20 % of its maximum speed. This procedure kept red blood cells intact while the branchial epithelium was disrupted, in a manner similar to previously published Dounce homogenisation techniques (Flik and Verbost, 1994; Bury et al. 1996b). The red blood cells and cellular debris were removed by centrifugation at 550 g for 10 min, and gill membranes were collected by centrifugation at 30 000 g for 30 min (Sorval RC-5B). The resulting pellet was resuspended in sucrose buffer containing 6 mmol l⁻¹ dithiothreitol with a Dounce-type homogenisation device (100 strokes). The resulting suspension was differentially centrifuged: 1000 g for 10 min, and 10 000 g for 10 min. The supernatant was then removed and centrifuged (30 000 g for 30 min). The pelleted membranes were resuspended in buffer (150 mmol l⁻¹ NaCl, 0.8 mmol l⁻¹ MgCl₂, 20 mmol l⁻¹ Heps/Tris, pH 7.4) by 10 passages through a 23 gauge needle fitted to a tuberculin syringe. This procedure produces a partly ressealed, vesiculated membrane preparation (Flik et al. 1985; Verbost et al. 1994). To obtain maximum K⁺-pNPPase activity, vesicles were permeabilised with saponin (0.2 mg ml⁻¹ at a membrane concentration of 1 mg ml⁻¹ bovine serum albumin equivalents) to ensure optimal substrate accessibility. The membrane protein content was estimated using a commercial kit (Bio-Rad); bovine serum albumin (BSA) was used as standard. Routinely, inhibition studies were performed using the material extracted from 23 mg of freeze-dried cyanobacterial material per milligram BSA equivalent of membrane protein, unless otherwise stated.

**K⁺-dependent pNPPase activity**

K⁺-dependent p-nitrophenol phosphatase (pNPPase) activity, which reflects the dephosphorylation step of the Na⁺/K⁺-ATPase reaction cycle, was determined as follows: toxin-treated or control membranes (10 µl) were mixed with 500 µl of either medium A or medium E and incubated for 20 min at 37 °C. Medium A contained 10 mmol l⁻¹ KCl, 7.5 mmol l⁻¹ MgCl₂, 1 mmol l⁻¹ trans-1,2-diaminocyclohexane-N,N',N''-tetra-acetic acid (CDTA) and 5 mmol l⁻¹ p-nitrophenolphosphate (pNPP), pH 7.4; medium E consisted of medium A to which 1 mmol l⁻¹ ouabain had been added and from which KCl had been omitted. The reaction was stopped by the addition of 1 ml of ice-cold 1 mol l⁻¹ NaOH. The K⁺-dependent ouabain-sensitive pNPPase activity was defined as the difference in the amount of p-nitrophenol (pNP) released in media A and E, measured at 420 nm and calculated using the equation:

\[
P_{\text{NPPase}} = \frac{AX}{AY} \times \frac{1}{\text{[protein]}} \times \frac{1}{t},
\]

where \( AX \) is the difference in absorbency at 420 nm between media A and E for experimental samples, \( AY \) is the difference in absorbency at 420 nm between a pNP standard and a blank sample, the concentration of pNP standard is in µmol l⁻¹; [protein] is the protein content of the experimental sample, and \( t \) is the duration of the assay (in h).

**Enzyme inhibition assays**

In the first series of experiments, membranes were either incubated on ice for 1 h with methanol extracts from *M. aeruginosa* PCC 7820 or CYA 43, or an equivalent quantity of cyanobacterial material was added to the membranes at the start of the assay. In this series of experiments, two concentrations of the methanol extracts from *M. aeruginosa*...
Hexane separation procedures were performed and details are described below.

The hexane layer was removed, and both fractions were left to dry in a fume hood. The residue from the hexane layer was resuspended in 75 μl of sucrose buffer, and the volume of the other layer was adjusted with sucrose buffer to an equivalent volume.

**Dichloromethane:methanol separation**

Methanol stock extracts from *M. aeruginosa* 7820 and CYA 43 (75 μl) were dried and resuspended in 75 μl of sucrose buffer. To this suspension, 0.73 ml of distilled water and 3 ml of dichloromethane:methanol (1:2) were added. This mixture was shaken for 15 min and, to obtain a two-phase system, a further 1 ml of dichloromethane and 1 ml of water were added and the solution was vigorously mixed. The layers were separated by centrifugation (550 g for 5 min), the dichloromethane layer was dried overnight in a fume hood, and the water/methanol layer was lyophilised. The residues were resuspended in 75 μl of sucrose buffer.

