Investigation of the mechanism of cell membrane-active compounds

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1. INTRODUCTION

The bacteria *Pseudomonas syringae* pv. *syringae* induce several plant diseases. Beside the plant pathogen effect, the toxins of the bacteria also possess antifungal and antibacterial activity that makes them promising compounds first of all to the antifungal therapy.

The target of the action of CLPs is the cell membrane. The CLPs induce pores in the membrane, which provide free diffusion for the ions between the extracellular and intracellular spaces. All investigated CLPs induced pores in red blood cells and model membranes, but the biological effects of the three compounds are different. Syringopeptines are thought to be responsible for the plant pathogenic effect, in this respect syringomycins and syringotoxin have weaker effect. While the antimicrobial effects can be related rather to syringomycins. The observed Gram negative bacteria were unaffected by the CLPs, but the Gram positive bacteria showed different sensitivity against the CLPs. All investigated CLPs inhibited the growth of fungi, but different fungal species showed different sensitivity. The lipid composition of the membrane, especially the sterol and sphingolipid content has crucial role in the effect of the CLPs. The spectrum of the activity and the effectiveness of the CLPs are different, which can be explained by their different structure.

On the base of their structure, CLPs can be divided into two groups: nonapeptides include syringomycins, syringotoxin, syringostatins and pseudomycins, while the other is the group of syringopeptines. All CLPs contain a polar head group and an apolar tail, but the size of the apolar part and the charge of the polar head group are different. We investigated the effects of three CLPs, the nonapeptide SRE and ST, and one member of the syringopeptins,
the syringopeptin22A (SP22A). SRE possesses a lactone ring with three positive and one negative charges linked to a 3-hydroxy-dodecilic acid tail. The hydrocarbon chain of ST is longer by two carbon atoms and has one less positive charge on the head group. The third observed CLP, the SP22A has two positive charges on the lactone ring built up by eight aminoacids, which is linked to the hydrocarbon chain by an apolar unit containing 14 aminoacids.

The pore forming properties of CLPs can be investigated on red blood cell (RBC) membrane and planar bimolecular lipidmembrane (BLM). The SRE, investigated earlier by our team, induced pores built up by a few monomers in RBCs membranes and caused partial lysis of the RBCs. The SRE channels inactivated in a temperature dependent manner, which suggested that the membrane fluidity plays role in the inactivation. The results obtained on BLM showed that the inactivation is the consequence of a decrease in the number of pores.

For the application of CLPs in human treatment a derivative with selective toxicity is required. In order to obtain such a compound knowledge of the linkage between the structure and the function is necessary. Recognition of the connection between the structure and the function is possible e.g.:

- mapping the properties of another CLP derivatives, or
- studying the molecular level interactions between the CLPs and the lipid membranes.

2. AIMS

The toxins produced by the phytopatogen *Pseudomonas syringae* pv. *Syringae* are cyclic lipodepsipeptides (CLPs). Beside the plant pathogen effect, CLPs also possess antifungal and antibacterial activity that makes them promising compounds, first of all, to the antifungal therapy. The spectrum and of the activity and the effectiveness of the different CLP derivatives are different, which can be explained by their different structure. In order to obtain a compound with selective toxicity the knowledge of relation between structure and function and the comparative investigation of the activity of CLPs is necessary. The most studied CLP is syringomycin E (SRE). Earlier studies of our team showed that SRE induce pores in red blood cell (RBC) and induced partial hemolysis. SRE pores are built up by some monomer and they inactivated in time at 37 °C and 20 °C. On the contrary at 8 °C, under the phase transition temperature of the main lipid components of the RBC membrane, inactivation could not be found. In order to obtain knowledge about the relation between the structure and function of CLPs, the effects of two another CLP derivatives, the syringopeptin22A (SP22A) and the syringotoxin (ST) were investigated. In the course of our work the followings were aimed:

I.1. to investigate the membrane permeabilizing effects of SP22A and ST on RBC;
I.2. to study the temperature and concentration dependence of the permeability increase;
I.3. to describe the pore inactivation by a transport kinetic model;
I.4. to study the pore forming properties of the CLPs, especially the inactivation and the oligomerization.

