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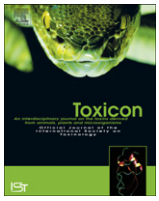

Mode of action of membrane-disruptive lytic compounds from the marine dinoflagellate *Alexandrium tamarense*

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ABSTRACT

Certain allelochemicals of the marine dinoflagellate *Alexandrium tamarense* cause lysis of a broad spectrum of target protist cells but the lytic mechanism is poorly defined. We first hypothesized that membrane sterols serve as molecular targets of these lytic compounds, and that differences in sterol composition among donor and target cells may cause insensitivity of *Alexandrium* and sensitivity of targets to lytic compounds. We investigated Ca^{2+} influx after application of lytic fractions to a model cell line PC12 derived from a pheochromocytoma of the rat adrenal medulla to establish how the lytic compounds affect ion flux associated with lysis of target membranes. The lytic compounds increased permeability of the cell membrane for Ca^{2+} ions even during blockade of Ca^{2+} channels with cadmium. Results of a liposome assay suggested that the lytic compounds did not lyse such target membranes non-specifically by means of detergent-like activity. Analysis of sterol composition of isolates of *A. tamarense* and of five target protistan species showed that both lytic and non-lytic *A. tamarense* strains contain cholesterol and dinosterol as major sterols, whereas none of the other tested species contain dinosterol. Adding sterols and phosphatidylcholine to a lysis bioassay with the cryptophyte *Rhodomonas salina* for evaluation of competitive binding indicated that the lytic compounds possessed apparent high affinity for free sterols and phosphatidylcholine. Lysis of protistan target cells was dose-dependently reduced by adding various sterols or phosphatidylcholine. For three tested sterols, the lytic compounds showed highest affinity towards cholesterol followed by ergosterol and brassicasterol. Cholesterol comprised a higher percentage of total sterols in plasma membrane fractions of *A. tamarense* than in corresponding whole cell fractions. We conclude therefore that although the molecular targets of the lytic compounds are likely to involve sterol components of membranes, *A. tamarense* must have a complex self-protective mechanism that still needs to be addressed.

1. Introduction

Marine dinoflagellates of the genus *Alexandrium* are known to produce secondary metabolites with widespread allelochemical effects on other photosynthetic (Blanco and Campos, 1988; Arzul et al., 1999; Fistarol et al., 2004; Tillmann et al., 2007, 2008a; Tillmann and Hansen, 2009) as well as heterotrophic protists (Hansen, 1989; Hansen et al., 1992; Tillmann and John, 2002; Tillmann et al., 2007, 2008a). Cell lysis is the most common reaction of target species when exposed to *Alexandrium* cell cultures, cell-free culture supernatant or filtrate (Tillmann et al., 2008b; Ma et al., 2009). The unknown allelochemicals from *Alexandrium* are therefore assumed to act destructively on the external plasma membrane of sensitive organisms.

The exact molecular composition of cell plasma membranes in eukaryotes varies widely among species. Nevertheless, because they share commonalities in structure

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Nomenclature

ATP	Adenosine-5′-triphosphate
ATPase	Adenosine triphosphatase
BCA	Bicinchoninic acid
CCD	Charge-coupled device
DSP	Diarrhetic shellfish poisoning
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis-(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
GC–MS	Gas chromatography–Mass spectrometry
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HILIC	Hydrophilic interaction ion-chromatography
MES	2-(N-morpholino) ethanesulfonic acid
OA	Okadaic acid
PC	Phosphatidylcholine
PEG	Polyethylene glycol
RPMI	Roswell Park Memorial Institute medium
SPE	Solid phase extraction
ZIC-HILIC	Zwitterionic-HILIC

and function according to recent models (Mayor and Rao, 2004), the membranes typically comprise similar general classes of metabolites, including phospholipids, sterols, transmembrane proteins, polysaccharides, etc. In many cases, however, it is still unknown how lytic compounds interact with the membrane of target cells and how the producing organisms protect their own membranes from destructive attack.

Research on membrane-lysing toxins isolated from microorganisms has been carried out for decades. For example, polyene antibiotics amphotericin B (AmB) and filipin are known to interact with sterols in biomembranes when lysing target cells, but they permeate the membrane by different mechanisms (Kotlerbrajtburg et al., 1979; Brajtburg et al., 1980; Knopik-Skrocka and Bielawski, 2002). Eight pairs of AmB/sterols comprise a complex similar to ion-channels in the membrane (Andreoli, 1974; de Kruijf and Demel, 1974), whereas filipin induces damage by large non-selective perforations thereby causing membrane disruption (Knopik-Skrocka and Bielawski, 2002). Selective toxicity of AmB is due to higher affinity for ergosterol than cholesterol, the principal fungal and mammalian plasma membrane sterols, respectively.

Marine dinoflagellates, including toxic species, are known for the production of unusual sterols, particularly 4-α-methyl derivatives such as gymnodinosterol and brevesterol (Giner et al., 2003), with poorly defined or unknown function. Furthermore, several studies on membrane permeation mechanisms of hemolytic and/or lytic polyene-polyhydroxy toxins from dinoflagellates are also reported to relate to membrane sterols. Hemolytic activity of amphidinols isolated from the dinoflagellate *Amphidinium klebsii* is apparently increased when cholesterol is present in the target membrane (Paul et al., 1996, 1997; Morsy et al., 2008a). Karlotoxins (KmTx) from the dinoflagellate *Karlodinium veneficum* show different binding affinities to conspecific sterols than to sterols of target species, indicating that the toxin producers may protect themselves by possessing special sterols, e.g. the 4-α-methyl sterol gymnodinosterol, which does not interact with the toxins (Deeds and Place, 2006).

Lytic compounds released by *Alexandrium tamarense* show functional if not structural similarities to the dinoflagellate polyene metabolites amphidinols and karlotoxins with respect to their effects on cell membranes. The membrane-active compounds from *A. tamarense* lyse membranes of sensitive protists, but microscopic observations have revealed no effect on cells of various strains of *A. tamarense*, of other species of the genus *Alexandrium*, or of the toxigenic prymnesiophyte *Prymnesium parvum* (Tillmann et al., 2007). Several studies on sterol composition of *Alexandrium* spp. have shown that the 4-methyl sterol dinosterol is one of the major components (Piretti et al., 1997; Leblond and Chapman, 2002). Similar to gymnodinosterol found in *Karlodinium*, certain specific sterols might protect membranes of *A. tamarense* cells from being lysed by their own lytic compounds. Given previous research on membrane-lytic activity in marine dinoflagellates, our study of the lytic activity of *A. tamarense* was designed accordingly to establish: (1) if cell lysis in target species is directly caused by membrane disruption; (2) if the mechanism of cell lysis is non-specific (e.g., "detergent-like"); (3) if the lytic compounds also interact with sterols; (4) if *Alexandrium* cells protect themselves by possessing special sterols; and (5) if the target protists have sterols with high affinity to the lytic compounds.

