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The lipopeptide toxins anabaenolysin A and B target biological membranes in a cholesterol-dependent manner

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ABSTRACT

The two novel cyanobacterial cyclic lipopeptides, anabaenolysin (Abl) A and B permeabilised mammalian cells, leading to necrotic death. Abl A was a more potent haemolysin than other known biodetergents, including digitonin, and induced discocyte–echinocyte transformation in erythrocytes. The mitochondria of the dead cells appeared intact with regard to both ultrastructure and membrane potential. Also isolated rat liver mitochondria were resistant to Abl, judged by their ultrastructure and lack of cytochrome *c* release. The sparing of the mitochondria could be related to the low cholesterol content of their outer membrane. In fact, a supplement of cholesterol in liposomes sensitised them to Abl. In contrast, the prokaryote-directed cyclic lipopeptide surfactin lysed preferentially non-cholesterol-containing membranes. *In silico* comparison of the positions of relevant functional chemical structures revealed that Abl A matched poorly with surfactin in spite of the common cyclic lipopeptide structure. Abl A and the plant-derived glycolipid digitonin had, however, predicted overlaps of functional groups, particularly in the cholesterol-binding tail of digitonin. This may suggest independent evolution of Abl and digitonin to target eukaryotic cholesterol-containing membranes. Sub-lytic concentrations of Abl A or B allowed influx of propidium iodide into cells without interfering with their long-term cell viability. The transient permeability increase allowed the influx of enough of the cyanobacterial cyclic peptide toxin nodularin to induce apoptosis. The anabaenolysins might therefore not only act solely as lysins, but also as cofactors for the internalisation of other toxins. They represent a potent alternative to digitonin to selectively disrupt cholesterol-containing biological membranes.

1. Introduction

Cyanobacteria represent a rich source of bioactive compounds, including an abundance of substances containing cyclic peptides not produced by higher eukaryotes [1]. Based on screens of aquatic cyanobacteria for cell death inducing compounds [2,3], we isolated two related cyclic lipopeptides (anabaenolysin A and B, Abl A and B; Figs. 1A and S1A) from marine benthic cyanobacteria of the genera *Anabaena* [4]. Their amino acid composition consists of four amino acids from which two are proteinogenic and two unusual; glycine[1], glycine[2], 2-(3-amino-5-oxytetrahydrofuran-2-yl)-2-hydroxyacetic acid[3] and a long unsaturated C_{18} β-amino acid[4] with a conjugated triene structure. These two amphipathic toxins are relatively compact (MW = 558 and 560), have no charge at physiological pH, and induced both cell necrosis and haemolysis [4].

Lipopeptides are highly abundant in cyanobacteria, and show a large variation in both structure and bioactivity [5]. They can cause membrane damage, but some can also cross membranes without disrupting them. Comparatively little is known about the amphipathic cyanobacterial lipopeptides. Some are believed to perturb membranes of organisms that either compete for space, nutrients and light, or grazers feeding on the cyanobacteria [5]. Membrane-damaging biological compounds [6] can be categorised as enzymatic, pore-forming or detergent-like [7,8]. The detergent-like toxins, like the plant-derived sugar-containing saponins and the bacterially derived lipopeptide surfactin, apparently destabilise and disrupt the integrity of membranes [9–11]. The amphipathic biodetergents with the highest potency, like the glycopeptide digitonin, may bind preferentially to subdomains of the outer membrane leaflet [12,13].

The present study was initiated to reveal the cellular effects by the unusual cyanobacterial lipopeptides Abl A and B [4]. We used both cells, and membrane models like liposomes to find the mode of action,

Abbreviations: Abl, anabaenolysin; GFP, green fluorescent protein; PI, propidium iodide; SEM, scanning electron microscopy; SPE, solid phase extraction; TEM, transmission electron microscopy

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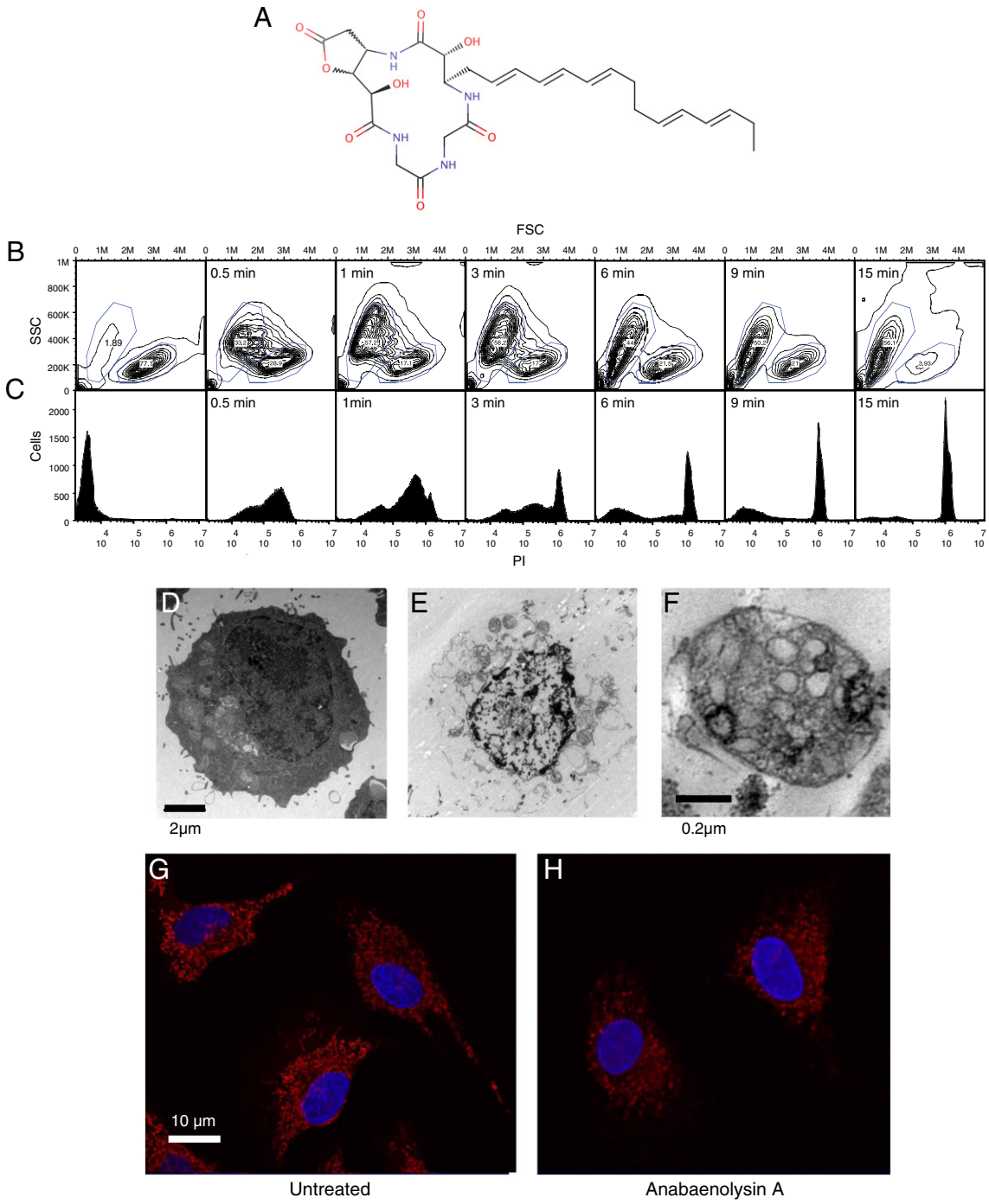

