SSM-based electrophysiology

Patrick Schulz, Juan J. Garcia-Celma, Klaus Fendler *

Department of Biophysical Chemistry, Max Planck Institute of Biophysics, Max von LAue Str. 3, D-60438 Frankfurt/Main, Germany

Abstract

An assay technique for the electrical characterization of electrogenic transport proteins on solid supported membranes is presented. Membrane vesicles, proteoliposomes or membrane fragments containing the transporter are adsorbed to the solid supported membrane and are activated by providing a substrate or a ligand via a rapid solution exchange. This technique opens up new possibilities where conventional electrophysiology fails like transporters or ion channels from bacteria and from intracellular compartments. Its rugged design and potential for automation make it suitable for drug screening.

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1. Introduction

Electrophysiology has evolved in the past from the simple detection of neural activity of excitable tissues to a powerful instrument for the investigation of transport processes on the molecular level. This was possible by the continuous improvement of the technology and the introduction of revolutionary concepts like the patch clamp technique. All these methods of "standard" electrophysiology have in common, that their principle tool is the electrolyte filled micro-pipette electrode. In this review, we introduce a technique that detects charge translocation via a solid supported membrane electrode, which for a number of applications offers an advantage over the standard electrophysiological approach.

Electrophysiological measurements based on solid supported membranes (SSM)1 have been used for the functional characterization of ion pumps and transporters. In this technique, proteoliposomes, membrane vesicles, or membrane fragments are adsorbed to an SSM and are activated using a rapid substrate concentration jump. Then charge translocation is measured via capacitive coupling of the supporting membrane. This method has the advantage of providing an aqueous environment on both sides of the membrane for the incorporated transport proteins.2 In addition, adsorption of proteoliposomes or membrane fragments allows a large number of transporters to be immobilized on the electrode in a simple spontaneous process. In contrast, direct incorporation of the proteins into the planar membrane is not very effective and requires complicated reconstitution procedures (compare e.g. [1]).

SSM-based electrophysiology is extremely useful in cases where conventional electrophysiology cannot be applied. Apart from a few rare exceptions bacterial transporters cannot be investigated using voltage clamp or patch clamp methods because of the small size of bacteria and because they are difficult to express in mammalian cells or oocytes. On the other hand, SSM-based electrophysiology was very successful in this field [2–6]. But also physiologically relevant mammalian transporters could be investigated [7,8]. In this case SSM-based electrophysiology is attractive for transporters from intracellular membranes and for screening applications in drug discovery because of its robustness and its potential for automation [9,10]. Furthermore, SSM-based electrophysiology can be employed for the investigation of ion channels [11].

Using standard electrophysiology, also time resolved characterization of transporters is challenging. Since the turnover of trans-
porters is low (<100 s⁻¹ in most cases) ~10^4–10^5 transporters are required to generate a measurable signal. This can only be obtained in a ‘giant patch’ or a ‘whole cell’ configuration which have an inherently low time resolution in a solution exchange experiment. The complication can be overcome using photolytic substrate release from protected (“caged”) substrates (see e.g. [12–14]). However, only a limited number of substrates are available in caged form. Here the rapid solution exchange at the SSM offers the unique opportunity to perform electrophysiological studies with a high time resolution using arbitrary substrates. Recently, it has been demonstrated that transporter currents can be measured with a time resolution of <4.5 ms [15].

2. Description of method

2.1. The instrument for SSM-based electrophysiology

2.1.1. SSM cuvette

The cuvette is made from Plexiglas (Fig. 1). After mounting of the sensor chip with the gold electrode, the cuvette base plate and the cuvette body are screwed together. Inlet and outlet bores have a diameter of 1 mm. The cuvette volume is cylindrical and 0.75 mm deep with an internal volume of 17 µl (net volume with O-ring mounted). When mounted, the circular active area of the electrode (diameter 1 mm) which is covered by the SSM is centered under the inlet bore. In the outlet pathway the reference electrode assembly is placed. The reference electrode is a Ag/AgCl electrode which is separated from the main fluid pathway by an acrylamide salt bridge saturated with 100 mM KCl. The gold electrode is connected to the current amplifier (Stanford Research, amplification 10^9 V/A), the reference electrode to the function generator or to ground (Fig. 2). For details of the set-up see also [3,7].