We therefore investigated the cytotoxicity of lytic compounds from *A. tamarense* on rat neuroendocrine PC12 cells, and determined an increased permeability of target cell membranes for Ca^{2+} ions despite blockade of Ca-channels, possibly by membrane disruption. Nevertheless, supernatant fractions from *Alexandrium* with lytic activity against the cryptophyte *Rhodomonas* did not lyse liposome membranes non-specifically, as would be expected for detergent-like activity. We also compared the whole cell as well as plasma membrane sterol composition of *A. tamarense* strains with those of five target strains of protists. Although sterols, specifically cholesterol, appear to be a molecular target of the lytic substances against liposomes, we propose that the lytic effects against protist cells cannot be accounted for merely by compositional variation and relative binding affinity among various sterol analogs.

2. Materials and methods

2.1. Protist culture

Two clones of *A. tamarense* (Alex2 and Alex5) were selected based on their different lytic capacity as quantified by a whole cell cryptophyte *Rhodomonas* bioassay (Tillmann et al., 2009). Alex2 had high lytic capacity whereas Alex5 had no measurable lytic effect on *Rhodomonas* and thus served as a negative control. Both *Alexandrium* strains were isolated from the North Sea coast of Scotland (Tillmann et al., 2009) and grown in K-medium (Keller et al., 1987), supplemented with selenite (Dahl et al., 1989), prepared from 0.2 μm sterile-filtered (VacuCap, Pall Life Sciences, Dreieich, Germany) North Sea seawater (salinity 32 psu). Cultures

were maintained in 1 L Erlenmeyer flasks under controlled conditions at 15 °C under cool-white fluorescent light at a photon flux density (PFD) of 100 μmol photons m^{-2} s^{-1} on a 16 h light: 8 h dark photocycle.

Five protist species were selected for target sensitivity experiments. The autotrophic cryptophyte *Rhodomonas salina* (Kalmar Culture Collection KAC 30), the chlorophyte *Dunaliella salina* (AWI culture collection), and the raphidophyte *Heterosigma akashiwo* (CCMP 2274) were cultured under the same conditions as *A. tamarense*. The heterotrophic dinoflagellates *Oxyrrhis marina* (Göttingen culture collection, strain B21.89) and *Gyrodinium dominans* (AWI culture collection) were grown at 20 °C under cool-white fluorescent light at a PFD of 20 μmol photons m^{-2} s^{-1} on a 16 h light: 8 h dark photocycle, with the chlorophyte *D. salina* as food. Cultures were starved and free of food algal cells (as checked by microscopy) before harvested for sterol analysis.

2.2. Determination of lytic compound targets with PC12 cells

2.2.1. Cell culture methods

PC12 cells derived from a pheochromocytoma of the rat adrenal medulla were obtained from the CLS (Cell Line Service, Eppelheim, Germany and German Collection of Microorganisms and Cell Cultures) and kept in culture medium containing RPMI 1640, 10% fetal calf serum, 5% horse serum. Cells were cultivated in an incubator at 37 °C, 90% humidity and 5% CO_2 with regular medium changes twice a week or when additionally necessary. Cells were grown in culture flasks and 1–2 days prior to the experiments were seeded into Petri dishes coated with collagen.

2.2.2. Preparation of lytic fractions

Supernatant fractions obtained by solid phase extraction (SPE) in 80% methanol and hydrophilic interaction ion-chromatography (HILIC) fractions were prepared from *A. tamarense* cell cultures of Alex2 and Alex5 in stationary growth phase, as previously described (Ma et al., 2009). In the current experiment, Alex5 fractions served as a negative control to ensure that any observed effect was induced by the allelochemicals from Alex2 fractions rather than by artifacts. For HILIC fractions, 50 μL SPE fraction, purified from approximately 600 mL supernatant, was separated through an analytical column (150 × 4.6 mm) packed with 5 μm ZIC-HILIC, 200 Å, (SeQuant, Haltern, Germany) and maintained at 25 °C. A pre-column with the same packing material was also used. The flow rate was 0.7 mL min^{-1} and gradient elution was performed with two eluants. Eluant A was 2 mM formic acid and 5 mM ammonium formate in 20% water and 80% acetonitrile; eluant B was 10 mM formic acid and 10 mM ammonium formate in water. The gradient was as follows: column equilibration with 0% eluant B until 20 min, then linear gradient to 100% B until 35 min, followed by isocratic elution with 100% eluant B until 45 min and finally a return to initial 0% eluant B until 46 min. A total of five runs was performed and fraction components with retention times of 7–9 min (according to preliminary results, data not shown) were collected. The fractions were pooled and dried under N_2, and finally dissolved in 1 mL K-medium and/or deionized water and stored at 4 °C for more than two days before use. Lytic activity of both supernatant and HILIC fractions were quantified as half maximal effective concentrations (EC_{50}, expressed as EC_{50} and 95% confidence interval) with a *R. salina* bioassay (Ma et al., 2009).

2.2.3. Fluorimetric measurements of intracellular Ca^{2+} changes

PC12 cells were incubated in buffer (in mM: 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 2.0 $CaCl_2$, 1.3 NaH_2PO_4, 30 glucose, 26 Na HEPES) containing 5 μM Fura II acetoxymethylester (Sigma–Aldrich, Deisenhofen, Germany) for 30 min at room temperature (22 ± 2 °C). The incubation buffer was removed from the cultured cells and replaced with 1 mL buffer. Fluorescence of cells was monitored by an imaging system (Visitron, Puchheim, Germany) and a CCD camera (Coolsnap) mounted on an inverted microscope (Zeiss Axiovert 100, Göttingen, Germany). Single PC12 cells (total: 8–30 cells) were simultaneously measured, and separated using "the region of interest" function of the software Metafluor (Meta Imaging Series, Downingtown, USA). Fluorescence was transmitted through a UV objective (Zeiss NeoFluar 20X, Göttingen, Germany). Data were obtained by division of two images (F340/380) - one at 340 nm and the other at 380 nm excitation (Grynkiewicz et al., 1985; Bickmeyer et al., 2004, 2010). Fluorescence ratios were not converted into Ca^{2+} concentrations. Statistics were performed using Prism from Graphpad and Igor (WaveMetrics, Portland, OR, USA). Values of a 2 min period before compound applications were averaged and compared (Student's *t*-test) to values of the last 2 min (18–20 min experiment duration).

Experiments on calcium influx from the extracellular buffer were performed with buffer as described above and buffer without calcium but supplemented with 2 mM EGTA (ethylene glycol bis-(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid). In this experiment, solution changes and compound application were performed by a continuous perfusion system. The Alex2 SPE 80% methanol fraction, purified from 200 mL supernatant, was applied at 1% by volume. In another set of experiments, samples of supernatant as well as HILIC fractions from both Alex2 and Alex5 cultures were used. Samples were pipetted as a total 0.5 mL, including 15 μL sample in buffer into Petri dishes containing 1 mL PC12 cells. Because of the lack of effect after adding the low sample concentration, 100 μL were added after several minutes. The changes of intracellular Ca^{2+} concentration expressed as fluorescence emission ratio were recorded. Mechanical disturbances during application of the compounds may lead to spurious cellular Ca^{2+} level responses due to mechanosensitive Ca^{2+} permeable ion-channels in PC12 cells, as in most other cells (Bickmeyer, 2005).