Fig. 1. Abl A causes necrotic cell death without mitochondrial damage. A: The structure of anabaenolysin A (Abl A) depicted with L-amino acids. B and C: Human NB4 leukaemia cells were treated with 15 μM Abl A from 0.5 to 15 min and analysed for changes in (B) apparent size (forward scatter: FSC) and granularity (side scatter: SSC), and (C) influx of propidium iodide (PI) by flow cytometry. D and E: IPC-81 rat leukaemia cells were incubated for 30 min without (D) or with (E) 18 μM Abl A, fixed and studied by transmission electron microscopy. F: A typical mitochondrion from the cell shown in panel E. G and H: NRK cells were exposed to vehicle (G) or 9 μM Abl A (H) in the presence of mitotracker (red fluorescence), which labels viable mitochondria with intact membrane potential. After 15 min the cells were fixed with 2% buffered formaldehyde, containing the DNA stain Hoechst 33342 (blue), and studied by fluorescence microscopy.

and hypotheses were supported by *in silico* modelling. We show that Abl A and B act like amphipathic biodetergents, Abl A being a more potent haemolysin than digitonin. Abl A and B permeabilise liposomes in a cholesterol-dependent manner. They induce necrotic death of mammalian cells while sparing their mitochondria, whose membrane has low cholesterol content. The anabaenolysins appear therefore to be targeted against eukaryotic membranes, in distinction to the bacterial lipopeptide surfactin, which shows preference for the non-cholesterol containing membranes found in prokaryotes.

2. Materials and methods

2.1. Materials

Pure anabaenolysin A and B were isolated as recently described [4]. Hoechst 33342, surfactin, digitonin, cholesterol, acetonitrile (Rathburn), propidium iodide (PI) and calcein were from Sigma-Aldrich, St. Louis, USA. Dulbecco's modified Eagle medium (DMEM), foetal calf serum and horse serum were supplied by EuroClone® Life Sciences Division,

Milano, Italy. RPMI 1640 Medium (Gibco) was from Invitrogen AS, Carlsbad, USA. Complete protease inhibitor cocktail tablets were from Roche Diagnostics, Mannheim, Germany. Osmium tetroxide and agar-100 resin were supplied by EMS, Hatfield, USA. Cytochrome *c* antibody (H-104) was from Santa Cruz, San Diego, USA. CDP®Star was from Tropix, Bedford, USA. Phosphatidylcholine isolate (95% PC, Emulmetik 930) was from Lucas Meyer cosmetics, Champlan, France, and hydrogenated egg phosphatidylcholine was from Lipoid AG (Steinhausen, Switzerland). All other chemicals were from VWR (West Chester, USA) and of analytical grade.

2.2. Maintenance of cell lines and experimental conditions

IPC-81 rat promyelocytic leukaemia cells [14] were cultured in Dulbecco's modified Eagle's medium with 10% (v/v) heat inactivated horse serum. Human NB4 promyelocytic leukaemia cells [15] were cultured in RPMI 1640 medium with 10% (v/v) heat inactivated foetal calf serum. Normal rat kidney fibroblasts (NRK, ATCC: CRL-6509) were cultured in Dulbecco's modified Eagle's medium with 10% (v/v) heat inactivated foetal calf serum. All cells were incubated at 37 °C in a humid atmosphere of 5% CO_2.

The plasma membrane permeability of non-fixed cells was assessed by microscopic or flow cytometric detection of propidium iodide (PI) influx. NB4 cell density was adjusted to 0.5×10^6 cells/ml before adding anabaenolysins or solvent in the presence of 0.1% PI. At least 50,000 non-gated cell events were collected for each sample on an AccuriC6 flow cytometer (BD, Accuri cytometry, MI, USA). Mitochondria of Abl A treated NRK cells were stained with MitoTracker® (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. The cells were then fixed in buffered formaldehyde and studied by fluorescence microscopy.

2.3. Scanning- and transmission electron microscopy

Transmission electron microscopy of Abl-treated NB4 cells or rat liver mitochondria was done as previously described [16], and studied with a Jeol JEM-1230 transmission electron microscope. For scanning electron microscopy NRK cells were cultured and treated with detergents on collagen coated coverslips before fixation. Fixed erythrocytes and mitochondria were spun onto collagen-coated coverslips. The specimens were washed with cacodylate buffer, post-fixed for 60 min with 1% osmium tetroxide and thereafter step-wise dehydrated in ethanol. Specimens were examined using a Jeol JSM-7400F scanning electron microscope.

2.4. Isolation of mitochondria from primary rat hepatocytes and detection of cytochrome c

Primary rat hepatocytes were isolated from male Wistar rats (80–150 g) using *in vitro* collagenase perfusion [17], as previously described [18]. The rat experiments were approved by the Norwegian Animal Research Authority and conducted according to the European Convention for the Protection of Vertebrates Used for Scientific Purposes. Primary rat hepatocytes (6×10^6) were homogenised in ice-cold homogenisation medium (0.25 M sucrose, 2 mM Hepes, 0.2 mM EGTA, 0.1% BSA) using a ball-bearing cell cracker (EMBL, Heidelberg, Germany). The homogenate was first centrifuged 1200×*g* for 10 min at 4 °C. Postnuclear supernatants were centrifuged at 12,000×*g* for 10 min at 4 °C and the pellet fraction containing the mitochondria were collected and dissolved in the mitochondrial respiration medium MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, 1 g/l bovine serum albumin). After incubation of isolated mitochondria with anabenolysins the mitochondria were centrifuged 12,000×*g* for 10 min at 4 °C and the supernatants and the mitochondria were collected. Proteins in supernatant or mitochondria were lysed in 10 mM K_2HPO_4, 10 mM KH_2PO_4, 1 mM EDTA (pH 6.8) containing 10 mM CHAPS, 50 mM NaF, 0.3 μM $NaVO_3$ supplemented with Complete protease inhibitor and separated using Tricine SDS-page. Proteins were blotted onto a Hybond polyvinyldifluoride membrane (GE Healthcare, Little Chalfont, UK) and cytochrome *c* was visualised by alkaline-phosphatase conjugated antibody and CDP®Star as enzyme substrate. Detection was performed in a LAS-3000 Fuji Imager.

