Scientific Report

concerning the implementation of project 68/01/10/2015 in the period October-December 2015

Investigation of prokaryotic Na^+/H^+ antiporters using Solid-Supported Membrane-based electrophysiology

Introduction

The regulation of intracellular pH and of the intracellular Na^+ concentration are a requirement for the continued survival of all living organisms [1]. The systems responsible for ensuring pH and Na^+ homeostasis are membrane proteins belonging to two categories: primary active transporters (ATPases) and secondary active transporters, the Na^+/H^+ exchangers or antiporters [2]. The Na^+/H^+ antiporters, present in virtually all living organisms, have the delicate role of responding to the challenge of either high intracellular Na^+ or abnormal intracellular pH (whether high or low) [3]. Therefore, a better understanding of their transport mechanism and regulation is essential. While elucidating the inner workings of the human Na^+/H^+ antiporters is the ultimate goal, investigation of prokaryotic exchangers is also of extreme relevance and importance, as they can either serve as model systems for the eukaryotic exchangers, or they can be important for the continued survival of certain pathogens.

The present project aims to investigate the Na^+/H^+ antiporter proteins present in such a pathogenic bacterium, *Klebsiella pneumoniae*, that is one of the major causes of nosocomial (hospital-acquired) infections [4]. Four such antiporters exist in *K. pneumoniae* belonging to three classes of antiporters, NhaA, NhaB (both electrogenic) and NhaP (electroneutral): KpNhaA1, KpNhaA2, KpNhaB and KpNhaP2. The results of the project will be twofold. First, we will elucidate the transport mechanism of these proteins, compare it to that of already characterized antiporters from other organisms and establish their potential of being used as models for the study of the Na^+/H^+ antiporter family. Second, we will establish whether these antiporters can be inhibited by some common Na^+/H^+ exchange inhibitors, that might be used as possible treatment against antibiotic-resistant *K. pneumoniae* infections.

The characterization of the 4 Na^+/H^+ exchangers in *K. pneumoniae* will be performed by the use of electrophysiological and fluorimetric investigation methods. The main technique used, solid-supported membrane based (SSM) electrophysiology is highly adapted to the investigation of prokaryotic transporters [5, 6] and has previously been used by the project director, among others, in the characterization of Na^+/H^+ exchangers from other organisms [3, 7-9]. This specialized technique is available in a small number of labs worldwide, and setting up the measurement setup for use in our lab in Bucharest was one of the main objectives of this first phase of the project.

Materials and Methods

Materials

Proteoliposomes containing the NhaP Na^+/H^+ antiporter from *Pyrococcus abyssi* (PaNhaP) reconstituted into *E. coli* polar lipid extract were kindly provided by Prof. Klaus Fendler (Max Planck Institute of Biophysics, Frankfurt).

Unless otherwise stated, chemicals were produced by Sigma Aldrich (Taufkirchen, Germany). 1,2-diphytanoyl-sn-glycero-3-phosphocholine was produced by Avanti Polar Lipids (Alabaster, Alabama, USA). Ultrapure water was produced using a Millipore MiliQ water purification system (Merck Millipore, Darmstadt, Germany).

SSM-based electrophysiology

The experimental setup for SSM-based electrophysiology requires the following components [5, 6]:

- SSM sensor chips prepared by photolithographic lift-off on a borofloat glass substrate, to which a gold layer is adsorbed;
- the cuvette, where the SSM sensors are inserted, which restricts solution flow to the surface of the SSM electrode;
- a gel bridge-reference electrode assembly;
- a solution exchange system capable of ensuring rapid solution switching between a non-activating (NA) and an activating solution, this is ensured by pressurized bottles with compressed air or nitrogen at 0.55 bar; flow is controlled via 2- or 3-way valves (NResearch Inc, West Caldwell, NJ, USA);
- a current amplifier;
- a function generator;
- a manometer;
- a source of pressurized N_2 or air;
- an analog-digital interface (National Instruments USB Analog/Digital I/O, National Instruments, Austin, TX, USA) connected to a computer running SURFE^{2}R One software (Nanion Technologies, Munich, Germany).

SSM-based electrophysiological measurements were performed essentially as described previously [3]. In brief, SSM electrodes were incubated in octadecanethiol, then a hybrid bilayer was formed by the addition of 1,2-diphytanoyl-sn-glycero-3-phosphocholine solution (15 mg/mL in *n*-decane). The sensor was then placed inside the SSM cuvette and washed with NA buffer solution containing 50 mM MES, 50 mM HEPES, 50 mM Tris, 300 mM choline chloride, 5 mM $MgCl_2$ and 1 mM dithiothreitol (DTT).