The effect of cadmium application to block calcium ion-channels (Weinsberg et al., 1995) on the potency of Alex2 lytic compounds was determined with 0.5 mL buffer including 90 μL Alex2 HILIC fraction in K-medium, as well as 3 μL $CdCl_2$ (from 50 mM stock solution), applied to PC12 cells. The change of fluorescence emission after excitation at 340 and 380 nm was recorded.

2.3. Liposome leakage assay

2.3.1. Preparation of liposome encapsulation of calcein

Liposomes were prepared according to a modified method of Pinnaduwage and Bruce (1996). 25 mg lipid

sample including 100% phosphatidylcholine (PC, Sigma–Aldrich, Steinheim, Germany) or PC/cholesterol (70:30) were dissolved in 2 mL chloroform/methanol (3:1). The organic solvent was removed under N_2 for 2 h. The lipid film formed was hydrated by 1 mL North Sea seawater containing 50 mM of the fluorescent probe calcein (Sigma–Aldrich, Steinheim, Germany). The mixture was ultrasonicated four times for 5 min at 35 min intervals in a sonicator bath (VWR, USC300T, Lutterworth, UK) to initiate encapsulation and formation of liposomes. Liposomes were collected from the supernatant by centrifugation (Eppendorf, 5415R, Hamburg, Germany) at 2500× g for 10 min at room temperature. Liposomes were separated from un-encapsulated calcein by size-exclusion chromatography on a Sephadex G50 (Sigma–Aldrich, Steinheim, Germany) column (ca. 1 × 20 cm), and were applied immediately for lysis determination.

2.3.2. Calcein release from liposomes induced by Triton X-100 and lytic compounds of A. tamarense

Calcein is self-quenching at high concentration in that the fluorescence intensity of calcein is low at high concentration. Triton X-100 induces the release of calcein from liposomes, and fluorescence therefore increases due to dilution of calcein in the medium. The leakage of liposomes was monitored fluorometrically at excitation and emission wavelengths of 490 and 515.4 nm, respectively, with a fluorescence spectrophotometer (Horiba Jobin-Yvon, FluoroLog®-3, Unterhaching, Germany).

The initial fluorescence intensities of 2.7 mL samples including North Sea seawater (blank), Triton X-100 (0.1%), or *R. salina*, Alex5 or Alex2 supernatant were measured. Then 0.3 mL liposome sample was added into each cuvette and the fluorescence intensities of each group were measured for 10 s at 5 min intervals until 30 min. Release of calcein was calculated from the following formula

$$\%\ leakage = 100(F - F_{0t})\ /(F_{Tt} - F_{0t})$$

Where F is the fluorescence after the addition of liposome to the sample, F_{0t} is the fluorescence at each measuring time point, and F_{Tt} is the fluorescence after liposomes were added to 0.1% Triton X-100 at each measuring time point.

2.4. Protist sterol composition

2.4.1. Cell pellet preparation

Protist cultures were centrifuged (Eppendorf 5810R, Hamburg, Germany) at 3220× g for 10 min at 15 °C to acquire cell pellets for direct sterol analysis or plasma membrane isolation. The numbers of cells harvested of whole cell and plasma membrane fraction, respectively, of each strain are as follows: Alex2 (9.8×10^5 and 4.4×10^6), Alex5 (1.1×10^6 and 3.3×10^6), *D. salina* (3.3×10^7 and 2.1×10^8), *H. akashiwo* (9.8×10^6 and 4.9×10^7), *O. marina* (1.4×10^6 and 1.0×10^7), *G. dominans* (4.4×10^5 and 1.7×10^6). For *R. salina*, the cell number was 4.1×10^8 for plasma membrane. The cell pellets for whole cell sterol composition analysis were stored at −20 °C before use, whereas those for plasma membrane preparation were further processed immediately after centrifugation.

2.4.2. Plasma membrane preparation and characterization

2.4.2.1. Plasma membrane enrichment. Cell pellets were re-suspended in 5 mL disruption buffer composed of 400 mM mannitol, 2 mM EDTA, 1 mM $MgCl_2$, 100 mM Na_3PO_4, pH 7.4 (Peeler et al., 1989). For each sample, the cell suspension was disrupted by a French Press at 16,000 psi. Each homogenized sample was centrifuged at 2960× g for 5 min at 4 °C. The supernatant was transferred to a 15 mL Sarstedt tube and further purified by two-phase partitioning. Both dextran T500 (13.4%, w/w) and polyethylene glycol (PEG) 3350 (26.8%, w/w) solution stocks were added to reach a final concentration of 6.7% (w/w), respectively. The mixture was inverted 40 times, and was immediately centrifuged at 660× g for 10 min at 4 °C. The upper PEG phase was ultracentrifuged at 121,000× g (Beckmann Coulter, Optimal™ LE-80K, Krefeld, Germany) for 1 h at 4 °C, and the supernatants were carefully removed by pipette. The residue was re-suspended with 2 mL disruption buffer, and again ultracentrifuged as above to pellet the membrane microsomes. The membrane samples were stored at −20 °C for sterol compositional analysis.

2.4.2.2. Vanadate-sensitive ATPase. Determination of vanadate-sensitive ATPase activity was performed with reference to the method of Gallagher and Leonard (1982). For analysis of plasma membrane purity, an Alex2 cell pellet (ca. 5.5×10^6 cells) was used to prepare cell membrane fractions as described above. After the first ultracentrifugation, the residue was washed with 2 mL 10 mM Tris–HCl buffer, pH 7.5, including 250 mM sucrose, and again ultracentrifuged. The pellet was re-suspended in 600 μL Tris–HCl buffer, and stored at −70 °C. One fifth of the dextran T500 fraction was diluted 10-fold with Tris–HCl buffer, and the following steps were the same as for the PEG fraction.

Total protein was quantified by the bicinchoninic acid (BCA) assay (Smith et al., 1985), performed as described in the BCA assay kit (Pierce, Thermo Fisher Scientific Bonn, Germany). Briefly, 50 μL sample from membrane fractions or albumin standard solutions were mixed with 1 mL working reagent containing 50 parts solution A, 1 part solution B, and 0.01% Triton X-100 (w/w). The mixtures were incubated in a water bath for 30 min at 60 °C, then cooled to room temperature and read spectrophometrically at 562 nm. The protein content of membrane samples was calculated according to the standard curve.

ATPase activity was determined according to Briskin et al. (1987). Assays were carried out in a 1 mL reaction volume with 20 μL membrane fraction and 980 μL reaction buffer containing 5 mM $MgSO_4$, 5 mM ATP Na_2, 30 mM Tris-MES (pH 6.5), 50 mM KCl (when present), and 50 μM Na_3VO_4 (when present). The reaction was performed at 38 °C for 1 h, and then terminated by adding 2.6 mL color development solution consisting of six parts 0.42% (w/v) ammonium molybdate in 1 N H_2SO_4 and 10% (w/v) ascorbic acid. The optical density was determined spectrophotometrically at 700 nm after incubation at room temperature for 20 min.