2.5. Preparation and determination of permeability of liposomes

Liposomes were prepared from soybean phosphatidylcholine (PC) isolate or pure hydrogenated egg phosphatidyl choline (HEPC). The PC or HEPC was dissolved in chloroform and evaporated in a rotary evaporator. Liposomes with cholesterol were prepared by adding cholesterol to the PC solution at molar ratio of 1.8:1 (5 mg PC and 1.2 mg cholesterol). The dry biofilm was hydrated with 60 mM calcein solution. The calcein solution was prepared by adding buffer (10 mM Tris–HCl with 1 mM EDTA) to a 300 mM calcein solution in 1 N NaOH until pH 8 was reached. After rehydration, the liposomes were extruded (LIPEX™ extruder; Northern lipids, Burnaby, Canada) ten times through a 0.2 μm filter (Whatman, Kent, UK) at 20 °C. The liposomes were transferred to a 10 mM Tris–HCl (pH 8) buffer with 100 mM NaCl and 1 mM EDTA by gel filtration on a Sephadex G-50 (medium grade; Sigma-Aldrich, St. Louis, USA) column. Membrane intactness was measured based on calcein release from the liposomes. Detergents were added to diluted liposome suspension, and the fluorescence (λ_{EX} 500, λ_{EM} 517) recorded with a Cary Eclipse fluorescence spectrophotometer (Varian, Santa Clara, CA, USA). Triton X-100 (final concentration 0.1%) was added to determine maximum calcein release.

2.6. Erythrocytes: isolation, experimental conditions, and measurement of haemolysis and release of adenosine nucleosides

Human full blood in acid citrate dextrose (11 mM citric acid, 13 mM sodium citrate, 17 mM glucose) was centrifuged 1750×*g* for 7 min and the erythrocytes collected and suspended in buffered (pH 7.4) NaCl solution (9.0 g/l). In each experiment, 2×10^7 erythrocytes were incubated for 1 h at 20 °C with the various agents to be tested.

For determination of haemolysis the erythrocytes were centrifuged at 300×*g* for 15 min at 20 °C and the absorbance of the supernatant read at 540 nm. For determination of maximum haemolysis the erythrocytes were incubated in buffered (pH 7.4) hypotonic NaCl solution (1.0 g/l).

To measure the release of adenosine nucleotides from erythrocytes, the supernatant from four experiments was combined and analysed on a Hitachi LaChrom HPLC system (VWR, West Chester, USA), equipped with a L-7455 diode array detector. Analytes in the supernatants were separated on a reversed phase chromatography column, Kromasil®KR100-5-C18 (250×4.6 mm (i.d.) from EKA chemicals, Bohus, Sweden) with a flow rate of 0.8 ml/min. A 5 min isocratic run with 0.1 M phosphate buffer, pH 7, was followed by a 10 min gradient to 70% acetonitrile. The area of the nucleotide peaks at 256 nm was used to determine their concentration.

2.7. Molecular modelling

Pharmacophore models were prepared with the Phase module of the Schrodinger® software (www.schrodinger.com). Low energy conformations of Abl A, cholesterol, digitonin and surfactin (PubMed compound IDs: 5997, 409778 and 65307 respectively) tautomers at neutral pH were generated (force field: OPLS_2005, solvation treatment: distance dependent dielectric, max. rel. energy diff: 10 kcal/mol, RMSD of 1.0 Å), and pharmacophore sites were defined using the default settings (definitions: min. intersite distance: 2.0 Å, max. tree depth: 5, initial box size: 32 Å, final box size: 1 Å, scoring: RMSD below 1.2 Å, vector scores above 0.5 Å, survival score formula: vector, site, volume, and

no. of matches all set to 1.0). The structures were then aligned based on the pharmacophores with the highest survival score.

3. Results and discussion

3.1. Features of the anabaenolysin-induced lysis of nucleated mammalian cells

The newly discovered [4] cyclic lipopeptide anabaenolysin A (Abl A, Fig. 1A) was studied for effects on NB4 leukaemia cells and hepatocytes. We noted that higher concentration of Abl A was required to produce cell lysis when the cell concentration was high (above 300,000 hepatocytes/ml or above 700,000 NB4 cells/ml) (not shown). All cell experiments reported here were therefore conducted at cell concentrations where the apparent EC_{50} for Abl A-induced lysis was independent of cell concentration.

NB4 cells incubated for 0.5 to 15 min with 15 μM Abl A were fixed and analysed by flow cytometry. Abl A decreased the apparent cell size (FSC) and increased the granularity (SSC; Fig. 1B) already after 0.5 min incubation. We also noted a drop in granularity at the later time-points (Fig. 1B). Abl A caused also influx of propidium iodide (PI, Fig. 1C), which is a sign of deficient surface membrane integrity.

Transmission electron microscopy revealed that the Abl A exposed cells had lost most of their electron dense intracellular material (Fig. 1D and E). This can explain the dramatic drop in size seen by flow cytometry (Fig. 1B), which could be caused by detection of nuclei only. A notable exception of the lysis of the cellular components was the mitochondria, which appeared morphologically intact (Fig. 1F). The functional intactness of the mitochondria was probed by incubating NRK cells with Mitotracker® after exposure to Abl A. The mitochondria remained well stained with Mitotracker® (Fig. 1G and H) even in cells incubated with enough Abl A to become permeabilised to PI, but not causing massive lysis. In line with this, we found that Abl A was equally potent against IPC-81 cells with enforced expression of the anti-apoptotic protein Bcl-2 [19], which prevents mitochondrial permeability transition pore opening (Supplementary Fig. S2), suggesting that the cell death was not coupled to typical apoptotic pathways involving the mitochondria.

We found that cells incubated continuously with sub-lytic (not causing membrane lysis observable by light or transmission electron microscopy) Abl A concentrations could show increased PI influx without microscopical or flow cytometric (FSC/SSC) indices of cell death (not shown). They had also an intact ability to undertake clathrin-dependent endocytosis of transferrin (Supplementary Fig. S3A). Furthermore, while leukaemia cells exposed for only 1 min to 6–8 μM Abl A had an increased PI content, this was not true if Abl A was removed from the cells before challenging the cells with PI. Such washed cells also continued to proliferate normally (not shown) suggesting that the early membrane permeation caused by Abl A can be reversible. We considered therefore that Abl might promote the internalisation of substances without killing the cells.