**Thin-layer chromatography**

Samples (20–60 μl) from the dichloromethane:methanol extracts from both strains of *M. aeruginosa* were dried and resuspended in an equivalent volume of methanol. In addition, the hydrophobic and inhibitory fractions obtained with the Pharmacia ‘Smart-System’ for either strain of *M. aeruginosa* were lyophilised and resuspended in a volume of methanol equivalent to that initially injected onto the column. Known volumes of these methanol suspensions were streaked along the origin of a Merck silica gel 60 (20 cmx20 cmx0.2 cm) plate (thin-layer chromatography, TLC, plates) and then exposed to a solvent system containing diethyl ether/isopropanol:formic acid (100:4:5:2.5).

TLC plates were divided into two batches, and one batch was sprayed with a sulphuric acid:methanol (1:1) solution. Regions of the silica gel plates were scraped off, including an area that had only been exposed to the solvent system to act as a control, and extracted three times with 2 ml of methanol. These extracts were centrifuged at 550 g to remove particulate matter, and the supernatant was dried in a water bath at 40 °C. The resulting residues were resuspended in half the volume of methanol that was initially streaked onto the silica gel plate. This was then dried, and the residues were resuspended in sucrose buffer prior to inhibition assays. The final concentration of cyanobacterial material used for the assay was equivalent to 46 mg dry mass mg⁻¹ membrane protein.

**Fatty-acid-free bovine serum albumin (FAF-BSA) treatment**

Samples (75 μl) of the dichloromethane:methanol extracts from *M. aeruginosa* PCC 7820 or CYA 43 were dried, resuspended in sucrose buffer to an equivalent volume and incubated for 1 h with 2 % (w/v) fatty-acid-free bovine serum albumin (FAF-BSA; Boehringer Mannheim, Germany). Samples from this solution were added to the membranes (equivalent to 30 mg dry mass mg⁻¹ membrane protein) and incubated for 1 h. Additionally, membranes were initially incubated with 2 % (w/v) FAF-BSA for 1 h and then exposed.
to an equivalent quantity of *M. aeruginosa* PCC 7820 or CYA 43. Appropriate controls using FAF-BSA were also run.

**Statistics**

Results are presented as mean ± s.e.m. Differences in the time course study and the concentration curves were assessed by analysis of variance (ANOVA) in combination with a Tukey's honestly significant differences (HSD) test. All other differences among groups were assessed using a paired *t*-test (SPSS-6 for Windows).

**Results**

**Enzyme inhibition assays**

Preincubation of membranes with extracts from the cyanobacteria *M. aeruginosa* 7820 or CYA 43 for 1 h prior to commencing the K*-dependent pNPPase assay caused 70% inhibition of activity compared with controls (Fig. 1B,D). Membranes that did not receive the extracts from the cyanobacteria at the beginning of the assay generally retained their enzyme activity at the level of controls (Fig. 1A,C). However, there was one exception to this: inhibition was seen in membranes that had received 23 mg dry mass of *M. aeruginosa* PCC 7820 per milligram membrane protein after 20 min of incubation (Fig. 1A).

An increase in the concentration of cyanobacterial extracts resulted in an increase in the level of inhibition of membrane K*-dependent pNPPase activity. However, the degree of inhibition depended on the concentration of membrane (Fig. 2A,B). Extracts of 150 μg of freeze-dried material of *M. aeruginosa* CYA 43 gave 99% inhibition at 1.875 μg of membrane protein (Fig. 2B), whilst *M. aeruginosa* PCC 7820 proved to be less potent (Fig. 2A); extracts from 200 μg of freeze-dried material of *M. aeruginosa* PCC 7820 gave 67% inhibition at the same membrane concentration. In comparison, 30 μg of membrane protein exposed to the same concentration of *M. aeruginosa* 7820 or CYA 43 showed 19% and 32% inhibition, respectively.