Membrane parameters (conductance and capacitance) were then measured to ensure proper formation of the hybrid bilayer. Proteoliposomes containing PaNhaP were then added to the sensor at a lipid to protein ratio (LPR) of 10 and a concentration of 3.3 mg/mL lipid. Na^+-dependent transient currents were recorded by rapidly switching between the NA solution and an activating (A) solution containing 50 mM MES, 50 mM HEPES, 50 mM Tris, 250 mM choline chloride, 50 mM NaCl, 5 mM $MgCl_2$ and 1 mM dithiothreitol (DTT). The solution flow protocol was of the form NA (for 500 ms) – A (500 ms) – NA (500 ms). Transporter-dependent currents were recorded when solution was switched between NA and A solution or between A and NA solution; however, due to the the existence of a non-zero membrane potential at the second switching event, only transient currents recorded for when the solution was switched from NA to A were considered.

Results and discussion

Objective 1: Project management

The initial goal of our project was to ensure the basis of the succesful implementation of our future tasks. To this end, we first set out to plan in detail the next phase of the project. In collaboration with our German partner, Prof. Klaus Fendler from the Max Planck Institute of Biophysics in Frankfurt, we have submitted an order for DNA plasmids containing the genes for the antiporters that will be investigated: KpNhaA1, KpNhaA2, KpNhaB and KpNhaP2. The genes will be codon optimized for expression in *E. coli* in order to ensure that the maximum amount of expressed protein can be obtained. Furthermore, we have mapped the immediate steps of the project, including the cloning strategy employed for cloning the genes from the plasmid they are supplied in (pET-21d) to the plasmid that we will use (pTrcHis2-TOPO) for expression in the *E. coli* strain deficient in Na^+/H^+ antiporters, *E. coli* KNabc. This step will be followed by expressing the four antiporters, purifying them and reconstituting them according to experimental protocols that have previously been used for the purification of NhaA [10], NhaB [11] and NhaP [3] Na^+/H^+ antiporters. Also, we have optimized the experimental protocols that will be used for the characterization of these antiporters, either by SSM-based electrophysiology or fluorimetry, in order to obtain the best results with a minimum consumption of laboratory materials.

As some of the members of the team have not had previous experience in the use of SSM-based electrophysiology, we have also arranged a seminar in which the project director presented this experimental technique to the project's team. The team members had the opportunity to see the theoretical basis of the SSM electrophysiological technique as well as the way it can be applied to characterize the function of prokaryotic Na^+/H^+ exchangers. Furthermore, team members were provided with hands-on experience in the operation of the SSM setup (see Objective 2). Further training will be given to the team members in the following year, after the proteoliposomes containing the *K. pneumoniae* Na^+/H^+ antiporters will be obtained.

Objective 2. Setting up the SSM installation and preliminary measurements.

Part of the components required for building the SSM setup were kindly provided to us by Prof. Klaus Fendler (Max Planck Institute of Biophysics Frankfurt). In addition, we have employed equipment already available in our department at UMF "Carol Davila" and we have also acquired through the funding available for the project a computing system to handle the data acquision and automatic control of the solution exchange system.

Once assembled, the experimental setup was tested to see if it can be reliably used for the characterization of Na^+/H^+ antiporters in the future phases of the project. To this end, several parameters vere verified.

First, we tested whether the hybrid bilayers produced on the system of the setup-cuvette that we used were adequate for electrophysiological experiments. Two parameters were tested – the conductance (G) and the capacitance (C) of the prepared bilayer.

The conductance of the SSM was tested by applying a 100 mV potential between the measurement and reference electrodes. Due to the capacitive behavior of the SSM, this leads to the appearance of a peak current that decreases as the capacitor (the membrane) is charged and stabilizes to a constant value after a brief period of time (~ 1 s). The remaining conductance (G) of the membrane can then be directly calculated using Ohm's law (I = GV). In order for the system to be considered adequate, maximum values of conductance should reach at most 50-100 nS/cm^2 [5]. Considering the surface of the sensor where the membrane is formed is of 0.8 mm^2, this would correspond to a maximum recorded value of 0.5-0.8 nS. In practice, membranes with G > 0.5 nS are discarded and the process of building the membrane is repeated on a different sensor. Our results showed that our system reliably produced membranes with G < 0.4 nS, being therefore more than adequate for further experimental procedures. A typical recording for the measurement of the membrane conductance is presented in Figure 1A.