2.5. Sterol compositional analysis

Cell pellet or plasma membrane fractions were lyophilized (Freeze Dryer, Osterode am Harz, Germany) overnight. Lipids were extracted by mixing 10 mL *n*-hexane per 2 mg lyophilizate and subsequent ultrasonication for 10 min, and then were left at room temperature for 3 h for complete solubilization of lipids. After centrifugation (Hettich, EBA 8S, Tuttlingen, Germany) at 855× *g*, the supernatant was removed and evaporated under N_2. Sterols were derivatized with N-methyl-n-trifluoroacetamide (Macherey–Nagel, Düren, Germany). For sample amounts up to 5 mg, 50 μL of derivatization reagent was added to the dry sample in a micro-sampling vial and stored for 2 h at 70 °C in a thermoblock.

The silylated sterols were analyzed by gas chromatography coupled to mass spectrometry (GC–MS). Gas chromatography was performed on a 3800 GC System (Varian, Palo Alto, CA, USA). Separation was achieved on a low-bleeding CPSil 8CB MS fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Chrompack, Middelburg, Netherlands). Detection was carried out by an MS/MS 2000 Ion-Trap Detector (Varian, Palo Alto, CA, USA) with GC–MS operation control and data processing by the Varian Saturn MS Workstation 6.9.2 software. The sample volume in the direct injection mode was 1 μL. Gas chromatographic analysis was started at 200 °C and held for 1 min and then increased to 320 °C at a heating rate of 40 °C per min, remaining constant for 36 min. The total run time was 40 min.

The sterol standards brassicasterol, desmosterol, ergosterol, cholesterol, stigmasterol, and stigmastanol were purchased from Sigma–Aldrich (Taufkirchen, Germany) and beta-sitosterol from BIOTREND Chemikalien GmbH (Cologne, Germany). Standard sterol solutions were run as reference and samples were quantified by external calibration. The amounts of campesterol, corbisterol and dinosterol were calculated as cholesterol equivalents. Only sterol peaks above the limit of quantitation (s/n ratio $>$ 10) were reported.

2.6. Lipid incubation bioassay

Three common and representative sterols, namely cholesterol, ergosterol and brassicasterol, are major sterols of *A. tamarense* and *O. marina*, *D. salina*, and *R. salina*, respectively, and therefore were selected for competitive binding assay. Phosphatidylcholine (PC) as a basic membrane component was also applied for the assay. Methanolic standard stock solutions of cholesterol, ergosterol, brassicasterol (Sigma–Aldrich, Taufkirchen, Germany) and PC at different concentrations (1×10^6, 10^5, 10^4, 10^3, and 10^2 nmol L^{-1}) were prepared for a supernatant incubation assay. For each concentration of each sterol, a five-point 4 mL bioassay (Ma et al., 2009) was performed in triplicate, containing 0.04 mL standard sterol stock solution; and 500, 250, 100, 50, 25 μL Alex2 culture supernatant. The PC binding assay was performed in an identical fashion but with a different batch of supernatant. An aliquot of 0.1 mL *R. salina* culture (final bioassay concentration: 10×10^3 mL^{-1}), and 0.2 μm filtered seawater made up the remaining volume. Supernatant was applied alone as a positive control of lytic activity. After 3 h incubation, the number of intact *R. salina* cells in each vial was quantified. The results were expressed as percentage relative to controls (intact *R. salina* cells in K-medium).

3. Results

3.1. Putative main target site of lytic compounds

In the lytic assay with *R. salina*, independent application of Alex2 supernatant and the HILIC fraction yielded EC_{50} values of 0.68% (0.61–0.77%) and 0.83% (0.68–1.02%), respectively. No lysis of *R. salina* cells was observed for any undiluted Alex5 fractions.

The SPE fractions of Alex2 increased intracellular Ca^{2+} levels significantly only in the presence of extracellular Ca^{2+}, whereas no alteration of intracellular Ca^{2+} concentration was detected when extracellular Ca^{2+} was absent (Fig. 1, right panel). This indicates that rather than directly affecting the intracellular Ca^{2+} pool, lytic compounds target PC12 cell plasma membranes, and either act directly on Ca^{2+} channels or damage membrane integrity.

Application of either Alex5 supernatant or HILIC fraction at high concentration (5.75%) did not alter intracellular Ca^{2+} concentration of PC12 cells (Fig. 1A and B). In contrast to treatments with Alex5 fractions, the intracellular Ca^{2+} concentration was increased after addition of Alex2 fractions. Although at a low concentration (1%) only weak or no alterations were observed in any group (Fig. 1D and E, left box), at high concentration (5.75%), both Alex2 supernatant and HILIC fraction in K-medium caused a significant increase in intracellular Ca^{2+} concentration (Fig. 1D and E, right box) within a short time (ca. 10 min). However, an Alex2 HILIC fraction taken up in deionized water, and subsequently applied at the same concentration as in K-medium, showed no effect on intracellular Ca^{2+} concentration (Fig. 1C). When $CdCl_2$, a blocker of Ca^{2+} channels, was present, the increase of intracellular Ca^{2+} concentration caused by the HILIC fraction of Alex2 was not inhibited (Fig. 1F), as the increase of intracellular Ca^{2+} was significant.

3.2. Liposome leakage assay

Both Alex2 and Alex5 supernatant (90% volume), and to a lesser extent supernatant from the cryptophyte *Rhodomonas*, were capable of lysing PC liposomes. The fact that the time course of calcein release was almost identical between the supernatant of the lytic (Alex2) versus the non-lytic strain (Alex5) (Fig. 2A), with lytic activity as defined with reference to effects on *Rhodomonas* cells, suggested that this effect against PC liposomes represents non-specific perhaps detergent-like activity.

In contrast, although both Alex2 and Alex5 supernatant (90% volume) also lysed PC/cholesterol liposomes, Alex2 supernatant caused more calcein release than Alex5 supernatant (Fig. 2B), and the time course of fluorescence increase was different. With Alex2 supernatant, fluorescence yield almost reached 100% of the Triton X maximum after 30 min incubation. This indicated that the lytic