**Characterisation of the compound(s)**

Combining fractions from the Pharmacia 'Smart-System' confirmed a previous study (Bury et al. 1996b): the compounds that inhibited Na*/K*-ATPase were primarily found in the more hydrophobic fractions (data not shown). However, inhibition studies on the individual fractions show that there were four regions showing significant inhibitory activity (Table 1). These inhibitory fractions did not correspond to individual protein or peptide peaks on the chromatogram as

<table>
<thead>
<tr>
<th>Fraction</th>
<th>pNPPase activity (μmol·mg⁻¹·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.38±0.88</td>
</tr>
<tr>
<td>24</td>
<td>5.89±0.92</td>
</tr>
<tr>
<td>25</td>
<td>6.09±0.77</td>
</tr>
<tr>
<td>26</td>
<td>6.08±0.81</td>
</tr>
<tr>
<td>27</td>
<td>6.42±0.88</td>
</tr>
<tr>
<td>28</td>
<td>5.88±0.83</td>
</tr>
<tr>
<td>29</td>
<td>5.22±0.93</td>
</tr>
<tr>
<td>30</td>
<td>4.75±0.48*</td>
</tr>
<tr>
<td>31</td>
<td>4.31±0.61*</td>
</tr>
<tr>
<td>32</td>
<td>3.31±0.36*</td>
</tr>
<tr>
<td>33</td>
<td>4.19±0.72*</td>
</tr>
<tr>
<td>34</td>
<td>4.93±0.50</td>
</tr>
<tr>
<td>35</td>
<td>5.89±0.68</td>
</tr>
</tbody>
</table>

See Materials and methods for details of fractionation procedure. Values are mean ± s.e.m., *N=4*. Asterisks indicate a significant difference from control values (paired *t*-test, *P*<0.05).

Activity is measured as μmol pNP mg⁻¹·membrane protein h⁻¹.
Table 2. The K*-dependent pNPPase activity of tilapia gill basolateral membranes treated with methanolic extracts of M. aeruginosa PCC 7820 or CYA 43 resuspended in sucrose buffer and heated to 90 °C for 30 min or treated with Concanavalin A sepharose beads.

<table>
<thead>
<tr>
<th></th>
<th>M. aeruginosa 7820</th>
<th>M. aeruginosa CYA 43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.66±0.44</td>
<td>4.2±0.46</td>
</tr>
<tr>
<td>Untreated</td>
<td>5.95±0.53</td>
<td>4.2±0.46</td>
</tr>
<tr>
<td>Heated</td>
<td>5.03±0.60*</td>
<td>3.26±0.457*</td>
</tr>
<tr>
<td>Control</td>
<td>5.45±0.194</td>
<td>3.72±0.56</td>
</tr>
<tr>
<td>Untreated</td>
<td>4.01±0.434</td>
<td>3.72±0.56</td>
</tr>
<tr>
<td>Concanavalin-A-treated</td>
<td>4.26±0.31*</td>
<td>4.67±0.28*</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.; N=9 for the heated samples and N=5 for those treated with Concanavalin A. In all cases, untreated and treated values are significantly different from controls; asterisks indicate a significant difference between treated and untreated values (paired t-test, P<0.05).

Activity is measured as μmol pNP/mg membrane protein h⁻¹.
Table 3. The \( K^+ \)-dependent \( pNPP \)ase activity of tilapia gill basolateral membranes treated with different regions from a thin-layer chromatography plate following the separation of dichloromethane:methanol extracts from \( M. \) aeruginosa PCC 7820 or CYA 43 with a solvent phase of diethylether:isopropanol:formic acid (100:4.5:2.5).

<table>
<thead>
<tr>
<th>( M. ) aeruginosa 7820</th>
<th>( M. ) aeruginosa CYA 43</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_F )</td>
<td>( pNPP )ase activity (( \mu )mol mg(^{-1}) h(^{-1}))</td>
</tr>
<tr>
<td>Control</td>
<td>4.43±0.45</td>
</tr>
<tr>
<td>1-0.96</td>
<td>4.67±0.50</td>
</tr>
<tr>
<td>0.96-0.91</td>
<td>4.94±0.64</td>
</tr>
<tr>
<td>0.91-0.76</td>
<td>3.78±0.49*</td>
</tr>
<tr>
<td>0.76-0.71</td>
<td>3.21±0.46*</td>
</tr>
<tr>
<td>0.71-0.56</td>
<td>2.56±0.50*</td>
</tr>
<tr>
<td>0.56-0.51</td>
<td>4.31±0.47</td>
</tr>
<tr>
<td>0.51-0.34</td>
<td>4.64±0.48</td>
</tr>
<tr>
<td>0.34-0.05</td>
<td>3.04±0.52*</td>
</tr>
<tr>
<td>0.05-0</td>
<td>1.74±0.53*</td>
</tr>
<tr>
<td>0.05-0</td>
<td>2.14±0.50*</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m., \( N=5 \).
Asterisks indicate a significant difference from control values (paired \( t \)-test, \( P<0.05 \)).
Activity is measured as \( \mu \)mol\( \mu \)mol\( pNPP \) mg\(^{-1}\) membrane protein h\(^{-1}\).