We tested first if sub-lytic concentrations of Abl A could allow the cyanobacterial cyclic peptide toxin nodularin to be internalised and induce apoptosis in cell types like NRK, which normally are impermeable to this type of toxins and therefore depend on nodularin microinjection to achieve apoptosis [20]. NRK cells treated with a combination of Abl A and nodularin underwent apoptosis similar to that seen after nodularin microinjection [20,21], whereas cells treated with nodularin alone retained their normal morphology (Supplementary Fig. S4A). We conclude that anabaenolysin can synergise with other cyanobacterial toxins like nodularin to induce eukaryotic cell death.

We tested next if Abl A could enhance the cellular uptake of macromolecules like the plasmid encoding green fluorescent protein (eGFP). For this purpose we used HEK293 cells, which hardly show any “spontaneous” uptake of plasmid, as judged by the lack of GFP positive cells (not shown). We did note, however, that a significant proportion of the cells expressed GFP if the plasmid was given together with a sub-lytic concentration of Abl A (Supplementary Fig. S4B). Although the transfection was more efficient with calcium phosphate (Supplementary Fig. S4C), the data show that sub-lytic concentrations of Abl A allowed not only the internalisation of plasmid into cells, but also avoided serious cell damage, since both the transcription and translation machinery had to be intact to produce enough eGFP to be detected microscopically.

To know more about the nature of the membrane damage inflicted by Abl A, we exposed NRK cells to high concentrations of Abl A as well as to the established biodetergent toxins surfactin and digitonin. Since Abl A appeared to cause most of it effects on membranes (Fig. 1), we chose to study the cell surface by scanning electron microscopy (SEM). This revealed that high concentrations of Alb A could solubilise completely the lipid component of the plasma membrane (Fig. 2 C and D), exposing the perimembraneous sub-cortical cytoskeletal web (Fig. 2 C and D). A similar morphology was observed for cells treated with Abl B (Supplementary Fig. S1D and E), digitonin (Fig. 2E and F), and surfactin (Fig. 2G and H), which both are known to have a detergent-like mechanism of action [10,13].

3.2. The haemolytic effect of anabaenolysins. Comparison with surfactin and digitonin

The single bilayer membrane of the red blood cell serves as model for eukaryotic cell membranes [22]. The haemolytic potency of Abl A was about 5 times higher than for digitonin and about 50-fold higher than for surfactin (Fig. 3G). It is also more than one order of magnitude higher than what has been reported for dirhamnolipids [23], and trehalose lipids [24].

We tested also if inhibitors directed against specific processes incriminated in erythrocyte lysis could interfere with Abl-induced haemolysis. We found that none of these inhibitors could antagonize the Abl A or Abl B-induced haemolysis (Supplementary Fig. S5). We considered therefore if the amphipathic anabaenolysins could act as membrane perturbing detergents and insert into the membranes. Many detergents induce either discocyte–echinocyte erythrocyte transformation or discocyte–stomatocyte transformation with formation of endocytic vesicles, depending on whether the detergent targets the positively curved (outer leaflet) or negatively curved (inner leaflet) of the erythrocyte membrane [25,26]. We found that the haemolysis caused by Abl A and B was accompanied by discocyte–echinocyte transformation (Fig. 3). The haemolysis induced by Abl A or B was coupled with release of adenine nucleotides (Fig. 3G). A similar coupling was noted for the detergents digitonin and surfactin (Fig. 3G). The discocyte–echinocyte transformation, together with a simultaneous release of haemoglobin and adenine nucleotides is typical for amphipathic detergents inserting into the outer membrane leaflet of erythrocytes [25–27]. In contrast, dirhamnolipid and trehalose lipid biosurfactants causes a mixed spherocyte/echinocyte morphology, suggested to be due to insertion into the outer membrane leaflet (echinocytosis), but also some osmotic lysis (spherocytosis) [23,24].

3.3. Anabaenolysins do not permeabilise isolated mitochondria and show cholesterol-dependent permeabilisation of liposomes

Although the anabaenolysins efficiently lyse the membranes of both nucleated (Figs. 1 and 2) and anucleated cells (Fig. 3), the mitochondria appeared functional (Fig. 1G and H). Further evidence for the mitochondrial resistance to Abl A was obtained by incubating isolated rat liver mitochondria with Abl A at concentrations above those required to induce complete cell lysis. The toxin had no visible effect on the shape, surface membrane or the internal membrane organisation of the mitochondria, (Fig. 4A–D). Neither did we observe any leakage of cytochrome *c* (Fig. 4E), which is an early marker of mitochondrial pore formation associated with apoptosis [28].