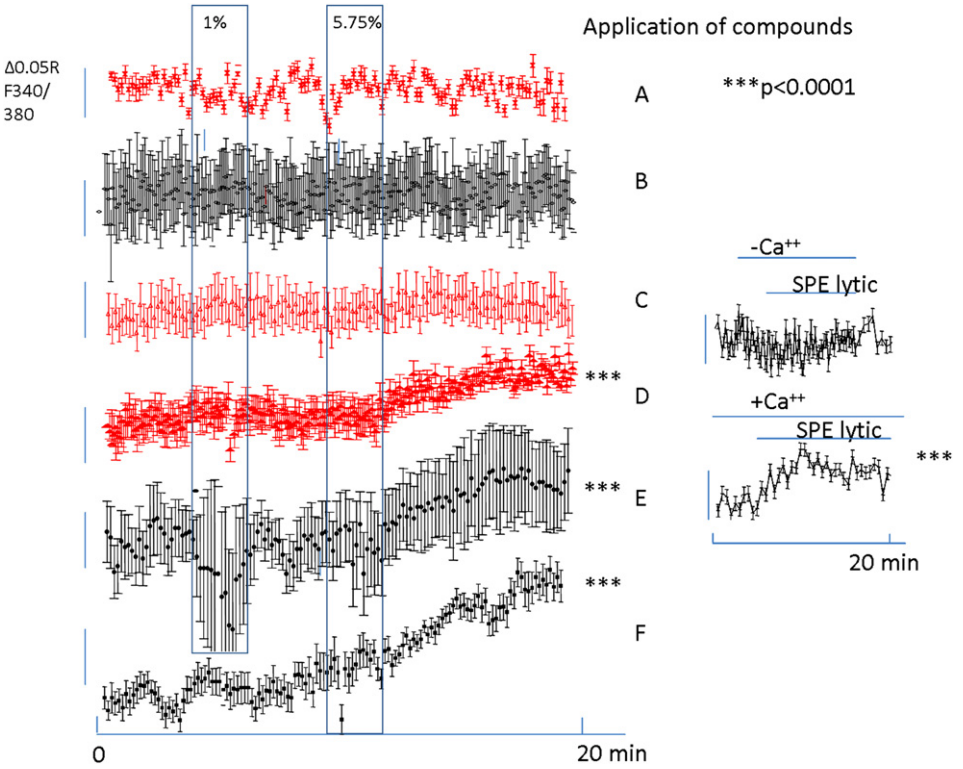

Fig. 1. Intracellular fluorescence of cells separated using the "region of interest" function of the software (Metafluor, Meta Imaging Series) obtained through a UV objective (Zeiss NeoFluar 20×). Data represent the fluorescence ratio of two images (F340/380), at 340 nm and 380 nm excitation. Increases in F340/380 are equivalent to rises in intracellular calcium levels. A ratio increase of 0.05 (scale bar) represents an elevation of free Ca^{2+} of roughly 50 nM. The long boxes indicate the time window during which samples at final concentrations of 1% and 5.75%, respectively, were added. A. Alex5 supernatant; B. Alex5 HILIC fraction; C. Alex2 HILIC fraction in water; D. Alex2 HILIC fraction in K-medium; E. Alex2 supernatant; F. 6% Alex2 HILIC fraction with 100 μM $CdCl_2$. 8–15 cells were measured simultaneously (error bars: SEM). $P < 0.0001$ = ***. The right part of the figure shows experiments demonstrating the compound-induced calcium influx only in the presence of extracellular calcium. Horizontal bars indicate time course of treatments.

compounds interacted with cholesterol within liposome membranes, thereby enhancing lysis and calcein release.

3.3. Control for plasma membrane isolation

The results of the ATPase activity with or without potassium or vanadate in fractions of the two phases are shown in Table 1. The presence of potassium did not significantly increase ATPase activity in either fraction. Vanadate significantly inhibited ATPase activity (Student's *t*-test, $p < 0.05$) in the PEG phase, whereas no significant inhibition of ATPase activity (Student's *t*-test, $p > 0.05$) was detected in the dextran phase. This indicated that plasma membrane-related vanadate-sensitive ATPase was preferentially partitioned into the PEG phase, and thus the isolated PEG fraction was enriched in plasma membrane.

3.4. Sterol composition

The sterol composition of whole cells (Table 2) of both *Alexandrium* strains Alex2 and Alex5 comprised mainly cholesterol (>50%) and the 4-methyl sterol dinosterol (>25%). The cryptophyte *R. salina* contained 100% brassicasterol, whereas the major sterols of the chlorophyte *D. salina* and the raphidophyte *H. akashiwo* were ergosterol (49%) and corbisterol (51%), and β-sitosterol (98%), respectively. The heterotrophic dinoflagellates *O. marina* and *G. dominans* fed on *D. salina* contained cholesterol and stigmastanol, respectively.

In most cases, plasma membrane fractions from both *A. tamarense* strains and five target protists enriched by PEG/dextran partitioning yielded different sterol compositions (Table 3) compared to those of whole cells. No compositional differences between whole cell extracts and plasma membrane fractions were observed only for *R. salina* (100% brassicasterol) and *H. akashiwo* (98% β-sitosterol). In contrast, the sterol ratios of the fractions of the other species differed, often dramatically. For example, plasma membrane fractions of Alex2 and Alex5 contained mainly cholesterol (>88%), but less than 10% dinosterol (Table 3), whereas the whole cell extract contained 26% and 42% dinosterol for Alex2 and Alex5, respectively (Table 2). Even more pronounced differences can be seen for the species *D. salina*, *O. marina*, and *G. dominans*. For example, plasma membrane fractions of these three species are enriched in desmosterol (Table 3), but this sterol component falls below the limit of quantitation in whole cell extracts (Table 2). In any case, none of the five target species, which are

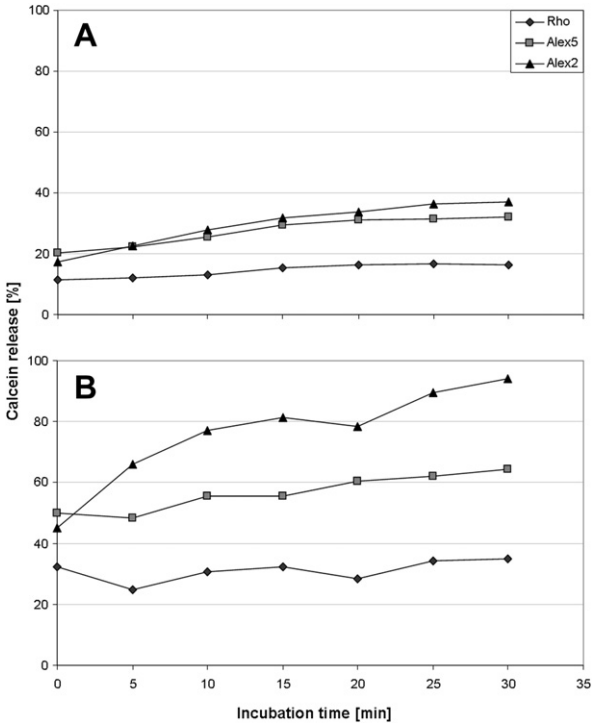

Fig. 2. Percentage of calcein released from liposomes induced by *R. salina* or *A. tamarense* supernatant (relative to maximum release by Triton X-100). A. PC liposome; B. PC/cholesterol (70/30) liposome. Alex2 = *A. tamarense* lytic strain; Alex5 = *A. tamarense* non-lytic strain; Rho = *R. salina*.

lysed by Alex2 exudates, contained any trace of the 4-methyl sterol dinosterol.