TLC plate relative to the solvent front) of 0.56-0.43 (Table 3). Separation of the hydrophobic region obtained from the Pharmacia ‘Smart-System’ by TLC revealed that \( M. \) aeruginosa PCC 7820 had two inhibitory regions and \( M. \) aeruginosa CYA 43 had three (Table 4). The additional region had an \( R_F \) value of 0.66-0.48 (Table 4). However, in the case of both separation procedures and for both cyanobacterium strains, the most potent region occurred in extracts taken from the origin of the plate (Tables 3, 4).

Incubation of the membranes with dichloromethane:methanol extracts from either \( M. \) aeruginosa PCC 7820 or CYA 43 followed by treatment with 2% (w/v) FAF-BSA significantly reduced the inhibitory activity of the extracts. The inhibition could be further and significantly reduced by pretreatment of the extracts with FAF-BSA (Fig. 4).

Discussion

The results from this study show that there are a number of cytotoxic compounds present in methanolic extracts of cyanobacteria that inhibit fish gill \( \text{Na}^+/\text{K}^+ \)-ATPase, other than the hepatotoxin microcystin-LR (MC-LR). Characterisation shows that these compounds must be lipids. These findings are in line with and extend results from a previous study (Bury et al. 1996). TLC analysis of the lipids present in \( M. \) aeruginosa shows a profile that includes monoglycosyl-diglyceride (MGDG),

Table 4. The \( K^+ \)-dependent \( pNPP \)ase activity of tilapia gill basolateral membranes treated with different regions of a thin-layer chromatography plate following separation of the most hydrophobic region collected from the Pharmacia ‘Smart-System’ for \( M. \) aeruginosa PCC 7820 or CYA 43 with a solvent phase of diethylether:isopropanol:formic acid (100:4.5:2.5).

<table>
<thead>
<tr>
<th>( M. ) aeruginosa 7820</th>
<th>( M. ) aeruginosa CYA 43</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_F )</td>
<td>( pNPP )ase activity (( \mu )mol mg(^{-1}) h(^{-1}))</td>
</tr>
<tr>
<td>1-0.94</td>
<td>6.20±0.865</td>
</tr>
<tr>
<td>0.94-0.81</td>
<td>6.18±0.95</td>
</tr>
<tr>
<td>0.81-0.61</td>
<td>5.94±0.89</td>
</tr>
<tr>
<td>0.61-0.45</td>
<td>6.02±0.91</td>
</tr>
<tr>
<td>0.45-0.26</td>
<td>6.09±0.86</td>
</tr>
<tr>
<td>0.26-0.05</td>
<td>5.27±0.89*</td>
</tr>
<tr>
<td>0.05-0</td>
<td>2.73±0.67*</td>
</tr>
</tbody>
</table>

Values are means ± s.e.m., \( N=5 \).
Asterisks indicate a significant difference from control values (paired \( t \)-test, \( P<0.05 \)).
Activity is measured as \( \mu \)mol\( \mu \)mol\( pNPP \) mg\(^{-1}\) membrane protein h\(^{-1}\).

\( R_F \), distance migrated from the origin relative to the solvent front.
diglycosyl-diglyceride (DGDG), phosphatidyl-glycerol and sulfoquinovosyl-diglyceride (sulphilipid), as well as orange, red and dark green pigments (Murata and Nishida, 1987; Ikawa et al. 1996; Walsh et al. 1997). It has previously been shown that lipids present in cyanobacteria may be bioactive (Murakami et al. 1991; Ikawa et al. 1994, 1996). MGDGs and DGDGs from Phormidium tenue have autolytic properties (Murakami et al. 1991), and unsaturated fatty acids (linoleic and oleic acids) extracted from Aphanizomenon flos-aquae and M. aeruginosa inhibit the growth of the green alga Chlorella (Ikawa et al. 1994, 1996). The present study indicates that the lipid compounds present in M. aeruginosa CYA 43 and M. aeruginosa PCC 7820 inhibit Na+/K+-ATPase activity in fish gills. In addition, the reduction in inhibition observed when methanolic extracts of M. aeruginosa are treated with Concanavalin A suggests that at least one of the compounds may possess a sugar moiety. The inhibition profiles of the thin-layer chromatograms for each strain of cyanobacterium differ, with M. aeruginosa CYA 43 possessing an additional inhibitory region compared with M. aeruginosa PCC 7820.