The formed hybrid bilayer should also display a reasonable capacitance. This can be measured by applying a triangular voltage change from -50 to +50 mV across the membrane. The capacitance of the membrane distorts this triangular voltage step to a rectangular signal, and allows calculation of the membrane capacitance by using the formula C = Q/V, where Q is the charge transferred (I·t) and V is the applied voltage. Typical values for the SSM membrane should be between 150-500 nF/cm^2, which for one SSM sensor corresponds to 1.2 – 4 nF. Membranes with lower or higher capacitance are typically discarded. We could observe that the membranes obtained using our setup were typically well within the allowed interval. A typical recording is presented in Figure 1B.

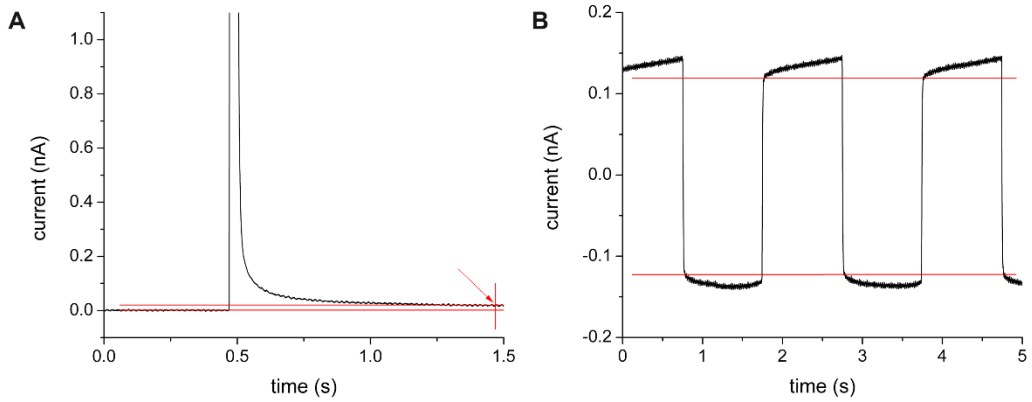

Figure 1. Measurement of the conductance (A) and capacitance (B) of the hybrid bilayer. A, Typical recording of the conductance of the hybrid bilayer. A voltage step of +100 mV was applied. The current difference between the trace and the baseline after 1 s since the application of the voltage step (red arrow) can be used to calculate the membrane conductance (see text). B, Typical recording of the capacitance of the hybrid bilayer. A triangular voltage step from -50 to +50 mV was applied. The current difference between the two horizontal red lines can be used to calculate the capacitance (see text).

Once a membrane with the adequate parameters was obtained, the response of the membrane to concentration jumps was tested. In response to the changing of the concentration of charged substrates at the surface, a transient current can typically be recorded on the "empty" membrane (without proteoliposomes added). These currents are typically much smaller than the currents recorded when proteoliposomes are added in response to transporter activation. As we plan to record signals corresponding to the activation of Na^+/H^+ antiporters by Na^+ concentration jumps, we recorded the response of the empty membrane to switching from a solution (NA) that contained 50 mM choline chloride to a solution of identical composition (A) containing 50 mM NaCl. Typically, the values recorded for such a concentration jump should not exceed 0.2-0.3 nA. We could observe (Figure 2) that, in our setup, the results fitted the expected range.

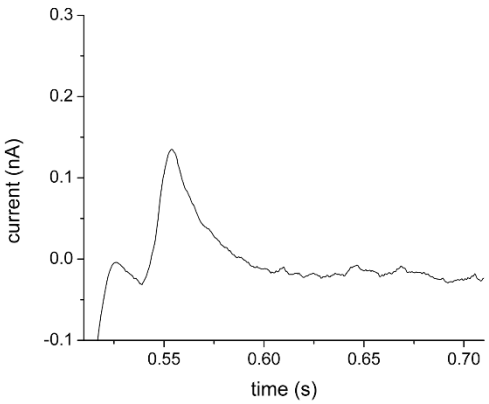

Figure 2. Measurement of a transient current resulting from a 50 mM Na^+ concentration jump on the empty SSM membrane. The solutions are switched at 0.5 s from NA (containing no Na^+) to A (containing 50 mM Na^+). The initial trace before ~ 0.55 s results from the switching of the valve. The transient current response is positive and has a pictured maximum of ~ 0.09 nA.

Finally, we tested the use of the experimental setup in the analysis of a sample Na^+/H^+ antiporter available, the NhaP transporter from *P. abyssi* (PaNhaP). Much like its homologue from *M. jannaschii* (MjNhaP1) that was previously characterized by the project director, PaNhaP is overall electroneutral, but the charge translocation of either Na^+ or H^+ ions is electrogenic and can be observed by the use of SSM-based electrophysiology. Thus, at pH 8, when PaNhaP proteoliposomes were added to the SSM, we could clearly detect a positive transient current following Na^+ concentration jumps (Figure 3, black), that was much higher in amplitude than the currents recorded on the empty membrane (Figure 2) or currents recorded when liposomes devoid of protein ("empty liposomes") were added (Figure 3, red).