3.5. Lipid incubation bioassay

Assays with three representative sterols, cholesterol, ergosterol and brassicasterol, and the basic membrane component PC, showed an increase in the percentage of intact *R. salina* cells when these sterols were incubated at high concentration together with supernatant from Alex2 (Fig. 3). Total lytic activity of Alex2 supernatant, which caused 100% lysis of *R. salina* in the lipid-free control treatment, was completely inhibited by all four lipids at the highest concentration (1×10^4 nmol L^{-1}, Fig. 3A–D). Nevertheless, at the next lower sterol concentration 1×10^3 nmol L^{-1} only cholesterol yielded 100% intact *R. salina* cells (Fig. 3A), suggesting higher affinity of lytic compounds for cholesterol than for the other sterols. Comparison of ergosterol and brassicasterol at a concentration of 100 nmol L^{-1} simultaneously applied with Alex2 supernatant (250 μL) showed higher affinity of lytic compounds for ergosterol than brassicasterol. At the two lowest concentrations (1 and 10 nmol L^{-1}), all three sterols reduced lytic activity in 100 μL sample volume, but differences among the effect of these sterols were not distinguishable. Furthermore, the two lowest concentrations of the same sterol reduced the lytic activity by almost the same extent. In summary, the lytic compounds showed binding effects to free sterols. The lytic compounds exhibited different affinities to the three tested sterols, with the highest binding to cholesterol followed by ergosterol and brassicasterol, but lytic activity was also reduced dose dependently by the presence of PC.

Table 1
Plasma membrane enzyme marker activities (μmol Pi mg protein^{-1} min^{-1}) in two membrane fractions in the presence or absence of co-factor/inhibitor (results expressed as mean ± s.d., $n = 3$).

ATPase	PEG phase (plasma membrane)	Dextran phase
$-K^+$	0.19 ± 0.04	0.38 ± 0.02
$+K^+$	0.21 ± 0.05	0.40 ± 0.06
$+K^+$, +vanadate	0.05 ± 0.01	0.28 ± 0.06

Table 2
Sterol composition (%) of whole cells of *A. tamarense* and target protistan species.

Sterol	Alex2	Alex5	*R. salina*	*D. salina*	*H. akashiwo*	*O. marina*	*G. dominans*
Cholesterol	73.0	55.6	0.0	0.0	0.0	68.4	0.0
Ergosterol	0.0	0.0	0.0	48.8	0.0	0.0	0.0
Brassicasterol	0.0	0.0	100.0[a]	0.0	0.0	0.0	0.0
Dinosterol	26.0	41.7	0.0	0.0	0.0	0.0	0.0
Stigmasterol	0.0	0.0	0.0	0.0	0.2	7.3	0.0
Desmosterol	0.0	0.0	0.0	0.0	0.0	0.0	0.0
β-Sitosterol	1.0	2.6	0.0	0.0	97.9	0.0	0.0
Stigmastanol	0.0	0.1	0.0	0.0	0.0	0.0	100.0
Campesterol	0.0	0.0	0.0	0.0	1.9	24.3	0.0
Corbisterol	0.0	0.0	0.0	51.2	0.0	0.0	0.0

[a] Data based on preliminary result acquired from a cell pellet for which cell number was not recorded.

4. Discussion

The ecological role of allelopathy among microalgae is believed to relate to bloom formation and succession of certain species via competitive interactions (Keating, 1977; Legrand et al., 2003; Kubanek et al., 2005). Among microalgae, allelopathy is reported to mainly influence the survival of target species by inhibition of growth, photosynthetic pathways, immobilization, or even causing death of target microalgae (Legrand et al., 2003; Granéli and Hansen, 2006; Tillmann et al., 2008b). Exposure of sensitive target organisms, e.g. the cryptophyte *R. salina*, to allelochemicals from the dinoflagellate *A. tamarense*, yields cell lysis in a relatively short time of several minutes. The

Table 3
Sterol composition (%) of plasma membrane-enriched fractions from *A. tamarense* and target protistan species.

Sterol	Alex2	Alex5	*R. salina*	*D. salina*	*H. akashiwo*	*O. marina*	*G. dominans*
Cholesterol	93.0	88.1	0.0	8.0	0.0	9.9	11.5
Ergosterol	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Brassicasterol	0.0	0.0	100.0	0.0	0.0	0.0	0.0
Dinosterol	2.9	8.1	0.0	0.0	0.0	0.0	0.0
Stigmasterol	0.0	0.0	0.0	0.0	0.3	6.2	18.5
Desmosterol	4.1	3.8	0.0	44.7	0.0	33.8	38.8
β-Sitosterol	0.0	0.0	0.0	32.3	97.9	50.1	0.0
Stigmastanol	0.0	0.0	0.0	15.0	0.0	0.0	31.2
Campesterol	0.0	0.0	0.0	0.0	1.8	0.0	0.0
Corbisterol	0.0	0.0	0.0	0.0	0.0	0.0	0.0

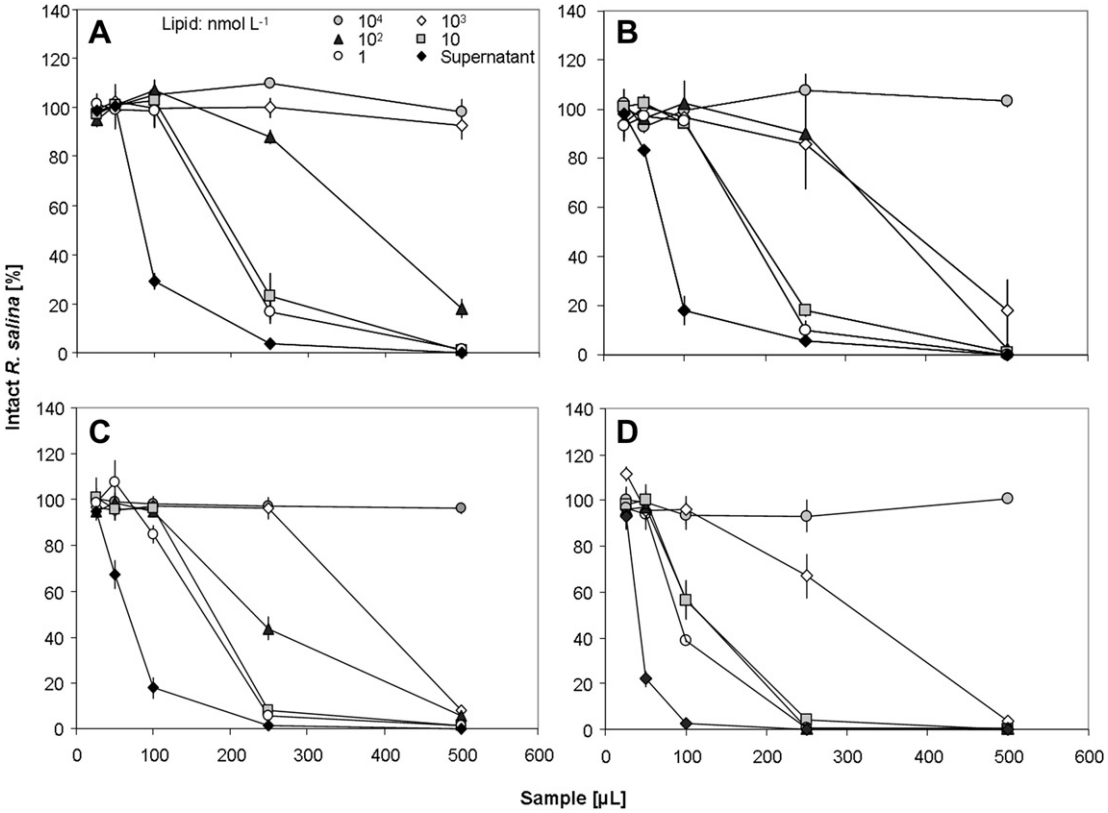

Fig. 3. Percentage (compared to control) of intact *R. salina* cells exposed to five volumes of Alex2 supernatant together with four different lipids at five concentrations. A. Cholesterol. B. Ergosterol. C. Brassicasterol. D. Phosphatidylcholine (PC). Results expressed as triplicate mean ± SD.