2.1.2. SSM set-up and flow protocol

The cuvette and the entire fluid pathway including solution containers and valves are mounted in a faraday cage (Figs. 2 and 3). The solution containers (A, NA, R in Figs. 2 and 3) are standard 100 ml polyethylene bottles which are pressurized with compressed nitrogen gas. Pressures of 0.2–1.0 bar were used in the experiments. The valves are switched using home made valve drivers which are controlled via computer. Solution flow is controlled via the valves V1 (NR225T011), V2 (NR360T331) and V3 (NR225T031, all valves from NResearch, West Caldwell, USA). For fast switching the valves are driven with a voltage of 17 V, which is slightly larger than the specified value (12 V). Also the current is recorded by computer. For both purposes an AD/DIO card PCI 6023E (National Instruments, Austin, TX, USA) and SURFE2R software (IonGate Biosciences, Frankfurt/Main, Germany) is employed.

Two different solution exchange protocols are generally employed for the experiments: a single and a double solution exchange protocol. In the single solution exchange protocol (Fig. 2A) two different solutions are sequentially conducted through the cuvette: the non-activating solution (NA) followed by the activating solution (A) containing the substrate of the transporter immobilized on the SSM. This produces a substrate concentration jump at the SSM initiating transport and concomitant charge displacement. Finally the activating solution is again replaced by non-activating solution, so that before and in between experiments, the SSM is incubated with non-activating solution. Note that V2 is a mechanically coupled 3 × 3-way valve. This design ensures that during the entire experiment both, activating and non-activating solutions are in motion. Electrical signals are observed at the concentration jumps taking place at the beginning (on-signal) and at the end of phase A (off-signal) (Fig. 4). Usually, only the on-signal (exchange from non-activating to activating solution) is used for analysis.

The double solution exchange experiment (Fig. 2B) requires an additional resting solution, which is conducted through the cuvette after the NA/A/NA solution exchange. Thus, in between experiments the SSM is incubated in resting solution allowing the establishment and/or maintenance of ion gradients. For example, for a ligand gated ion channel, the resting solution is free of permeant ions while the non-activating and the activating solutions contain the permeant ions. In addition, the activating solution contains the ligand. Therefore, in the first concentration jump, resting versus non-activating solution, a concentration gradient of the permeant ion is established. Subsequently, upon non-activating versus activating solution exchange, transport is activated by the ligand concentration jump. Note that in this solution exchange protocol the SSM is incubated before and in between experiments with resting solution to remove the permeant ion from the internal volume of the proteoliposomes/membrane vesicles.

2.1.3. Commercial instruments

In the meantime commercial instruments for SSM-base electrophysiology are available which are adapted to the requirements of industrial drug screening. Initial developments focused on a single-channel, semi-automated analysis system (SURFE2R One, IonGate Biosciences, Frankfurt, Germany) with a closed flow cell comparable to the laboratory set-up outlined above. A critical evaluation of
the application of this instrument for drug screening can be found in Ref. [10]. The demand for a higher throughput and lower reagent consumption triggered the development of a product family of fully automated SURFE2R Workstations. Unlike the semi-automated system, the Workstations operate in an open configuration with 96-sensor plates in a standard microtiter plate format (Fig. 5). A movable fluidic unit, the IonJet, collects solutions for each measurement and injects them on the sensor surface in individual wells of a 96-well plate, thus generating a rapid solution exchange. The SURFE2R Workstation 500 performs 400–600 measurements per day. Further parallelization of the technology has been achieved recently (eight parallel channels, SURFE2R Workstation 5000) and provides a significantly higher throughput.

2.2. Materials

2.2.1. Lipid solution for formation of the SSM

375 μl of a stock solution of diphytanoyl-phosphatidylcholine (20 mg/ml in chloroform) together with 25 μl of octadecylamine (5 mg/ml in chloroform) is added to a small vial and the solvent is removed by a gentle stream of nitrogen gas. Then 500 μl of n-decane are added yielding a lipid solution of 15 mg/ml and 1:60 (w/w) octadecylamine.

2.2.2. Sensor chips

The sensor chips are prepared by a photolithographic lift-off process on a 4 inch 1 mm thick borofloat glass substrate (Fraunhofer Institut für Schicht und Oberflächentechnik, Braunschweig, Germany). The 100 nm thick gold structure on a 10 nm adhesive layer consists of a 1 mm diameter circular active area which is connected to a contact pad via a thin contact strip (Fig. 1). After cutting, the glass chips (9/22 mm) carrying the electrode are incubated for 6 h in an ethanolic solution of 1 mM octadecanethiol. For long time storage the sensor chips are kept in nitrogen atmosphere and shielded from light.

