Azobenzene-substituted phosphate amphiphiles
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Chapter 4

Effect of isomerisation of azobenzene-substituted dialkyl phosphates on the function of MscL*

4.1 Introduction

The function of MscL (mechanosensitive channel of large conductance) has been assessed by patch clamp techniques,\textsuperscript{1} the ability to allow growth of cells under low osmotic conditions,\textsuperscript{2} detection of released proteins\textsuperscript{3,4} and release of small fluorescent molecules.\textsuperscript{5} Another type of method is the use of site-directed spin labeling together with electron paramagnetic resonance spectroscopy (EPR)\textsuperscript{6,7} in studies of the structure and dynamics of MscL. However, patch clamp is the most important technique for studying the functional properties of MS proteins.

4.1.1 Patch clamp

The patch clamp technique has been developed by Neher and Sakmann,\textsuperscript{8} who won the Nobel Prize for medicine in 1991 for this invention. They demonstrated with the use of patch clamp that ion channels exist in muscle fibres of a frog and how they function. For patch clamp a thin glass micropipette with a diameter of thousandths of a millimeter is employed and the current flowing through the pipette is measured. The pipette is gently pressed against the membrane and a seal is formed (Figure 4.1, left), which can be followed by light microscopy.\textsuperscript{9} Upon gentle suction inside the pipette (Figure 4.1, right) the seal starts to enter the pipette and forms a so-called giga-ohm seal.\textsuperscript{10} This refers to the resistance of the seal, which is in the order of giga ohms. A high resistance is necessary to reduce the background noise in the recording.

The formed seal (or patch) is now in a fixed position in the pipette and the curvature can be varied by increasing or decreasing the suction power. Release of the suction leads to flattening of the patch and an increase in suction induces (more) curvature.

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Figure 4.1 Video images of the patch clamp technique. For an explanation, see text.

In contrast with spheroplasts derived from *E.coli* cells, membrane patches of reconstituted systems have the tendency to creep up the wall of the pipette, leading to a change in size and shape of the patch. At this point measurements can be performed which are called ‘cell attached’ or ‘on cell patch’. In this setting, the pipette is still attached to the rest of the membrane, e.g. the ion channels in the surface of neurons could be studied in this manner. Another setting can be reached by strong suction so that the patch is destroyed but the cell is still attached to the pipette. Whole cell recordings can now be achieved.

The most frequently used setting at the moment is the inside out mode. After the formation of the giga-seal the rest of the membrane/cell is removed from the pipette. Now single channel ion currents can be recorded in the patch. Another advantage is that the solution composition can be changed during the measurement. In Figure 4.2 a typical trace is shown of a single channel recording of an inner membrane of *E.coli*. In time, the suction pressure was first slowly increased and later slowly decreased.

The first change in current, which is observed, is due to opening of two channels, which are called MscS (mechanosensitive channel of small conductance). Upon increasing the suction pressure also the opening of MscL channels is visible. When the traces of the two types of channels are compared, four differences are observed. For MscS a smaller conductance (20 pA) is observed, that means a smaller pore is formed. Secondly, MscL (mechanosensitive channel of large conductance) has ‘sub-conductance states’. Some ion channels have more than one open conformation, and these conformations have different conductances.
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Figure 4.2 Typical trace of a single channel recording. The trace is recorded as a function of time and the suction pressure was slowly increased. Openings of two types of channels are visible. The scales for time, pressure (P) and current are given.

MscS and MscL also have different open probabilities (P(o)). P(o) is the chance that, at a certain moment, the channel will be found open under a given set of conditions, and P(o) can have values between 0 and 1 if one channel is present. The value of P(o) can be obtained in various ways but only the method used in this chapter will be discussed. Each data point measured represents one amplitude value for each unit of recording time. First the different levels of openings (no openings, one channel open, two openings, etc.) are ascribed to certain values of the current. Then threshold values are set to divide all data in subsequent categories of openings. If the current passes a certain threshold value it is called an event. P(o) can now be calculated from the data. Often a graph is constructed with P(o) depending on the applied pressure, a so called open probability curve. In Figure 4.3 typical examples are given, often an S-shaped dependence on the pressure is found. When a channel opens easier, that is, less pressure is needed, the open
probability curve shifts to the left. Vice versa the same argumentation holds. In this typical example (Figure 4.3), the open probability curve shifts to the left if the membrane thickness is decreased. Thus, it is easier to open the channel in a thinner membrane (see also section 4.1.2).