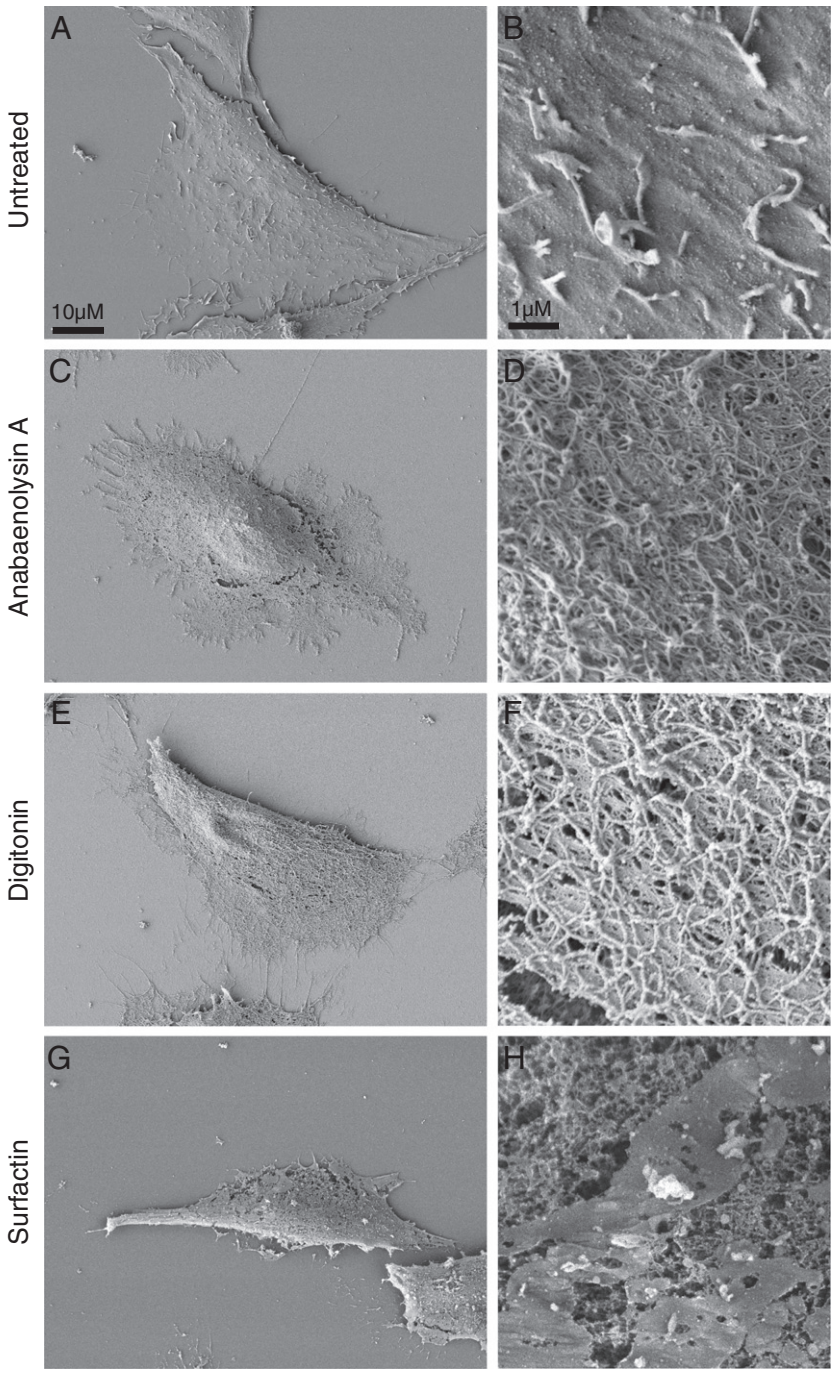

Fig. 2. The surface morphology of NRK cells exposed to Abl A, surfactin or digitonin. NRK cells were treated with vehicle (A and B), 15 μM Abl A (C and D), 20 μM digitonin (E and F) or 25 μM surfactin (G and H) for 5 min, fixed and processed for scanning electron microscopy. Panels A, C, E and G show low magnification overviews, and panels B, D, F and H show details of the surface structure. Note that all toxins had extracted the membrane surface lipids, allowing a direct view of the perimembraneous cortical web.

The outer mitochondrial membrane contains only about 5% cholesterol, whereas the cellular surface membranes contain about 30% [29]. We noted also that erythrocytes when partially depleted of cholesterol by cyclodextrin pre-incubation [30] became more resistant to Abl A and digitonin, but less resistant to surfactin (data not shown). These observations suggest that the anabaenolysins could depend at least in part on the cholesterol component of the membrane for their perturbing action. To test this more rigorously we compared the ability of Abl A and B to induce release of fluorescent calcein from liposomes composed of phosphatidylcholine with or without cholesterol. Both Alb A (Fig. 5 A and B) and Alb B (Supplementary Fig. S1) were more potent disruptors of liposomes containing cholesterol. The 5% cholesterol present in the mitochondrial membrane appears to be insufficient for Abl A to cause membrane permeabilisation (Fig. 4E). Interestingly, Abl-A had equal activity against liposomes made from soy phosphatidylcholine (Fig. 5A and B) and liposomes made from hydrogenated phosphatidylcholine (data not shown), both with and without cholesterol. This suggests that Abl A has a high ability to insert between the acyl-chains and cholesterol in the hydrophobic core of the membranes.

As expected [9] the plant-derived biodetergent digitonin also had a preference for cholesterol-containing membranes, even more