2.2.3. Protein preparations

A number of different electrogenic proteins have been tested on the SSM. These proteins come in many different preparations: purified and reconstituted into proteoliposomes, in membrane vesicles from native tissue, in enriched membrane fragments from native tissue, recombinantly expressed in bacteria and mammalian cell lines. Table 1 summarizes the various preparations and proteins. A short compendium of the most common preparations is given below.

2.2.4. Membrane fragments and membrane vesicles

Recombinantly expressed protein can be studied in bacteria and mammalian cell lines. Protein expressing bacterial strains are grown to an appropriate OD600 (e.g. 1.0), collected by centrifugation and lysed by three passages through a French press in a DNAase and protease-inhibitor containing buffer (see e.g. [16]). After ultracentrifugation the resulting pellet contains the membrane fraction with the target protein, which can be directly adsorbed to the SSM. Membranes are frozen and stored at −80 °C. Mammalian cells are mostly grown adherently, collected by trypsinization.
2.2.5. Purified and reconstituted proteins

His-tagged proteins are purified from membrane vesicles by Ni²⁺–NTA affinity chromatography as described elsewhere [3,5,6,17]. The reconstitution process is performed in three parts: liposomes are formed by extrusion from different lipids (we use routinely total or polar Escherichia coli lipid extract) with a final concentration of 10 mg/mL of lipid in the reconstitution buffer. Detergent is added in order to destabilize or dissolve the liposomes. Solubilized protein is added in a defined mass ratio to the lipid content and incubated for at least 15 min. Removal of the detergent is accomplished by the addition of polystyrene beads (Bio-Beads, Bio-Rad, München, Germany). The amounts and intervals of addition of the bio-beads are adjusted for every different protein. Typically, incubation overnight and exchange of the bio-beads afterwards is required to achieve a complete removal of the detergent (for reconstitution procedures see e.g. [5,6,16,17]).

2.3. Measurement procedure

2.3.1. Mounting of the sensor chip

Before use, the stored sensor chips are regenerated by incubation for 1 h in a 1 mM octadecanethiol solution in ethanol. The lipid monolayer is deposited using a painting technique where a small amount (2 µl) of the lipid solution is applied to the electrode for the formation of the SSM. Then the sensor chip is mounted into the cuvette. The monolayer forms spontaneously when the electrode is rinsed with buffer in the cuvette. The planar membrane has an area of ~0.8 mm².

The experiments were carried out at room temperature (22 °C). After the formation of the SSM its capacitance and conductance were determined until they became constant after a waiting time of ~90 min. Typical values were 300–500 nF/cm² for the capacitance and 50–100 nS/cm² for the conductance.

2.3.2. Addition of the protein sample

A proteoliposome suspension suitable for the SSM measurement has a typical lipid concentration of ~10 mg/mL and a lipid to protein ratio of ~5–10 (w/w). Membranes from native tissue or from bacterial or mammalian cells were prepared at a total protein concentration of ~2–8 mg/mL. Care was taken that the buffer osmolarity in the preparations was comparable to that of the measurement buffers. The samples were stored in liquid nitrogen or in a freezer at ~80 °C. Before use, the proteoliposomes were thawed and briefly sonicated in a bath (three sonication cycles of 10 s with 20 s cooling intervals on ice in between). The natural membranes were diluted after thawing to a final protein concentration of 1–2 mg/ml and sonicated using a tip sonicator (UP50H, 50 W, 30 kHz, 1 mm sonotrode diameter, intensity 20%, cycle 0.5, Dr. Hielscher, Teltow, Germany). Then, 30 µl of the suspension was applied to the SSM using a standard pipette via the exit bore of the cuvette with the reference electrode assembly removed. The membranes were allowed to adsorb to the SSM for 1 h. Finally, the SSM was rinsed with non-activating buffer and was ready for the measurement.

2.3.3. Signal recording

Signals are recorded using a specific solution exchange protocol as discussed above. The solution flow rate is controlled by adjusting the pressure on the solution containers. Typically the flow rate was 0.5–2 ml/s. The solution exchange protocols for a representative single and double exchange experiment and the resulting transient currents are shown in Fig. 4. In Fig. 4A a transient current generated by the NhaA Na⁺/H⁺-exchanger from Helicobacter pylori is shown. Transport is initiated by addition of 100 mM Na⁺ in the activating solution (A). This protein transports 2 H⁺ for 1 Na⁺.