Figure 4.3 Typical graph of open probability curves, showing the pressure dependence of the open probability $P(o)$. Here the open probability curves are given for MscL in membranes with different thickness (phosphatidyl choline with C16, C16:1 (PC16), C18, C18:1 (PC18), C20, C20:1 (PC20)).

Another value, which can be obtained from a trace, as depicted in Figure 4.2, is the dwell time, the time in which channel is in the open conformation. Channels can have the same $P(o)$ but shorter or longer opening times (compare MscS and MscL). For a more detailed discussion about dwell time analysis see reference 12.

4.1.2 Recent literature

To study MscL two types of variations are possible, mutagenesis of the protein and changes in the physical state of the membrane. Random mutagenesis has been used to find residues, which are important for functioning of MscL. A large number of mutants have been screened and mutations that affected the function were divided into two classes of phenotypes: gain-of-function (GOF) and loss-of-function (LOF) mutants. GOF mutants have channels that open more easily and LOF mutants show inhibition of channel opening. In this way α-amino acid residues were found which are important for gating mechanism/ stability of the closed state, fine tuning of mechanosensitivity and lipid-protein interactions.

The function of MscL was studied as a function of the lipid chain length, addition of amphiphiles, and temperature. An increase in
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temperature from 30°C to 37°C led to opening of the channel.\(^5\) After a decrease in temperature to 0°C, openings were no longer observed. It was suggested that an increase in temperature might induce an increase of the membrane tension and leading to opening of MscL. The efflux through MscL was monitored using endogenously produced fluorophores.

Perozo et al.\(^7\) investigated the function of MscL as a function of the membrane thickness (section 4.1.1). They obtained patch clamp results for PC liposomes with C16, C18 and C20 chains (Figure 4.3). Channel reconstitution was inhibited by C22. When compared to C18, MscL opened with a lower activation threshold in liposomes with C16 and an opposite behaviour was found for C20. Thus, in a thinner membrane the channel opens much easier and the open probability curve shifts to lower pressures.

The effect of amphiphiles on the function of MscL has been described in three articles. Higher gating tensions were necessary when DOPE was incorporated into the membrane.\(^{23}\) Recently, Perozo et al.\(^7\) found that asymmetric addition (addition to one leaflet) of 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC, 3 \(\mu\)M, Figure 4.4) to a patch, without extra applied tension, induced opening of the channel after an incubation time of ca. 10 min. Martinac et al.\(^{22}\) studied the effect of eight different amphiphiles on the opening of MscL. The amphiphiles were either neutral, positive or negatively charged. Except one, all induced a shift of the open probability curve to lower pressure. Upon the addition of chlorpromazine (CPZ, 20 \(\mu\)M, Figure 4.4), no extra applied pressure was necessary to open the channels (incubation time 60 min.). Perozo et al.\(^7\) proposes that the asymmetry in the lateral pressure profile between the two leaflets of the bilayer, induced by asymmetric addition of LPC, initiates the opening of the channel.

![Figure 4.4 Structures of chlorpromazine (left, pKa=9.3) and LPC (right).](image)

**Figure 4.4** Structures of chlorpromazine (left, pKa=9.3) and LPC (right).

### 4.1.3 Calcein efflux experiments

Calcein\(^{24}\) (Figure 4.5) and also carboxyfluorescein (CF)\(^{25}\) are often used for the study of vesicle stability and fusion of vesicles.\(^{26,27}\) Calcein exhibits self-quenching at high concentrations and is fluorescent at low
concentrations. After encapsulation of calcein at high concentration in a vesicle and removal of the non-encapsulated calcein, the leakage of calcein can be monitored by fluorescence spectroscopy. Although, to the best of our knowledge, no publication yet appeared in which the activity of membrane proteins is monitored by calcein efflux measurements, the method is often used in our laboratories.