Lipids have been shown to inhibit a number of ion-transporting enzymes in mammals, such as Na+/K+-ATPase (Kelly et al. 1986; Swarts et al. 1990), H+/K+-ATPase (Bin Im and Blakeman, 1982; Swarts et al. 1991; Beil et al. 1994), smooth endoplasmic reticulum Ca2+-ATPases (SERCA ATPases) (Kim and LaBella, 1988) and Zn2+-dependent ATPase (Ronquist and Frithz, 1992). In the brine shrimp Artemia salina, Morohashi et al. (1991) identified long-chain fatty acids as endogenous inhibitors of Na+/K+-ATPase. In most cases, inhibition is due to unsaturated long-chain fatty acids, rather than to the methylated or saturated forms (e.g. Davis et al. 1987; Swann, 1984; Swarts et al. 1990, 1991). Combining these findings with the known lipid profiles from M. aeruginosa, it is apparent that these cyanobacteria may produce unsaturated fatty acids inhibiting Na+/K+-ATPase. This hypothesis is corroborated by the observation that incubation of the dichloromethane:methanol extracts from M. aeruginosa PCC 7820 or CYA 43 with fatty-acid-free bovine serum albumin (FAF-BSA) reduced the extent of inhibition. However, the inhibition was not completely prevented, probably as a result of the concentrations of cyanobacterial extract used. Treatment of the branchial plasma membranes with FAF-BSA, following incubation with either strain of M. aeruginosa, only partially restored enzyme activity, indicating an avid binding of the inhibitor to the enzyme complex. Similar observations have been made for gastric H+/K+-ATPase activity after incubation with linoleic acid (Bin Im and Blakeman, 1982) and for Na+/K+-ATPase activity after incubation with oleic acid (Swarts et al. 1990).

Methanol extracts from M. aeruginosa have been shown to inhibit P-type and SERCA-type ATPases, as well as mitochondrial Ca2+-sequestering mechanisms (either via the H+/ATPase or the mitochondrial Ca2+ channel; Bury et al. 1996b). The use of assay conditions specific to the dephosphorylation step of the Na+/K+-ATPase reaction cycle indicates that lipids from the cyanobacterial extracts inhibit at the K+-binding site of the enzyme. Studies have shown that unsaturated fatty acids may displace ouabain from the Na+/K+-ATPase and that this may be one of the mechanisms by which fatty acids inhibit Na+/K+-ATPase activity (Lamera and Hulsmann, 1977; Swann, 1984; Tamura et al. 1983; Swarts et al. 1990).

The degree of inhibition of the fish gill Na+/K+-ATPase by lipid compounds extracted from M. aeruginosa was dependent on the reaction conditions, i.e. the concentrations of enzyme and inhibitor (Fig. 2), as well as on incubation time (Fig. 1). Similar results have been observed by Swarts et al. (1990) for the inhibition of Na+/K+-ATPase activity by unsaturated fatty acids. The convex inhibition curves obtained when the enzyme was exposed to increasing concentrations of cyanobacterial extract, and at different concentrations of membrane protein, are characteristic of tight-binding inhibition (Morrison, 1969; Williams and Morrison, 1979). Furthermore, the time taken for the inhibition to occur (1 h) and the nature of this inhibition suggest ‘slow, tight-binding’ inhibition (Morrison and Walsh, 1988; Szedlezek and Duggleby, 1995). However, we cannot yet further define the substances that cause inhibition and the kinetics of this inhibition because of the number of inhibitory compounds (see Tables 3, 4) extracted from both strains of M. aeruginosa.

What is the ecotoxicological importance of cyanobacterial lipids? They may reduce the growth of other phytoplankton (Ikawa et al. 1994, 1996), but it is not clear whether they adversely affect higher vertebrates when present in an aquatic environment.
environment. Immersion trials, in which tilapia were exposed to methanol extracts of *M. aeruginosa*, showed inhibition of whole-body Ca\(^{2+}\) influx (Bury et al. 1996b). Our results suggest that lipids, rather than MC-LR, from cyanobacteria interfere with gill basolateral membrane ion-extrusion mechanisms and thus may contribute to the fish deaths seen after lysis of a cyanobacterial bloom.

This study was supported by a fellowship to N.R.B. from the Research School Environmental Chemistry and Toxicology (M&T), The Netherlands. We thank K. A. Beattie and M. Herberigs for their technical assistance.

References