In order to get a better insight into the molecular mechanism of CLPs, we aimed to study on liposome systems:

II.1. the effect of CLPs on the membrane fluidity in case of small unilamellar vesicles (SUV) and multilamellar vesicles (MLV) contain saturated phospholipids;
II.2. the change of what molecular dynamic parameters correspond to the change of membrane fluidity;

II.3. the activity of CLPs as the consequence of structural analogies and differences, as well as role of CLPs in the effect of CLPs;

II.4. how are the unsaturated bonds of the fatty acid tail of membrane lipids and cholesterol content of the membrane influence the interactions of CLPs with liposomes;

II.5. what structural changes of lipid bilayer correspond to the irreversible fluidity change induced by CLPs.

3. METHODS

Cyclic lipodepsipeptides were gift from Dr. Jon Y. Takemoto (Department of Biology, Utah State University, Logan, UT, USA). Pore forming properties of CLPs were studied with transport-kinetic method on human red blood cell treded with Na-citrate as anticoagulant. For the ion transport measurement $^{86}\text{Rb}^+$ isotope ($\gamma$-energy: 1,078 MeV) was used as radioactive tracer. The rubidium ion is the analog of the physiologically more important potassium ion. The blood suspension was incubated at a given temperature, 37 °C, 20 °C, or 8 °C with cautious shaking. At definite time intervals samples from the suspensions were taken and RBCs were separated from the extracellular (EC) solution by centrifugation. The activity of the supernatant was determined by scintillation counter (Gamma Counter, Hungary). The activity of the supernatant is proportional to the amount of $^{86}\text{Rb}^+$, transported to the EC space, thus it is suitable for the characterization of the transport.

The samples were centrifuged for the determination of the hemoglobin (monomer) effluxed from the RBCs, and the hemoglobin (Hgb) concentration of the supernatant was measured with a hematology automata (Cobas Micros OT). The Hgb concentrations, less, then 1.3 mmol/l could not be measured with the automata. Thus for the determination of Hgb concentrations, less than 1.3 mmol/l an analog method was used: the hemoglobin was converted to cyano-methemoglobin by 2-3 drops of Quicksilver II reagent. The optical density of the solutions were proportional to the Hgb concentration and measured spectrophotometrically (Perkin-ElmerLambda UV-VIS spectrophotometer). The Hgb concentrations were read of from the calibration curve produced with the help of the reference blood of the hematology automata.

The lysis was calculated as the difference of the RBC number of the suspension before and after the addition of the toxin; the number of RBCs was determined by hematological automata (Cobas Micros OT).

The interactions between CLPs and membrane lipids were studied on small unilamellar vesicles (SUV) and multilamellar vesicles (MLV). SUVs were prepared by ultrasound sonication (Sonyprep 150 MSE) and MLVs were prepared by vortexing. Synthetic dimyristoyl-L-$\alpha$-phosphatidyl-choline, dioleoyl-phosphatidyl-choline, dipalmitoyl-L-$\alpha$-phosphatidyl-choline and cholesterol were used for the preparation of liposomes, obtained from Sigma Chemical Co. The purity of compounds was minimum 99%.

The change of the fluidity induced by CLPs, and the interactions between CLPs and membrane lipids were studied by electron spin resonance spectroscopy (ESR). The ESR spectra were registered by a Bruker-EMX-6 on line, X-band (9-10 GHz) spectrometer. The liposome membrane was studied at different depths: at the level of the head-groups, at the middle-, and at the end of the hydrocarbon chain with different spinlabels. 5-doxyl-, 7-doxyl-, and 12-doxyl-stearicacid (ICN Biomedicals Inc Ohio), and 16-doxyl-stearicacid
(Signa Chem. Co) and 4-(N,N-dimethyl-N-hexadecyl)-ammonium-2,2',6,6'-tetrametilpiperidinoxyll-iodid (Molecular Probes Inc., OR, USA) spinlabels were used. For a more detailed characterization of the changes in the molecular interactions, spectrum simulations were done. For the simulations of ESR spectra the NLLS (nonlinear-least-squares) program was used developed by J.H. Freed and his group.