**Figure 4.5 Structure of calcein.**

In this chapter the function of MscL is studied as a function of trans-cis and cis-trans isomerisation of double-tailed azobenzene-substituted phosphates with the use of patch clamp and calcein efflux experiments.

### 4.2 Calcein efflux experiments

#### 4.2.1 Preparation of the vesicles

In Figure 4.6 an overview of the method used for purification and reconstitution of MscL is given. After harvesting and washing of the bacteria (*Escherichia coli* and *Lactococcus lactus*), they were broken up in smaller parts with a French Press. After two steps of centrifugation a membrane fraction was collected. The membrane fraction was resuspended and solubilised with n-octyl glucoside. Detergent-lipid-protein mixed micelles were formed. After centrifugation, to remove unsolubilised parts, the solubilised material was incubated with a Ni$^{2+}$-nitrilotriacetic acid (NTA) resin. The MscL channel used in our work was modified at the carboxyl terminus with six histidines (6-histag)$^{28}$ Histidine is an α-amino acid with in the side chain two amines that are only partly positive at neutral pH. Due to the availability of the lone pairs of the nitrogens, histidine has a great affinity for Ni$^{2+}$.

After incubation with Ni$^{2+}$-NTA resin the MscL channel was bound to the resin via Ni$^{2+}$. The mixture was poured into an empty column and washed with a buffer containing low concentrations of imidazole. The MscL channels were eluted with a high concentration of imidazole that acted as a competitor for the 6-histag in its interaction with Ni$^{2+}$.

In the meantime liposomes were prepared of the desired lipid composition. They were titrated with Triton X-100 until the onset of solubilisation, which was followed by turbidity measurements.
Figure 4.6 Schematic overview of the purification and reconstitution of MscL.

The detergent destabilised liposomes and the purified proteins were mixed, incubated and then Biobeads were added. Biobeads are macroporous polymeric beads made of polystyrene with a large surface area for adsorbing apolar materials. Detergents like Triton X-100 can be removed from solution by Biobeads. After removal of Triton X-100, reconstitution provided vesicles with membrane-incorporated MscL channels. At this point calcein could be added and the vesicles were subjected to freeze-thaw cycles. At this stage the sample could be stored for a long period in liquid nitrogen. Addition of calcein was also possible one stage earlier, during the reconstitution. At the day of the fluorescence measurements, the samples were extruded and filtered over a sephadex column to remove non-encapsulated calcein (size exclusion chromatography).

Two types of MscL channels were used for the reconstitution, wild type (WT) and G22C which is a mutant of the wild type, in which the glycine at place 22 is substituted by a cysteine. Cysteine reacts with MTSET and forms a disulfide bond (Figure 4.7). The positive charge induces opening of the MscL channel. The WT channel does not open upon addition of MTSET.
4.2.2 Results of the calcein efflux experiments

Several setups have been used during the calcein efflux experiments, the first experimental setup had the following features:

(1) an amphiphile ratio of 20 mol% of DT Azo-5P and 80 mol% of DOPC (DOPC structure, see chapter 3), (2) the proteoliposomes were extruded through membranes of 400 nm, (3) isomerisation cycle: 7 min. irradiation at 365 nm, 5 min. dark, 90 s. irradiation at 436 nm, (4) control sample: only 90 s. irradiation at 436 nm, (5) the WT MscL channel was used if not otherwise stated, (6) control sample: without MscL.

With this setup it was found that there was a decrease in fluorescence of the master sample from the column (SEC) in time. Application of aluminium foil to avoid exposure to daylight led to a stable signal.

The membranes with and without channels had a stable fluorescence signal in time (hours-days), there was no leakage of calcein, and therefore it was concluded that the channels stayed in the closed conformation.

When the trans azobenzenes were switched to the cis conformation, an increase in fluorescence was observed but after subsequent irradiation back to the trans isomer the original fluorescence signal was observed (without irradiation). The increase in fluorescence, after switching to the cis conformation, is not due to release of calcein but due to an artefact. Therefore, only fluorescence measurements were carried out on samples, which contained azobenzene-substituted phosphates in the trans conformation.

Sometimes also a higher fluorescence signal was found for the samples which were subjected to a complete cycle (trans-cis-trans). When a control (trans) sample was irradiated with light of 436 nm, no isomerisation to the cis isomer took place, but a small increase in the fluorescence was often observed. In order to avoid misinterpretations, a control sample was always irradiated with light of 436 nm.