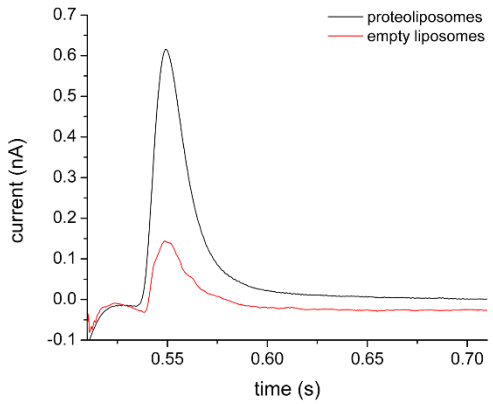

Figure 3. Measurement of a transient current resulting from a 50 mM Na^+ concentration jump on PaNhaP proteoliposomes (black trace) or empty liposomes (red trace). The solutions are switched at 0.5 s from NA (containing no Na^+) to A (containing 50 mM Na^+). The initial signal before ~ 0.55 s results from the switching of the valve. The transient current response was positive for proteoliposomes and had a maximum of ~ 0.6 nA.

Overall, the results we obtained using our SSM setup show that the setup is ready to use and works within optimal parameters. Further measurements will be performed on the *K. pneumoniae* Na^+/H^+ antiporters once they are expressed, purified and reconstituted.

Objective 3. Result dissemination.

In this initial phase of the project we have also set up the website dedicated to the project, which can be reached at the following addresses:

http://te-604.adius.ro/

or

http://biofizica-umfcd.ro/research/te-604/index.html

The website lists the abstract of the project and the project team. It will contain updated information regarding the results obtained in the course of the project (in the form of the yearly scientific reports), as well as a full list of publications and conference attendances by members of the team in the topic of the present project.

Final conclusions

In this first phase, we have established the groundwork for the successful implementation of the project, and we have fulfiled the objectives set out in the achievement plan. In the next phase, we will express the *K. pneumoniae* Na^+/H^+ exchangers in *E. coli*, test their functionality and obtain the purified and reconstituted proteins that will serve as the basis of most future experimental procedures.

References

1. Calinescu, O. and K. Fendler, *A universal mechanism for transport and regulation of CPA sodium proton exchangers.* Biol Chem, 2015. **396**(9-10): p. 1091-6.
2. Krulwich, T.A., G. Sachs, and E. Padan, *Molecular aspects of bacterial pH sensing and homeostasis.* Nat Rev Microbiol, 2011. **9**(5): p. 330-43.
3. Calinescu, O., et al., *Keeping it simple, transport mechanism and pH regulation in Na+/H+ exchangers.* J Biol Chem, 2014. **289**(19): p. 13168-76.
4. Hoppe, S., F.F. Bier, and M. von Nickisch-Rosenegk, *Identification of antigenic proteins of the nosocomial pathogen Klebsiella pneumoniae.* PLoS One, 2014. **9**(10): p. e110703.
5. Schulz, P., J.J. Garcia-Celma, and K. Fendler, *SSM-based electrophysiology.* Methods, 2008. **46**(2): p. 97-103.
6. Bazzone, A., et al., *Introduction to solid supported membrane based electrophysiology.* J Vis Exp, 2013(75): p. e50230.
7. Calinescu, O., et al., *Species differences in bacterial NhaA Na+/H+ exchangers.* FEBS Lett, 2014. **588**(17): p. 3111-6.
8. Alkoby, D., et al., *NhaA Na+/H+ antiporter mutants that hardly react to the membrane potential.* PLoS One, 2014. **9**(4): p. e93200.
9. Ganea, C. and K. Fendler, *Bacterial transporters: charge translocation and mechanism.* Biochim Biophys Acta, 2009. **1787**(6): p. 706-13.
10. Mager, T., et al., *Transport mechanism and pH regulation of the Na+/H+ antiporter NhaA from Escherichia coli: an electrophysiological study.* J Biol Chem, 2011. **286**(26): p. 23570-81.
11. Pinner, E., E. Padan, and S. Schuldiner, *Kinetic properties of NhaB, a Na+/H+ antiporter from Escherichia coli.* J Biol Chem, 1994. **269**(42): p. 26274-9.

Project director,

Dr. Octavian Călinescu