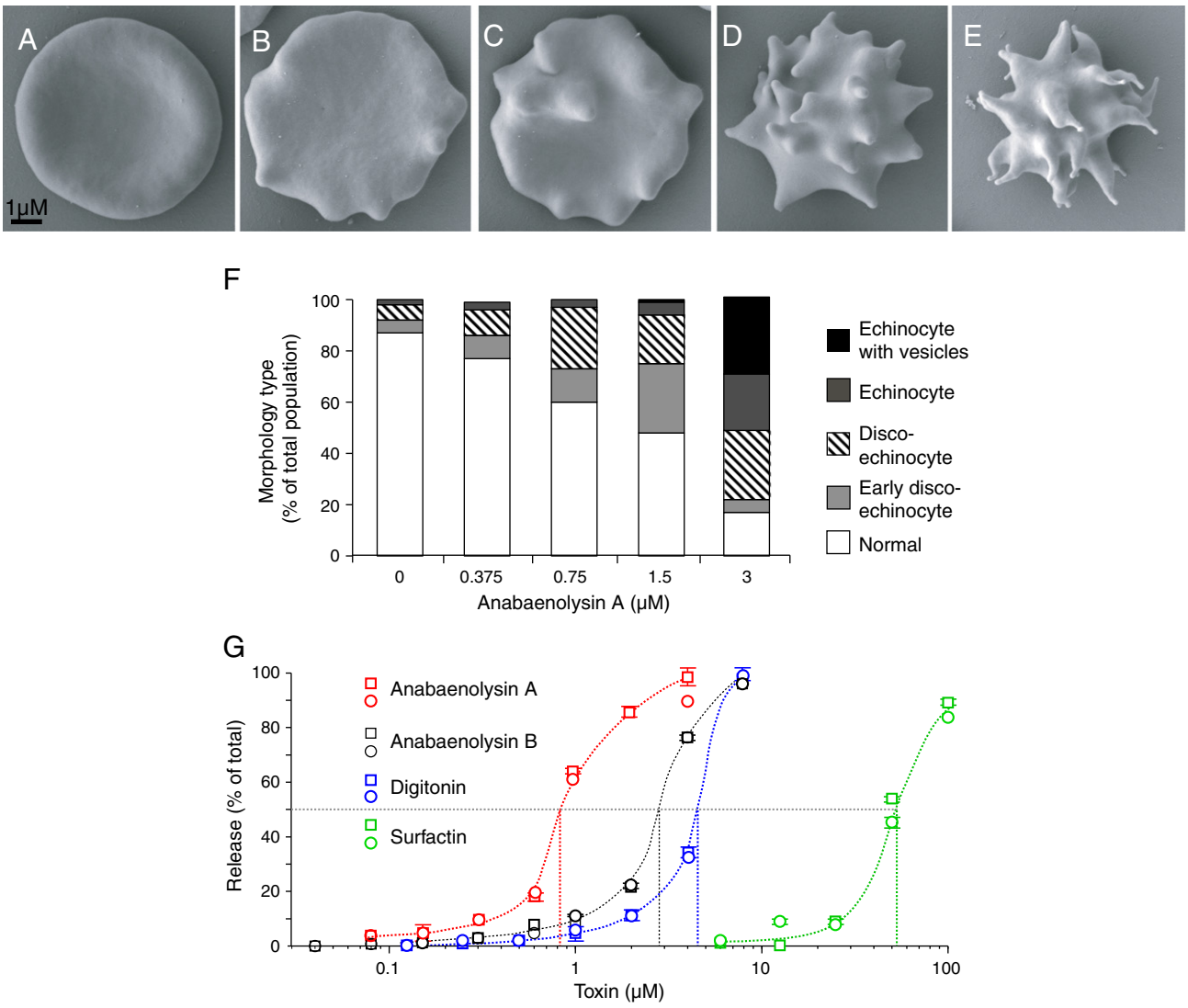

Fig. 3. Abl A causes a concerted release of erythrocyte haemoglobin and adenine nucleotides, associated with discocyte–echinocyte transformation. A–F: Erythrocytes were incubated for 1 h with 0 (vehicle only), 0.38, 0.75, 1.5 or 3 μM Abl A, fixed and the surface morphology analysed by scanning electron microscopy. Typical appearance is shown for a normal red blood cell (A), early discoechinocyte (B), discoechinocyte (C), echinocyte (D), and echinocyte with vesicles (E). Panel F shows the relative abundance (% of total population) of each morphology shown in panels A–E at the various Abl A concentrations. G: The release of haemoglobin (□) and adenine nucleotides (○) to the medium (phosphate buffered physiological saline) is shown for human erythrocytes incubated for 1 h with various concentrations of Abl A (red), Abl B (black), digitonin (blue) or surfactin (green). The data in G are average ± SEM of three separate experiments.

pronounced than Abl A (Fig. 5C and D). Importantly, surfactin, which like Abl is a cyclic lipopeptide, lysed preferentially cholesterol-free liposomes (Fig. 5E and F). The behaviour of Abl A, Abl B and digitonin is in contrast to biodetergents like surfactin [31], dirhamnolipids [23], and trehalose lipids [32], which preferentially lyse membranes that do not contain cholesterol. The preference for membranes not containing cholesterol may explain why surfactin has been promoted as an anti-bacterial/anti-fouling agent [31], since prokaryotic membranes lack cholesterol [29].

We next compared *in silico* the pharmacophore sites of Abl A with those of digitonin and surfactin. We noted high-survival pharmacophore constellations matching both Abl A and digitonin (Fig. 6A and B). This suggests that Abl A and digitonin may bind similarly to molecular targets, like membrane components. When the Phase module of Schrödinger™ was set to align Abl A, digitonin and cholesterol we noted again a close overlap of high-survival pharmacophore constellations (Fig. 6C). A close association was noted for both the hydrophobic and electrophilic centres between cholesterol and the known cholesterol-binding digitonin [9]. The similar association for Abl A is compatible with, although not proof of, similar cholesterol binding ability also for Abl.

When set to align Abl A and surfactin based on common pharmacophores, the Phase module of Schrödinger™ was unable to obtain any extensive high survival alignment. One obvious difference is the presence of two charged carboxyl moieties in surfactin against no charged residues in Abl. This difference may have functional significance, since we noted a significant drop in activity of Abl A towards both cells and cholesterol-containing liposomes when the lactone-moiety of Abl A or B was hydrolysed, creating a carboxy-group (not shown). Possibly, the charged moieties resist the partial insertion of the polar head molecules into the membrane [33]. We noted also poor overlap between the hydrophobic tails of surfactin and Abl A (Supplementary Fig. S6). This may be related to the shorter length of the hydrocarbon chain of surfactin (11 carbon atoms as compared to 15 carbon atoms in Abl A and B; [4]).

We conclude that the potency of Abl A to lyse cholesterol-containing membranes rivals that of digitonin in potency and selectivity between mammalian cell surface and mitochondrial membranes. In spite of their dissimilar origin and chemical composition Abl A and digitonin share a number of crucial pharmacophore sites, suggesting at least partially common molecular targets.

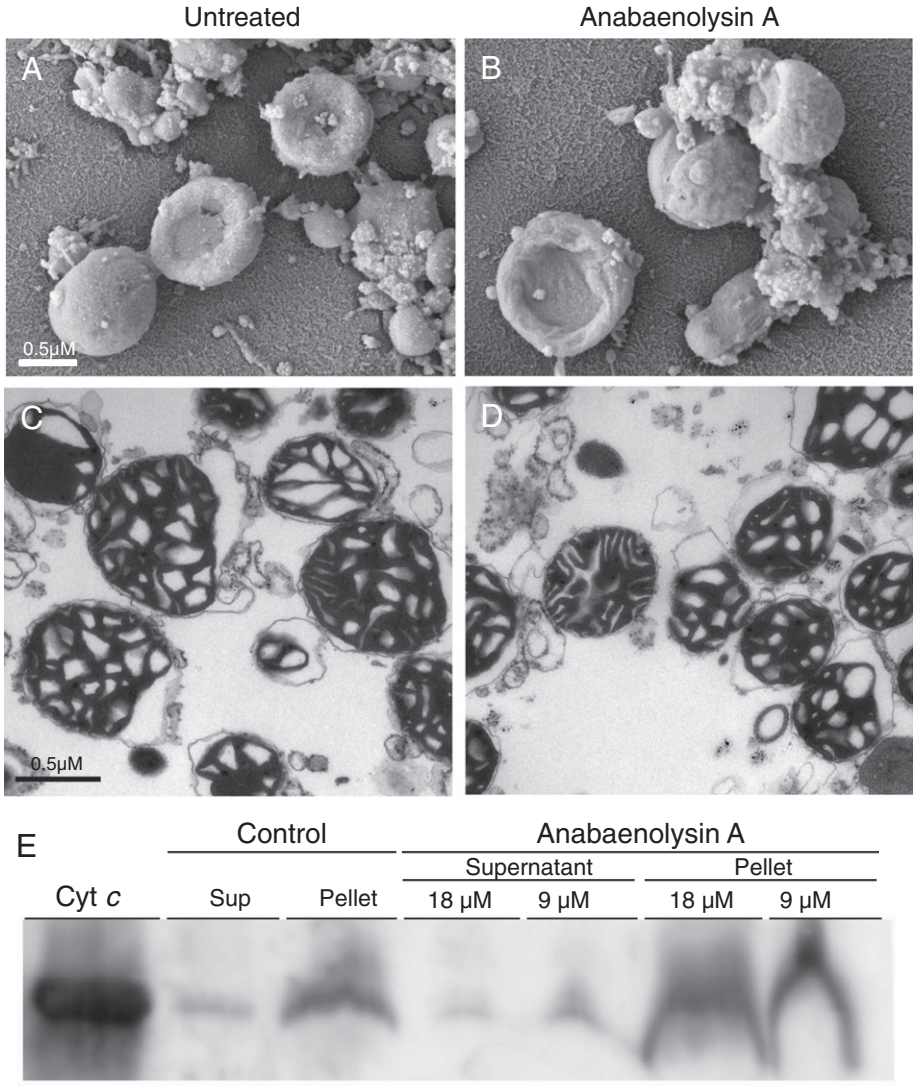

Fig. 4. Abl A does not permeabilise the membrane of isolated mitochondria. A–D: Mitochondria were isolated from primary hepatocytes, incubated with vehicle (A and C) or 18 μM Abl A (B and D) for 1 h, fixed and processed for scanning (A and B) or transmission (C and D) electron microscopy. E: The isolated mitochondria were incubated with vehicle, 18 or 9 μM Abl A for 1 h, and the supernatant and mitochondrial pellet separated. The mitochondrial pellet and supernatant were next subjected to SDS electrophoresis and immunoblotting with anti-cytochrome *c* antibody. Pure cytochrome *c* was included as standard. Note that Abl A failed to cause either morphological changes of, or release of cytochrome *c* from the mitochondria.