The size-distribution of SUVs was checked by dynamic light scattering (DLS) with ALV Goniometer. The source was a He-Ne laser (Spectrophysics 124B), worked at 632 nm, with 10 mW power.

4. RESULTS AND CONCLUSIONS

In order to elucidate the relations between the structure and functions of CLPs, the effects of two CLP derivatives, the SP22A and the ST were studied on RBC membrane as well as on liposomes of different composition. The SRE, studied earlier by our team, induced pores in RBC membranes and caused partial lysis of RBCs. The SRE pores built up by a few monomers and the pores inactivated at 37 °C and 20 °C. However, at 8 °C, under the phase transition temperature of the main lipid components of the RBC membrane, inactivation could not be found. As the results of our transport-kinetic measurement, the followings were verified:

I.1. The two CLP derivatives (SP22A and SRE) caused only partial hemolysis. In case of SP22A there was no detectable lysis at the toxin concentrations used for the transport measurement; in case of ST the measured lysis was less, then 6%. Both SP22A and ST increased the permeability of RBC membrane for $^{86}$Rb$^+$. On the basis of the $^{86}$Rb$^+$ transport kinetic results we concluded that SP22A and ST induce pores in RBC membrane and the size of the pores render the efflux of $^{86}$Rb$^+$ and hemoglobin monomers.

I.2. The increase of the membrane permeability was proportional to the applied CLP concentration for both toxins, but the effectiveness of the two toxins was different. Among the three CLP derivatives the SP22A has the highest pore forming activity, the earlier studied SRE is weaker and the weakest is the ST according to permeability increasing effect on RBC membrane.

In case of ST, well before the equilibrium distribution of the $^{86}$Rb$^+$ and the Hgb the increased permeability decreased to the level of the control sample at 37 °C and 20 °C, but not at 8 °C.

From the above findings we concluded, that the ST pores inactivated at 37 °C and 20 °C, but at 8 °C the pores are stable during the measurement. In case of SP22A the increased permeability of the membrane did not decreased even at 37 °C.

I.3. A transport kinetic model was worked out in order to characterize the $^{86}$Rb$^+$-efflux through the CLP pores. The mean life-time of the ST pores and the rate constants of $^{86}$Rb$^+$-transports were determined by the model.

I.4. According to our calculations the pores’ mean life-time at 37 °C is shorter, than 2 minutes, at 20 °C it is longer, than 2 minutes and at 8 °C it is longer, than the time interval of the measurement. On the basis of the temperature dependent inactivation we concluded, that the speed of the inactivation is influenced by the fluidity: the bigger the fluidity, the shorter the pores’ mean life-time. SP22A pores did not inactivated even at 37 °C, which suggest, that SP22A create much stable channel, than ST and the earlier studied SRE.
With the help of the model the rate constants ($k_p$) of $^{86}$Rb$^+$-transports across ST pores were determined. From the rate constants ($k_p$) the permeability coefficients ($p_p$) of pores for $^{86}$Rb$^+$ were calculated. The concentration dependence of $p_p$ in Hill representation showed that ST create pores built up by a few monomers in RBC membrane similarly to SP22A and earlier studied SRE.

From the pore forming activity and the different inactivation properties of the three compounds we concluded, that the greater hydrophobic part offer greater pore forming activity to the SP22A and stabilize the pore in the membrane. Beside the hydrophobic character the charge of the toxin also has a role in the stability: the SRE pores possess two net positive charges and have longer life-time than the ST pores, which have only one net positive charge; more positive charges also stabilize the channel.