intuitive hypothesis that such rapid cell lysis of target microalgae is caused by direct membrane disruption, as opposed to the induction of other catabolic cascades within the cell, however, remained to be proven.

The choice of a mammalian cell model, PC 12 cells, rather than more ecologically relevant putative target species, to investigate the mode of action of the lytic compounds produced by *A. tamarense* was based upon pragmatic considerations. Membrane systems of mammalian cells have been much more intensively studied, and in any case all eukaryotic cell membranes share basic structural similarities.

Research on the mode of action of cytotoxicity, including hemolytic effects, of known phycotoxins on a variety of cell types has demonstrated two main mechanisms of cell lysis - some toxins induce apoptosis, whereas others directly disrupt plasma membranes. For example, microcystins produced by some strains of the cyanobacteria *Microcystis*, *Anabaena*, and *Oscillatoria* (Sivonen et al., 1990; Fujiki and Suganuma, 1993; Bell and Codd, 1996; Fawell et al., 1999; Chorus et al., 2000), and diarrhetic shellfish poisoning (DSP) toxins, such as okadaic acid (OA) and dinophysistoxin-1, produced by dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum* (Yasumoto et al., 1979, 1984) belong to the first type. Although these toxin groups are structurally and biosynthetically unrelated, they share the capacity to act as potent protein phosphatase inhibitors in mammalian cells, and also exert allelopathic effects on certain macroalgae (Pflugmacher, 2002) and microalgae (Windust et al., 1996, 1997). With respect to cytotoxicity to mammalian cell lines, these toxins were reported to induce apoptosis of target cells, e.g. microcystins bind and inhibit catalytic subunits of the well-conserved intracellular serine/threonine protein phosphatase (PP) 1 and 2A (Holmes and Boland, 1993; Goldberg et al., 1995), and consequently cause a series of chain reactions, and finally apoptosis of target cells. By a similar mechanism, OA and its analogs also induce apoptosis of target cells (Tergau et al., 1997; Rossini, 2000; Klumpp and Krieglstein, 2002). Here it is important to note that the allelochemical lytic activity of *Alexandrium* is unrelated to phosphatase inhibition activity, at least as mediated by these toxins (they are absent). Furthermore, the lytic activity it also apparently unrelated to the presence or content of tetrahydropurine neurotoxins (saxitoxin and analogs) with Na-channel blocking activity often found within this genus (Tillmann and John, 2002).

In virtually all eukaryotic cell types, Ca^{2+} is an important intracellular messenger, inducing death signals if delivered in unsuitable circumstances (Berridge et al., 2000; Hajnoczky et al., 2000). Both Ca^{2+} influx through plasma membrane channels and release from endoplasmic reticulum can stimulate cell apoptosis. In our experiments, when the PC 12 cells were exposed to lytic compounds

from *Alexandrium*, the intracellular Ca^{2+} concentration increased and this enhancement was proven to originate from the extracellular medium, as no alteration of intracellular Ca^{2+} was observed when extracellular Ca^{2+} was absent. We interpret this to indicate that the lytic compounds do not interact with intracellular Ca^{2+} stores, but rather act directly on the plasma membrane, either by interacting with Ca^{2+} channels or by forming pores or causing disruption of the plasma membrane. By comparison, the large polyether maitotoxin (MTX) produced by the dinoflagellate *Gambierdiscus toxicus* (Yokoyama et al., 1988) elicits Ca^{2+} influx in virtually all cells and tissues by activation of ion-channels (Gusovsky et al., 1989), and subsequently causing a intracellular cascade of events.

The use of the Ca^{2+} channel blocker $CdCl_2$ assisted in the discrimination of ion channel activation, as it has been described for MTX, from an alternative mode of action for the lytic compounds from *Alexandrium*. The $CdCl_2$ inhibition assay, in which Ca^{2+} channels in the plasma membrane were blocked, showed that intracellular Ca^{2+} concentrations continued to increase. This effect indicates that the lytic compounds directly disrupt the plasma membrane rather than inducing apoptosis by activation of ion-channels. We interpret the increase in intracellular Ca^{2+} to be the result of enhanced influx of Ca^{2+} through damaged sites in the membrane.

Significantly, there was no influx of Ca^{2+} into PC12 cells when the lytic Alex2 HILIC fraction had previously been taken up in deionized water. This is consistent with our previous observation that the lytic compounds had less lytic effect on *R. salina* cells when re-suspended in deionized water than in seawater (Ma et al., 2009). We speculated that the bioactivity against the cell membrane targets of the lytic compounds is dependent upon a critical secondary or tertiary conformational structure, which is maintained in saline environments, such as in natural seawater including with nutrient enrichments, e.g. K-culture medium, but is lost in fractions in organic solvents or deionized water.

Phosphatidylcholine is a basic component of bilayer biomembranes. The lytic compounds showed affinity to PC, indicating that the lysis of target membrane could be caused by interaction of lytic compounds with membrane PC. However, the result of the liposome assay, for which the presence of the lytic compounds in the supernatant did not enhance or alter the time course of PC liposome lysis, suggests that the mechanism of membrane lysis of sensitive protist cells by lytic compounds of *Alexandrium* is not a non-specific detergent-like activity. The different calcein release potencies and time course of fluorescence yield of PC/cholesterol liposomes exposed to Alex2 versus Alex5 supernatant appear to be related to the presence of cholesterol in liposome membrane. We hypothesize that specific activity of the lytic compounds is a function of cholesterol in the membrane, with cholesterol likely serving as a molecular target of the lytic compounds.