As a control, proteoliposomes were made with G22C. Addition of MTSET led to opening of the MscL channels as indicated by calcein efflux.
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experiments, and therefore it can be concluded that the reconstitution was successful.

With the experimental setup as described above no evidence for calcein release after a full cycle of irradiation was obtained, with or without the channel. Therefore, variations in the setup were made.

Addition of tetracain

Martinac et al.\textsuperscript{22} showed that the open probability curve of MscL shifts to lower pressure as tetracain is added (0.3 mM, Figure 4.8).

\begin{center}
\includegraphics[width=0.5\textwidth]{tetracain_structure.png}
\end{center}

\textbf{Figure 4.8} Structure of tetracain. $pK_a=8.5$.

When tetracain was added at different concentrations, only at 1.0 mM a small effect was observed (Figure 4.9). The precise calculation of the percentage of dequenching of calcein (percentage dequenching) can be found in the experimental section. A dequenching of 0% means that all fluorescence of the calcein is completely quenched. A value of 0% will never be found in this type of experiments because there is always some calcein present outside the proteoliposomes, which gives rise to fluorescence.

\begin{center}
\includegraphics[width=0.5\textwidth]{dequenching_chart.png}
\end{center}

\textbf{Figure 4.9} Effect of 1 mM tetracain on the dequenching value of calcein. Legend: empty: without MscL; WT: with MscL (Wild Type); ctrl pre: control before irradiation; cycle: value after a full cycle of irradiation; ctrl 436 nm: control, only irradiated with 436 nm; ctrl post: control without irradiation after the experiment. The ctrl post of the WT measurement is not available. The values where measured in duplo and the average was taken.
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Apparently, the filtration over the column is not completely effective and calcein often sticks to the outside of the proteoliposomes. A dequenching value of 100% means that there is no quenching of fluorescence, hence no calcein is present at a high selfquenching concentration (complete release of calcein).

As can be seen from the results in Figure 4.9, an extra release of only 10% is observed. These results do not convincingly proof that the isomerisation of the azobenzene groups of DT Azo-5P leads to opening of the channels. Note that there is a small release in time of calcein due to tetracain (with and without MscL). In the experiments described below always 1 mM of tetracain was added.

Isomerisation cycle

The waiting time (dark period) after the isomerisation from trans to cis was varied to monitor if this has any effect on the function of the channel. The waiting time was varied from 5 min. to 30 min. and no significant effect was observed.

Size of the proteoliposomes

The tension (T) developed within a bilayer as a function of the radius of the vesicle (r) and pressure across the bilayer (P) is defined by Laplace’s Law:

\[ T = \frac{P \cdot r}{2} \]  

(1)

For example: In a patch clamp experiment the pressure (P) across the bilayer is varied by the intensity of the suction. The radius of the bilayer (r) can be determined when the patch is considered to be a part of a complete vesicle. The tension within the bilayer (T) can then be calculated.

The function of the MscL is thought to be dependent on the tension in the bilayer and therefore also on the radius of the vesicle.

Samples were prepared in which the vesicle size ranged from 200-800 nm by extrusion through the appropriate filters, but no extra release of calcein was observed upon isomerisation. For the 400 nm sample, which was taken as a control, again a 10% release of calcein was observed (reproduction of earlier results). Also tip sonicated proteoliposomes were tested (vesicle size <50 nm) and there was no release of calcein upon isomerisation. No relationship was found between the function of the channel and variation in the radius (r) of the proteoliposomes in combination with isomerisation of the azobenzene-substituted phosphate, DT Azo-5P (in the experimental setup used here). However, one should recognise that vesicles extruded through filters with different pore diameters generally have wide size distributions. Vesicles extruded
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through, for instance, filters with 100 nm pores have typically a mean diameter of 120-140 nm.\(^\text{30}\)

Lipid composition

The amphiphile composition was changed by adding DOPS or DOPE (for structures, see Figure 4.10). At a ratio of 50 mol\% of DOPS, 30 mol\% of DOPC and 20 mol\% of DT Azo-5P, similar results were obtained as for the samples without DOPS. In Figure 4.11 the results are given for proteoliposomes with 50 mol\% of DOPE, 30 mol\% of DOPC and 20 mol\% of DT Azo-5P. A release of 30\% was found for the liposomes containing MscL channels but also in the control experiment (without channel) a larger release was observed (20\%). This taken into account, again an additional 10\% release was found for liposomes with MscL channels. The release found in the control experiment could be due to the decreased stability of the membrane when DOPE (50 mol\%) was present.