In order to get a better insight into the molecular mechanism of CLPs the effects of the three CLP derivatives, SP22A, SRE and ST were investigated on liposomes of different compositions. On the basis of our results we drawn the following conclusions:

II.1. All the three CLPs decreased the fluidity of liposome prepared by saturated lipids. The fluidity decreasing effect was observable in the whole depth of the membrane. SP22A and SRE decreased the fluidity significantly even at quite low concentration: at 1/400 toxin/lipid molar ratio. From the change of the fluidity and the concentration dependence of the CLP activity we concluded that CLPs involve the lipids into the pore formation, they create the pores together with the lipid molecules. According to our results SP22A has the highest effect to decrease the fluidity, SRE is weaker and the weakest is the ST.

II.2. On the basis of the results obtained with spectrum-simulations we concluded that the fluidity decrease induced by the CLPs is connected with a decrease of the motional- and rotational freedom of the lipids, an increase of the ordering of lipid molecules.

II.3. The results obtained with SP22A and SRE at different temperatures showed, that they induce an irreversible structural change close to the pre-transition temperature, and in case of SRE higher temperature is required for similar change. This phenomenon also supports our conclusion: the hydrophobicity and the number of positive charges influence the activity of CLPs. The irreversible structural changes around the pre-transition temperature, and the fact that the effect of SP22A was much weaker on MLV, than on SUV suggests, that appropriate fluidity increase the effects of CLPs. To get the maximal effect, SP22A and SRE required appropriate low temperature, too. Our conclusion was that for the maximal effect of the CLPs an appropriate rigid state of the membrane is also required.

II.4. The influence of unsaturated lipids in the effect of CLPs was studied on DPPC-DOPC mixed liposomes. The difference of fluidity between the control and the treated (with CLP) liposomes was less than in case of pure DPPC vesicles. The difference decreased as the DOPC content increased, which suggested, that the double bond decreases the effect of CLPs. On DPPC-cholesterol liposomes the effect of the SP22A also decreased in a SP22A concentration dependent manner. Our results obtained on DPPC-DOPC and DPPC-cholesterol mixed liposomes suggest that the inhomogenity of the membrane reduce the effect of CLPs, and in accordance with the literature, the targets of the CLPs are likely the lipid-rafts of the membrane.

II.5. In case of SUVs prepared by DPPC, the irreversible structural change induced by the SP22A was observable at 31 °C. Above 31 °C the fluidity of SUVs treated by
SP22A were approximately the same as the fluidity of MLV, which suggested, that the irreversible structural change create similar structure as the MLV. Similarly ordered structure, like the MLV, can be formed e.g. with the sticking of lipid bilayers as the consequence of aggregation, or with the formation of large unilamellar vesicles as the consequence of fusion, or coalescence. The size distribution of vesicles was determined by dynamic light scattering. In accordance with the above results, the size distribution of liposomes became broader. In the samples treated with the CLPs much greater particles were found, than in the control sample, which suggests that \textit{CLPs induced coalescence, fusion, and/or aggregation}. In case of living cell, this phenomenon could lead to \textbf{successful bacterial attack}: in that case the bacteria acquire intracellular compounds of the target cell.

On the basis of our results with the SP22A we found such a molecule, that possess great activity, induce stable pores in the membrane and at the same time it does not cause hemolysis in that concentration where the permeability increasing effect is significant in comparison to SRE and ST. We succeeded to obtain information about the role of the two main part of the CLPs, the lacton ring and the peptide chain in the activity of CLPs. We also got information about the role of the lipid composition of the membrane in the defense and resistance of the target cells. With our results we got closer to the long-range plan: to find a CLP derivative with selective toxicity for the human therapy, that would be enough effective on fungal cells, but would not damage the human cells.