As putative allelochemical(s), it is therefore intriguing that the lytic compounds of *Alexandrium* are capable of disrupting the cell plasma membrane of target microalgal cells. Similar membrane-disruptive effects of dinoflagellate secondary metabolites have been reported for species of the genus *Amphidinium* (Houdai et al., 2004) and *Karlodinium* (Deeds and Place, 2006). The amphipathic hemolytic toxins, e.g. amphidinols with hairpin structures, are believed to interact with sterols located in the membrane, and to increase the membrane permeability by forming pores in the membrane (Houdai et al., 2005). Karlotoxins (KmTxs) are another group of toxic dinoflagellate metabolites, with structures similar to amphidinols (Van Wagoner et al., 2008). KmTx-2 exhibited different affinities to gymnodinosterol, a 4-methyl sterol from *Karlodinium veneficum*, cholesterol (found in erythrocytes as well as *O. marina*), and ergosterol, a fungal sterol (Deeds and Place, 2006). The latter authors concluded that *Karlodinium* protects itself from being lysed by the karlotoxins it produces by the presence of 4-methylated gymnodinosterol in its plasma membrane, which thus remained intact. In other words, karlotoxins are capable of lysing other cells, such as erythrocytes, as well as fungi and protists, by specifically binding to non-4-methyl sterols, such as cholesterol or ergosterol on target membranes. Such sterol-specific bioactivity is similar to the action of some antibiotics, e.g. the polyene amphotericin B, which specially bind to ergosterol but not to cholesterol on membranes, thus can be used on human cells whose membranes contain cholesterol.

Similarly, the lytic compounds of *Alexandrium* could conceivably behave in a sterol-binding selective manner. The fact that *A. tamarense* lytic compounds bind to non-4-methyl sterols and that this species contains the 4-methyl sterol dinosterol is consistent with the karlotoxin model system, whereby dinosterol could be a candidate for a self-protective agent against the lytic compounds produced by *A. tamarense*. Unfortunately we were not able to test the specific binding affinity of lytic compounds from *Alexandrium* to dinosterol due to unavailability of this purified compound. In any case, the sterol profile of *K. micrum* consists only of 4-methyl sterols (Leblond and Chapman, 2002), whereas *A. tamarense* contains both cholesterol and the 4-methylated dinosterol (26 and 41.7% of total sterol content for two different strains tested, respectively). The presence of the former sterol in *A. tamarense* does not support this sterol-specific hypothesis, because cholesterol showed a high binding affinity to *A. tamarense* lytic compounds and thus would counteract the protective role of dinosterol.

Sterols are present not only in the plasma membrane but also in other organelles and membranes inside the cell. Nevertheless, it is apparent that only sterols present in the plasma membrane will directly encounter lytic compounds, and therefore the sterol composition of the plasma membrane is critical for understanding the mode of action and cellular target of lytic compounds, and whether or not sterol-binding is involved. Unexpectedly, in the enriched plasma membrane fractions of both *Alexandrium* strains, the cholesterol component was an even higher percentage (>88%) of total sterols, than in the whole cell fraction. Among the target species, *R. salina* contained 100% brassicasterol, and other species contained various sterols in their plasma membranes, but none possessed dinosterol (Table 3). This practically rules out the self-protective function of dinosterol for *Alexandrium* against its lytic compounds because if sterols are the binding target of

these lytic compounds *Alexandrium* cells would also be lysed. *Alexandrium* cells contain a high percentage of cholesterol in the plasma membrane, and free cholesterol has the highest affinity for lytic compounds.

If sterols really are the molecular targets of lytic compounds, there are two possible explanations for the apparent contradictory results. One is that the sterol composition listed (Table 3) does not accurately represent that of the outer membrane surface of *A. tamarense*. The outer layer of thecate dinoflagellates is complex and difficult to define because dinoflagellates possess an amphiesma consisting of multiple layers, including the outermost membrane, amphiesmal vesicles, which for thecate dinoflagellates contain cellulose plates, and the innermost membrane layer in contact with the cytoplasm (Morrill, 1984). In *A. tamarense*, if the sterol composition of the outermost membrane layers is inconsistent with that of the basal membrane ("plasmalemma"), then the possible contribution of 4-methyl sterol to self-protective mechanisms cannot be excluded. To completely exclude sterols as the active target of lytic compounds, the actual location of sterols must be determined.

Other possibilities are that *A. tamarense* cells produce protective molecules which shield cholesterol from its lytic compounds or that prevent the lytic compounds from reaching critical intracellular targets. Similar protective modes were found in some prokaryotic organisms. For example, lipopolysaccharides that determine the serotype of *Pseudomonas aeruginosa* strains may prevent access of R-pyocins, the bacteriocins produced by *P. aeruginosa*, to their receptor sites on the producers (Köhler et al., 2010). Some nitrogen-fixing cyanobacteria, e.g. *Nostoc* spp., also produce antibiotic substances like bacteriocin against other cyanobacteria, and simultaneously produce a masking protein that inhibits bacteriocin, thereby rendering the producing strain immune to the effects of the bacteriocin (Flores and Wolk, 1986).

Components other than sterols in the plasma membrane may also be the targets of lytic compounds. For example, maitotoxin does not elicit Ca^{2+} influx into artificial phospholipid vesicles (Takahashi et al., 1982; Murata et al., 1992), but does induce Ca^{2+} influx into erythrocyte ghosts (Konoki et al., 1999), whose membranes are much more complex. The membrane permeabilizing activity of amphidinols (AM2 and AM3) was remarkably enhanced by the presence of sterols (cholesterol) (Morsy et al., 2008a), or transmembrane protein (glycophorin A) (Morsy et al., 2008b) in artificial liposomes composed of saturated lipids. The possibility that other yet unstudied membrane components serve as the target of lytic compounds cannot be excluded. Although cholesterol in PC/cholesterol liposomes appeared to act as a molecular target of lytic compounds from *Alexandrium*, other membrane components including other sterols were not tested in this assay and therefore cannot be ruled out with respect to their function in binding and membrane susceptibility. Most likely both the total amount and distribution of sterols and transmembrane components determine the particular fluidity and stability of membranes, and then various combinations determine the differential sensitivity of membranes to lytic compounds. The specific sterol composition of the outermost membrane layer and the presence of other protective agents in *A. tamarense* still need to be resolved.

5. Conclusion

Allelochemicals of *A. tamarense* cause lysis of protistan cells, apparently by direct damage to external membranes and loss of cell integrity. The molecular targets of the lytic compounds do not appear to be ion-channels, such as those for Ca^{2+}, but rather other membrane components. Nevertheless, on the basis of liposome membrane assays, the activity of the lytic compounds is not a non-specific detergent-like effect. Lytic compounds possess affinity for free sterols and PC, and the lysis of protistan targets is dose-dependently reduced by adding various sterols and PC. Lytic and non-lytic *A. tamarense* cell membranes contain mainly cholesterol, the sterol with highest affinity to lytic compounds. Different sterol composition of the outermost membrane and the "plasma membrane" fraction of *Alexandrium* enriched in this study may account for the seemingly contradictory results. Although the chemical structures of the lytic compounds from *Alexandrium* have not been completely defined, they are probably not structurally related to the membrane-disruptive polyene toxins. The superficial similarities in the lytic responses of target cells to polyene toxins and the lytic substances produced by *Alexandrium*, and the characteristics of the self-protective mechanism, are therefore unlikely to be totally mediated by the same interaction between specific sterols and/or transmembrane proteins of external membranes.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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