![Figure 4.10](structures.png)

Figure 4.10 Structures of DOPS (above) and DOPE (below).

In sum, the calcein efflux experiments were found to be experimentally laborious. A lot of factors, which can influence the fluorescence, have to be taken into account. To avoid misinterpretations, control experiments for leakage in time, quenching of calcein by light, etc. have to be made.

The highest extra release of calcein in the presence of MscL channels upon isomerisation of the azobenzenes of DT Azo-5P was approximately 10\%. Several parameters were varied but no really compelling evidence for the opening of MscL upon isomerisation of the azobenzene-substituted phosphate DT Azo-5P was obtained by the calcein efflux experiments.
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Figure 4.11 Results of the calcein efflux experiments in proteoliposomes consisting of 30 mol% of DOPC, 50 mol% of DOPE and 20 mol% of DT Azo-5P. Legends see Figure 4.9.

4.3 Patch clamp experiments

For these experiments, liposomes were used containing 80 mol% of DOPC and 20 mol% of DT Azo-5P and MscL from *E. coli* or *L. lactis*. As a control, liposomes were taken consisting of 80 mol% of DOPC, 20 mol% of DOPS and MscL. When a patch with channels was clamped, the pressure was set at around 10 mm Hg and the conductance was continuously recorded. The open probabilities were calculated for a time period of 10 s following the schedule presented in Figure 4.12 (control experiment, without irradiation) and Figure 4.13 (with irradiation). In the control experiments the P(o) was calculated at 0, 30, 130 and at 190 s.

Figure 4.12 Timeline for patch clamp experiments without irradiation. The bars indicate the time periods used to calculate the open probability (P(o)).

In the experiments with irradiation, first the P(o) was determined before irradiation. Then the sample was irradiated with light of 365 nm and the P(o) was determined at 30 and 130 s. At 140 s. the filter was changed to 436 nm and at 190 s. the P(o) was again determined.
Before presenting the results of the \( P(o) \) determination, first some typical patch clamp traces are shown in Figure 4.14. Traces A, B and C in Figure 4.14 belong to a sample containing 80 mol% of DOPC, 20 mol% of DT Azo-5P and MscL. Trace A was recorded before irradiation of the sample. Trace B after irradiation for 30 s. with light of 365 nm and trace C after subsequent irradiation with light of 436 nm for 60 s. Traces D and E correspond to proteoliposomes with the same lipid composition, but now the sample was not irradiated. Trace D is recorded directly after seal formation and trace E after 60 s. Traces F and G were recorded with a sample containing 80 mol% of DOPC, 20 mol% of DOPS and MscL.

Traces H and I belong to a sample which contained 80 mol% of DOPC and 20 mol% of DT Azo-5P but without MscL. F and H were recorded directly after formation and G and I after irradiation with light of 365 nm for 30 s.

Visual evaluation of the traces indicated an increase in MscL activity upon isomerisation of the azobenzene functionalities with light of 365 nm. Upon subsequent isomerisation with light of 436 nm a decrease in MscL activity was observed. Without irradiation no significant increase was observed (D,E). Also in the control experiments, without DT Azo-5P or without MscL, no significant increase was observed (F-I). The results are summarised in Figure 4.15, and \( P(o) \) values at discrete time periods (Figure 4.12 and Figure 4.13) were calculated.