4. Conclusions

The novel cyclic lipopeptides Abl A and B (Figs. 1A and S1A; [4]) caused haemolysis at a lower concentration than previously described biodetergents (Fig. 3), like the plant saponin digitonin and the *B. subtilis*-derived cyclic lipopeptide surfactin [34]. Both digitonin and surfactin have unusual low lytic concentration compared to many synthetic detergents [13,35], which act as general membrane perturbants [36]. The higher lytic potency of biodetergents has been ascribed to heterogeneous membrane perturbation, with the creation of local membrane defects that leave most of the membrane unaffected [36]. The high potency of Abl A and B suggests strongly that they also target membrane subdomains. Abl A and B resemble several other biodetergents by inducing discocyte–echinocyte erythrocyte transformation (Fig. 3), and by solubilising the lipid component of the surface membrane (Fig. 2). The anabaenolysins appear therefore to be new and highly potent members of the biodetergent family.

Abl A and B and surfactin share a polar cyclic peptide head and a hydrophobic hydrocarbon chain, but still differed strikingly with regard to membrane selectivity. While Abl A and B preferentially destabilise cholesterol-containing membranes, surfactin preferentially lyses cholesterol-free liposomes (Fig. 5). Both Abl A and B lack charged residues in their polar head (Figs. 1 and 6, and Supplementary Figs. S1 and S6), while surfactin has two carboxyl-moieties and a shorter hydrophobic tail than Abl A and B. These features will combine to counteract any deep insertion of surfactin into the hydrophobic interior of thick biomembranes. In line with this, we noted that biodetergents with a sugar-based polar head differ in activity based on charge. Digitonin is uncharged at physiological pH, whereas rhamnolipids and trehalose lipids both are negatively charged at physiological pH [23,32]. It thus appears that a neutral polar head causes cholesterol dependency, whereas a negative charge inverts the cholesterol dependency (Fig. 5E and F) [23,32]. In line with this, we noted a marked drop in Abl A activity if the lactone-bond was hydrolysed to form a carboxy moiety (not shown). Another distinguishing feature of the hydrophobic tails is the lack of double bonds in surfactin.

The double bonds of Abl A serve to allow its hydrophobic tail to align well with either digitonin or cholesterol (Fig. 6). Digitonin binds cholesterol and related steroids [9,37]. The presence of a hydrogen-bond acceptor (cholesterol, Fig. 6C, red sphere) and donor (Abl A and digitonin, Fig. 6A, blue sphere) together with the hydrophobic centres (green spheres) is in line with the observed binding of cholesterol to digitonin