\textbf{Abbreviations}

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CLP</td>
<td>cyclic lipodepsipeptides</td>
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<tr>
<td>DMPC</td>
<td>dimyristoyl-L-α-phoshatidyl-choline</td>
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<tr>
<td>DOPC</td>
<td>dioleoyl-phosphatidyl-choline</td>
</tr>
<tr>
<td>DPPC</td>
<td>dipalmitoyl-L-α-phosphatidyl-choline</td>
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<tr>
<td>EPR, ESR</td>
<td>electron paramagnetic resonance</td>
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<td>LUV</td>
<td>large unilamellar vesicles</td>
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<tr>
<td>MLV</td>
<td>multilamellar vesicles</td>
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<tr>
<td>NLLS</td>
<td>nonlinear least-squares method/program</td>
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<tr>
<td>RBC</td>
<td>red blood cell</td>
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<tr>
<td>SL-12</td>
<td>12-doxyll-stearic acid spin label</td>
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<tr>
<td>SL-16</td>
<td>16-stearic acid spin label</td>
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<td>SL-5</td>
<td>5-stearic acid spin label</td>
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<tr>
<td>SL-7</td>
<td>7-stearic acid spin label</td>
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<tr>
<td>SP22A</td>
<td>syringopeptin22A</td>
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<td>SRE</td>
<td>syringomycin E</td>
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<tr>
<td>ST</td>
<td>syringotoxin</td>
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<tr>
<td>SUV</td>
<td>small unilamellar vesicles</td>
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5. \textbf{SUMMARY}

The cyclic lipodepsipeptides produced by \textit{Pseudomonas syringae} pv. \textit{syringae} possess fungicide properties. They inhibit much of the cell functions, presumably on the basis of their pore-forming activity. The syringomycin E, studied earlier by our research group, formed pores on human red blood cells (RBC), and caused also partially hemolysis. To reach selective toxicity, knowledge of the relationship between the structure and function of the
CLPs is required. To fulfill this requirement, the poreforming properties of two other CLPs, the syringopeptin22A (SP22A) and the syringotoxin (ST), were investigated on RBCs. On the basis of our transport kinetic measurements on RBC, the SP22A and the ST formed pores built up by few monomers. ST pores inactivated at 37 °C, and at 20 °C, however, they remained stable at 8 °C, similarly to the SRE. Inactivation of the SRE and ST pores was faster at 37 °C than at 20 °C. SP22A pores did not inactivate even at 37 °C. On the basis of our results, we concluded, that fluidity has a crucial role in the process and the dimension of the hydrophobic part and the number of the positive charges of the polar headgroup influence the stability of the pores.

To study the interactions of CLPs with membranes on molecular level we applied EPR spectroscopy and spectral simulation methods for liposomes of different composition. CLPs decreased the fluidity at all depths of the membrane: the motional and rotational freedom of the lipid molecules decreased, while their ordering increased. These observations in conjunction with the concentration dependence of the CLP’s action suggested that CLPs form pores involving the lipids, too. Investigation of the temperature dependence of the CLPs’ action showed that to get their complete effect a given temperature range is required. It corroborated that the fluidity has a crucial role in the interactions between CLPs and membrane lipids. From the results, obtained with DPPC-DOPC or DPPC-cholesterol liposomes we concluded that the membrane-inhomogeneity decreases the effectiveness of the CLPs.

Dynamic light scattering measurements were used to determine the size-distribution of the liposomes. According to our experiences CLPs provoke fusion and/or aggregation, which can have a role in the cell-killing properties of the CLPs.

6. PUBLICATIONS IN THE FIELD OF THE THESIS

PAPERS:


Zsófia Szabó, Marianna Budai, Katalin Blaskó, Pál Gróf Molecular Dynamics of the CLP Action Studied on Model Membranes: Effects of Syringopeptin22A, Syringomycin E and Syringotoxin studied by EPR technique. Submitted for publication to BBA Biomembranes; April, 2003.

ORAL REPRESENTATIONS:


POSTERS:


Szabó Zsófia, Gróf Pál, Blaskó Katalin Ciklikus lipodepszipeptidek hatásainak sejti és molekuláris szintű vizsgálata XXXI. Membrán-Transzport Konferencia, Sümeg, 2001. 05. 22-25.