The open probability increased with a factor 4 upon isomerisation with light of 365 nm. and decreased upon isomerisation with light of 436 nm. In Table 4.1 a summary of \( P(o) \) values is presented. Upon isomerisation from cis to trans a decrease in activity was observed. The \( P(o) \) after trans-cis-trans isomerisation (1.34) was higher than the starting value (1.00). This can be due to migration of the patch inside the pipette.\(^{11}\)

It can be concluded that the activity of MscL increases upon isomerisation from trans to cis of the azobenzene group of DT Azo-5P.
Figure 4.14 Traces of the patch clamp experiments. For details see text.
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Figure 4.15 The open probability \(P(o)\) for the different time periods as indicated in Figure 4.12 (without irradiation, light bars) and Figure 4.13 (with irradiation, dark bars). The open probability for the time period 0 to 10 s. was indexed at 1 for each trace. The sample consisted of 80 mol% of DOPC, 20 mol% of DT Azo-5P and MscL.

Table 4.1 Open probabilities \(P(o)\) of the patches with composition: 80 mol% of DOPC, 20 mol% of DT Azo-5P (or DOPS) and MscL.

<table>
<thead>
<tr>
<th>Time period (s)</th>
<th>Patches with irradiation</th>
<th>Patches without irradiation</th>
<th>Patches without switchable lipid with irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>A</td>
<td>1.00</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>30-40</td>
<td>B</td>
<td>4.11</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>n.a.</td>
</tr>
<tr>
<td>130-140</td>
<td>C</td>
<td>4.15</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>n.a.</td>
</tr>
<tr>
<td>190-200</td>
<td>D</td>
<td>1.34</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

The open probability for the time period 0 to 10 s. was indexed at 1 for each trace. The numbers A-D refer to the letters used in Figure 4.12 and Figure 4.13. s.e.m.: standard error of the mean, n: number of time periods.

4.4 Discussion

The different results of the two methods, calcein efflux and patch clamp, are remarkable. Apparently, in the patch clamp experiments better conditions for activation of the channel are created. There are some significant differences between the two techniques. Using patch clamp only a small membrane part is examined, in contrast with the calcein efflux experiments where whole liposomes are studied. Secondly, an additional pressure can be applied during the patch clamp experiments. Third, there is a difference in the nature of the solutes that pass the channel, namely, ions of inorganic salts for the patch clamp experiments and a charged organic molecule for the calcein efflux experiments.
In patch clamp experiments, MscL channels can be activated by only increasing the pressure (Figure 4.2). In a typical patch clamp experiment the activation of the MscL channels starts at 30-50 mm Hg and full openings are recorded at ca. 100 mm Hg. The values depend on the lipid compositions, type of channel and the shape and position of the patch in the pipet. In the present experiments only a pressure of 10 mm Hg was applied, well below the pressures needed for activation. The data indicate that the isomerisation of the azobenzene groups of DT azo-5P induced a change in the membrane large enough to reach activation of the channels.

In the literature also activation of channels has been reported without applying pressure. Asymmetric addition of micelle-forming amphiphiles e.g. LPC (Figure 4.4) induced opening of MscL after incubation for ca. 10 min. It was proposed that the induced asymmetry in the lateral pressure profile initiated the opening. Upon addition of LPC, a decrease in the lateral pressure in the hydrophobic part of the membrane was suggested. This finding is strengthened by the fact that a shift of the open probability curve to higher pressure was observed upon increasing the concentration of DOPE in the membrane. (Figure 4.10) is a nonbilayer-forming lipid with the shape of a inverted cone. These literature results appear to be in contrast with the patch clamp results described in this chapter. The trans isomer of DT Azo-5P has a cylindrical shape but the cis isomer has the shape of an inverted cone. So, activation of MscL is more complicated than assumed by Perozo and Martinac. On their work some remarks can be made. Perozo carried out two types of patch clamp experiments with LPC: 1. 1.5 μM LPC and 70 mm Hg; 2. 3 μM LPC and no additional pressure. In the first experiment extra activation was observed after 25 s. but in the second experiment activation was observed after an incubation period of more than 10 min. No explanations are given for these results. Important in this discussion is the rate of binding and the flip-flop rate. A reasonable rate constant of binding for lyso phospholipids is approximately 0.2 s⁻¹. That means that within 5 s. already half of the LPC is bound to the liposomes and that also the equilibrium of partitioning is reached within seconds.