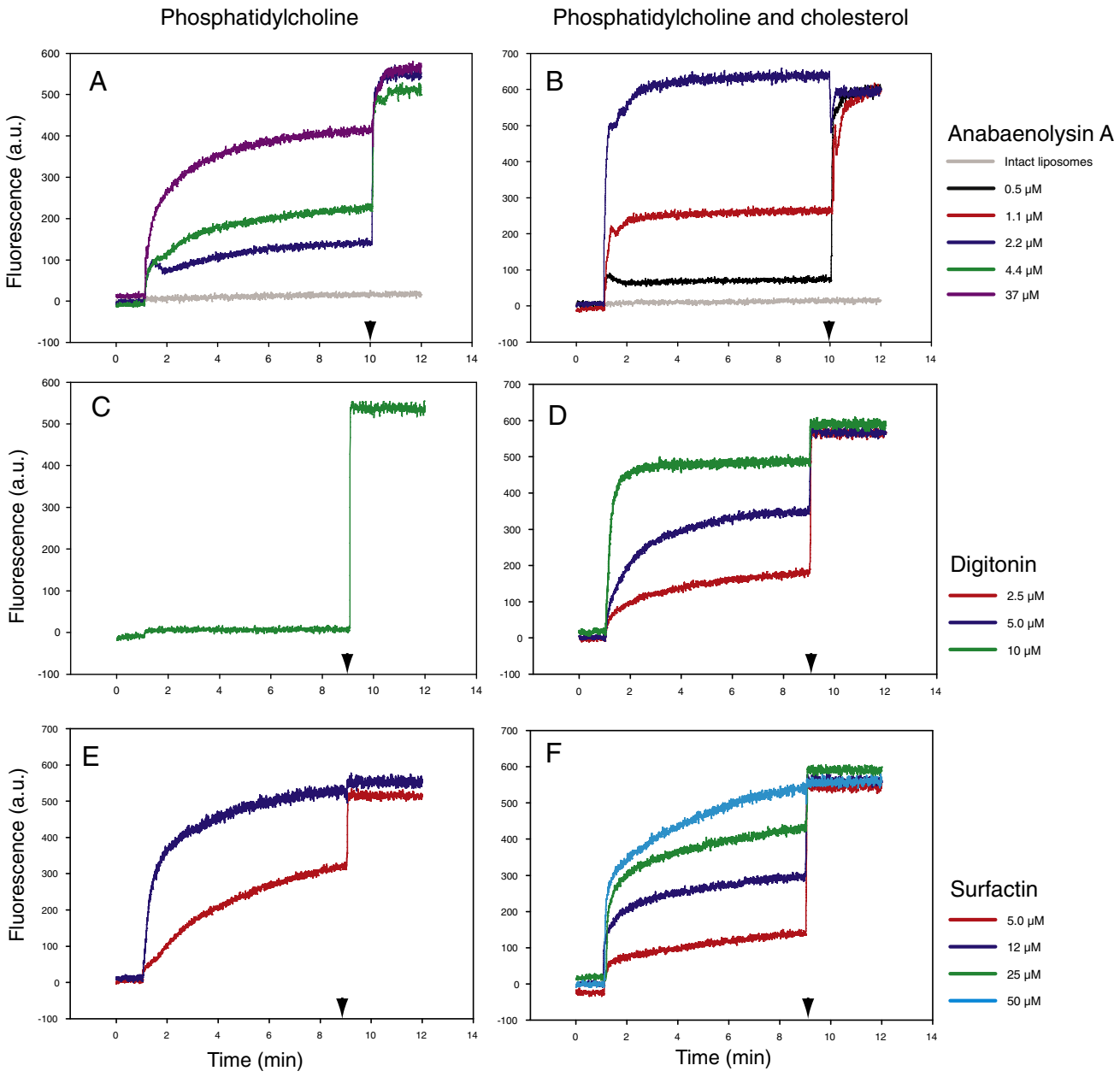

Fig. 5. Abl A permeabilises preferentially cholesterol-containing liposomes. Phosphatidylcholine liposomes without (A, C and E) or with (B, D and F) cholesterol containing the fluorescent marker calcein were incubated with the given concentrations of Abl A (A and B), digitonin (C and D) or surfactin (E and F) for 9 min before Triton X-100 (0.1% final concentration) was added (arrow) to achieve maximum calcein release. See the Materials and methods section for details on liposome preparation and experimental conditions. Note that Abl A was more potent against the cholesterol-containing liposomes (A) than either digitonin (D) or surfactin (F), and that Abl A, like digitonin, but opposite to surfactin, induced permeabilisation of liposomes in a cholesterol-dependent manner.

[9], and compatible with binding of cholesterol also to Abl A, which could contribute to the observed cholesterol-dependence of the Abl lytic action. Another option is that Abl A inserts into membranes, but instead of binding to cholesterol, unfavourable interactions causes repulsion leading to segregation of the membrane components. This has been suggested to be the case for surfactin-mediated destabilisation of membranes [38], but in this case repulsion was between negatively charged lipids and the negative polar head of surfactin. Although there is a high degree of similarity between digitonin and Abl A (Fig. 6C), suggesting that favourable interactions between cholesterol and Abl A represent the major cause of membrane destabilisation, as is the case with digitonin [9,37], we cannot exclude that repulsing forces destabilise cholesterol-containing membranes.

Cyanobacterial non-ribosomal cyclic peptides appeared early in evolution [39], and their purpose at the time of origin is still unknown. Some of them may have undergone modifications to selectively target grazers such as zooplankton, like the phosphatase inhibitors microcystins and nodularin [40], protease inhibitors [41], and inhibitors of cellular transport of small metabolites [42,43]. The anabaenolysins may represent another prokaryote-derived eukaryote-directed toxin, since they target cholesterol-containing membranes, typically found in eukaryotes. The observation that Abl A at sublytic concentrations enhanced the uptake/apoptotic effect of the cyanobacterial toxin nodularin against mammalian NRK cells (Fig. S3) suggests that a possible side-function of Abl is to enhance the penetration of other substances. It should be noted that nodularin and microcystin do not penetrate most cell membranes even when their charged moieties are neutralised [20].

In conclusion, the anabaenolysins target cellular membranes containing cholesterol. Their high potency makes them suited for

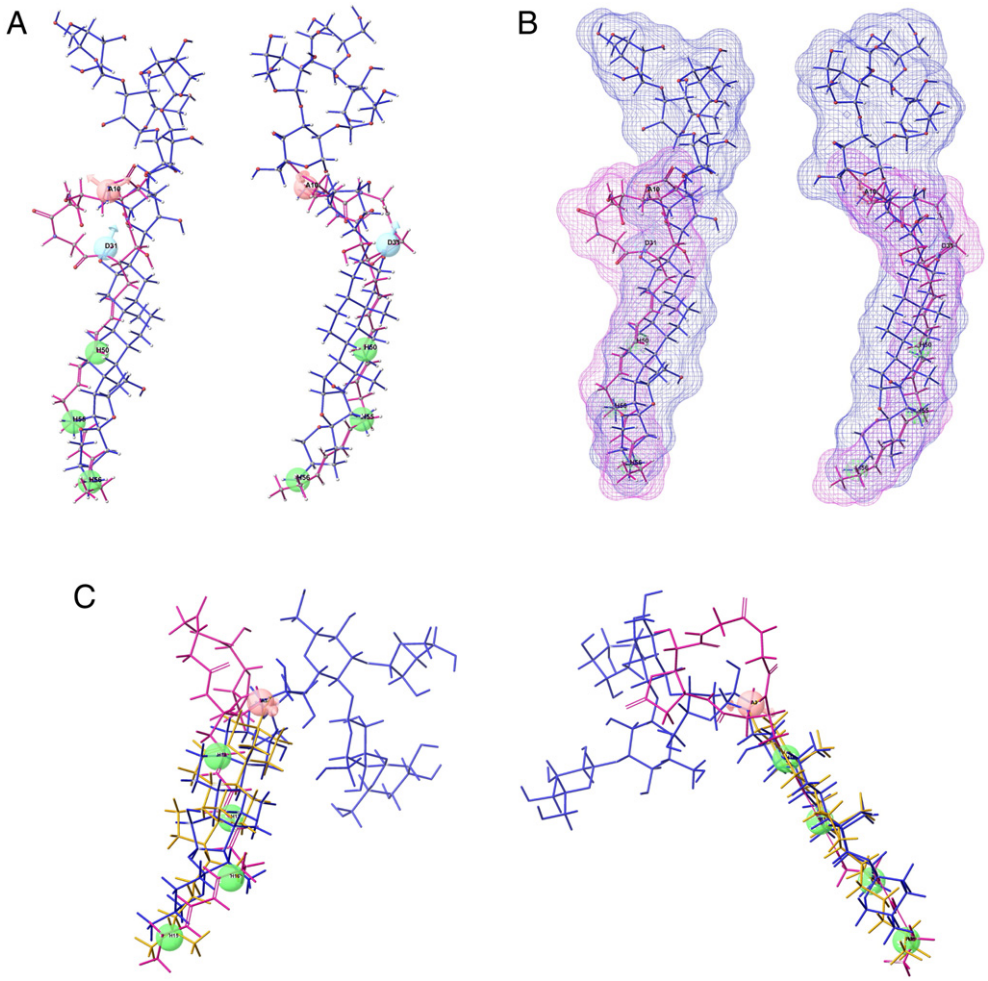

Fig. 6. Abl A shares structural and functional moieties with digitonin and cholesterol. Low energy conformations of Abl A, digitonin and cholesterol tautomers at neutral pH were generated and pharmacophore sites were defined using the Phase module of the Schrodinger™ software (www.schrodinger.com). A and B: Alignment of Abl A (magenta) and digitonin (blue) based on the highest scored pharmacophore constellation. A: Tube representation, the right panel is rotated 90° clockwise along the z-axis. B: As in A, but with surface representation (grid). C: Alignment of Abl A, digitonin and cholesterol based on the highest scored pharmacophore constellation. Colours as in A, cholesterol is in orange.

selective lysis of surface and non-mitochondrial organelle membranes. They should also be considered for future studies aiming for the selective perturbations of membrane subdomains or the selective solubilisation of subsets of membrane constituents.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.07.015.

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