For flip-flop rate for LPC was reported to be on the order of several hours (t½>12 h.) as measured by NMR spectroscopy measurements. With the use of micropipet method, a flip-flop rate constant of 0.0019 s⁻¹. (t½= ca. 6 min.) was reported. Apparently, the rate of flip-flop depends strongly on the used technique and besides, it is known that the flip-flop rate also depends on the composition of the membrane. A more precise determination of the flip-flop rate of LPC is important for the discussion of the results of Perozo. If it is assumed that the rate is 0.0019 s⁻¹, then the flip-flop is only a matter of
minutes. This number would then contradict with the hypothesis of Perozo, who assumes slower flip-flop.

Also Martinac needed long incubation times to measure a significant increase in activity of MscL upon addition of the amphiphiles. Furthermore, both positively- and negatively-charged amphiphiles showed the same effect on the open probability curve of MscL. Martinac used an *E.coli* membrane which was negatively charged inside and outside. Due to repulsions in the head group area with the negatively charged amphiphiles, a different change in (the lateral pressure of) the membrane will be accomplished in comparison with the positively charged amphiphiles (attraction).

Returning to the results of our own patch clamp experiments, we note that because cis DT Azo-5P has the shape of an inverted cone, a shift of the open probability curve to higher pressure could have been expected. Surprisingly, a decrease was observed, but this result is in line with the conclusions of Gawrisch and Holte. Introduction of unsaturations in the alkyl tails leads to less chain order and therefore lower lateral pressure.

Despite these considerations, activation of MscL by (intrinsic) membrane curvature seems to be reasonable according to the literature results. This does not exclude the fact that other types of changes in the membrane may contribute to the activation, as discussed earlier in this chapter. Every change in the setup, which shifts the open probability curve to lower pressure, can lead to opening of the channel.

In the patch clamp experiments, the combination of a small pressure and isomerisation of the azobenzene groups from trans to cis was enough for activation. In the calcein efflux experiments the effect of the isomerisation (in combination with the addition of tetracain) was not enough to reach an activation. A reason for the small leakage of calcein (ca. 10%) could be that the channels were not equally distributed over the proteoliposomes. Experiments with the G22C mutant (and activated by labelling with MTSET) showed that ca. 90% of the proteoliposomes contained MscL, indicating a relatively even distribution of the MscL channels over the vesicles.

The terms membrane tension, curvature and lateral pressure may lead to some confusion. The terms tension and curvature, related in Laplace’s law, are being used since the first measurements were performed on MscL channels. The tension applied during the patch clamp experiments leads to a change in curvature and therefore the channel is activated. In more recent years, the concept of lateral pressure profile became fashionable. The curvature is a part of this lateral pressure profile. The lateral pressure profile concept is still not generally accepted, there are ‘believers’ and ‘non-believers’.

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4.5 Conclusions

Two methods were used to study the activity of MscL upon isomerisation of the azobenzene groups of DT Azo-5P. Calcein efflux experiments showed that only a 10% extra release was observed upon isomerisation. The patch clamp experiments revealed a four times higher open probability upon trans-cis isomerisation.

4.6 Acknowledgement

Joost Folgering is gratefully acknowledged for his contributions to this chapter.

4.7 Experimental section

Isolation of the membrane. The MscL proteins from *E.coli* and *L. lactis* contained 6 histidine moieties at the carboxyl terminus. They were amplified in *E.coli* PB104, using the pB10b expression vector. The membrane fraction was obtained via a literature procedure.

Purification of MscL. The membrane fraction (10-20 mg/ml protein) was mixed with solubilisation buffer (1:7.5 (ml/ml), 300 mM NaCl, 50 mM KPi (pH=8.0), 35 mM imidazole) and incubated for 30 min at 4°C. The mixture was centrifuged for 45 min. at 80000 rpm at 4°C. The supernatant was taken and was combined with the already washed Ni²⁺NTA resin (2 ml of resin for 4 ml of solubilised membranes) and incubated for 30 min. at 4°C with gentle agitation. The mixture was poured into an empty column. The column was washed with 4 column volumes of wash buffer (300 mM NaCl, 50 mM KPi (pH=8.0), 35 mM imidazole, 0.5% (g/ml) Triton X-100) to remove all non-histaged proteins and lipids. MscL was eluted from the column with elution buffer (first: wash buffer + 50 mM histidine, later: wash buffer + 235 mM histidine) and collected in fractions of 1 ml. Eluted samples were analyzed with SDS-PAGE followed by staining with Coomassie Blue.

The amount of purified protein was determined by the use of the Bradford reagent.

Preparation of the liposomes. Stock solutions of the amphiphiles were prepared in methanol (1-2 mg/ml). The appropriate amounts of the amphiphile stock solutions were mixed in methanol. These solutions were rotary-evaporated in tubes to yield thin lipid films, which were dried in vacuum for at least 90 min. The appropriate amount of buffer (10 mM KPi, pH=8.0) was added to the vial, and the sample was vortexed for 30 s. The solutions were extruded 11 times through 200 nm filters at room temperature if not stated otherwise.

Preparation of the detergent-destabilised liposomes. The vesicles were titrated with Triton X-100 (10% v/v). The titration was followed by
turbidity measurements with an UV-vis spectrometer at 540 nm. First the absorption increased and, when a drop in the turbidity was observed (onset of solubilisation), the titration was ended.

**Reconstitution of MscL.** The membrane reconstitution was performed as described by Knol et al.\(^4\)\(^4\) Shortly, the purified MscL channels were incubated with the detergent-destabilised liposomes for 40 min. at 4°C with gentle shaking. The ratio amphiphile/protein varied for each reconstitution (indication: 0.012 mg protein/ 1.2 mg amphiphile). To remove the detergent Biobeads were added (20 mg Biobeads/ 1.2 mg amphiphile) and the mixture was incubated at 4°C for 1 h. An extra amount of Biobeads was added (80 mg/ 1.2 mg amphiphile) and the mixture was incubated at 4°C overnight. The proteoliposomes were collected by centrifugation and diluted in a fresh buffer (10 mM KPi, pH=8.0). Calcein (80 mM or 40 mM, 2 mM EDTA, 10 mM KPi, pH=8.0)) was added at a 1:1 ratio so that the final concentration of calcein became 40 mM (or 20 mM). The sample was subjected to three freeze-thaw cycles and was stored in liquid nitrogen.

**Calcein efflux experiments.** The sample was slowly warmed up to room temperature and extruded 11 times through 400 nm filters at room temperature if not stated otherwise. The sample was placed on a sephadex column (Sephadex G-75, particle size: 40-120 µ) and the sample was eluted with a buffer containing 10 mM KPi (pH=8.0), 1 mM EDTA, 29 mM NaCl (in case of 20 mM calcein) or 58 mM NaCl (in case of 40 mM calcein). The vesicle fraction was collected and stored in the dark for further use. The fluorescence was measured as described in chapter 3.

**Calculation of the percentage dequenching.** The fluorescent value of a sample consist of the fluorescence of calcein which resides outside the vesicles (\(F_{\text{extern}}\)) and the fluorescence of calcein inside the vesicle (\(F_{\text{intern}}\)). These values can be determined by filtration over a suitable membrane (under suction). From the fluorescence value after filtration \(F_{\text{extern}}\) and before filtration after addition of Triton (\(F_{\text{Triton}}\), the percentage of \(F_{\text{extern}}\) of the total fluorescence (%\(F_{\text{Triton}}\)) can be calculated:

\[
\%F_{\text{extern}} = \frac{F_{\text{extern}}}{F_{\text{Triton}}} \cdot 100\%
\]

The percentage of dequenching is calculated with the following equation\(^25\):
Herein, $F_{\text{sample}}$ is the fluorescence of the sample and $F_{\text{Triton}}$ is the fluorescence of the sample after addition of an excess of Triton X-100, corrected for dilution.

In the first equation, the amount of calcein outside the vesicles is calculated as a ratio of the total amount of calcein. In the second equation the ratio is multiplied by the total fluorescence, the result is the fluorescence due to calcein outside the vesicle. The fluorescence of calcein originally outside is subtracted from the measured fluorescence ($F_{\text{sample}}$).

**Patch clamp experiments.**

Joost Folgering performed the patch clamp experiments and the detailed experimental details have been published.\textsuperscript{31}

### 4.8 References

PHOTOCHEMICALLY-INDUCED OPENING/CLOSING OF MSCL

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