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and centrifugation and subsequently disrupted in a Parr Bomb (Parr Instruments Deutschland GmbH, Frankfurt/Main, Germany). The membrane fraction is collected by ultracentrifugation and the plasma membranes are isolated by density gradient centrifugation (sucrose-gradient: 9–45%, 3 h/100,000 g). For long term storage, membranes can be flash frozen in resting buffer with 10% glycerol and stored at −80 °C for months. These membranes contain membrane fragments and/or membrane vesicles with in general unknown orientation (see Section 2.4.4).

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behavior of the valves is not well defined and because the proteoliposome is charged when removal of the substrate occurs.

A double exchange experiment is shown in Fig. 4B. Typically, double exchange experiments are applied when ion gradients are required and transport is initiated by addition of a second substrate or a ligand. The example shown in the figure is the nicotinic acetylcholine receptor (nAChR) from Torpedo marmorata electric organ. Here first a Na⁺ gradient is established and then transport is initiated by addition of the ligand carbamylcholine. The Na⁺ gradient is established when the Na⁺ free resting solution (R), which is in contact with the SSM before the experiment, is replaced by the Na⁺ containing non-activating solution (NA). The solution exchange produces the biphasic artifact at t ~ 0.5 s. Then the ligand carbamylcholine is added and channel opening allows the inflow of cations into the membrane vesicles creating a transient current (indicated by the arrow). This transient current shows an EC₅₀ for carbamylcholine of 13 μM and is inhibited by the acetylcholine receptor specific antagonist α-bungarotoxin with an IC₅₀ of ~1 nM [11].

### 2.4.1. The compound membrane and capacitive coupling

The adsorbed membrane fragments, membrane vesicles or proteoliposomes and the underlying planar membrane form a compound membrane. This system and the respective current detection principle (called capacitive coupling) merits some special consideration.

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**Table 1**

<table>
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<tr>
<th>Protein Preparations Measured on the SSM</th>
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<tr>
<td><strong>Protein</strong></td>
</tr>
<tr>
<td>Ca⁺-ATPase rabbit</td>
</tr>
<tr>
<td>Nicotinic acetylcholine receptor Torpedo californica</td>
</tr>
<tr>
<td>MelB melibiose permease Escherichia coli</td>
</tr>
<tr>
<td>Glt aspartate transporter* Pyrococcus horikoshii</td>
</tr>
<tr>
<td>mEAAC1 glutamate transporter mouse</td>
</tr>
<tr>
<td>hNCX1 Ca⁺/H⁺ exchanger Homo sapiens</td>
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* This putative glutamate transporter [6] has been later identified as an aspartate transporter with low glutamate affinity [31].

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The electrical behavior of the compound membrane can be described and analyzed by an equivalent circuit (Fig. 6) [18,19]. Note that the equivalent circuit and consequently the electrical behavior of the system is essentially the same whether membrane fragments or membrane vesicles or liposomes are used (see Fig. 6). The transporter incorporated in the adsorbed membrane is represented by a current source in the equivalent circuit. It is clear from the equivalent circuit, that the charge displacement in the transport protein is transmitted to the measuring circuit via the capacitance of the planar membrane. The equivalent circuit is characterized by the so called system time constant which is determined by the components of the circuit:

\[ \tau_0 = \frac{C_m + C_p}{C_m + G_p} \]

In general, the conductance of the SSM \( G_m \) can be neglected and \( G_p \) represents the leak conductance of the liposome or the adsorbed membrane fragment. Values for \( \tau_0 \) determined on black lipid membranes and on SSMS are approximately 100–300 ms [7,18,20].

A qualitative understanding of the electrical signals measured by capacitive coupling may be obtained from Fig. 6C showing the pump current \( I_p(t) \) generated by the transporter and the current \( I(t) \) measured on the SSM. The two limiting cases are displayed in the figure.

(1) The pump current is a transient current with a time constant much smaller than the system time constant. In this case an additional negative component of time constant \( \tau_0 \) appears in the measured current but the time constant of \( I_p(t) \) is preserved and the measured amplitude is approximately proportional to that of \( I_p(t) \) [19]. (2) The pump current is a stationary current. In this case the measured current is a transient decaying with a time constant \( \tau \) which is smaller than \( \tau_0 \) and depends on the stationary current [18]. Here the amplitude of \( I(t) \) is proportional to the stationary current \( I_p \). Both cases were indeed experimentally observed: for example a rapid charge displacement generated by the NaK-ATPase [7] and stationary turnover of the NhaA Na+/H+-exchanger from E. coli [5]. But also the mixed case was found for the melibiose permease, MelB, from E. coli [21], where a rapid electrogenic conformational transition is followed by stationary transport.

In conclusion, rapid pre-steady-state charge displacements as well as stationary pump currents may be reliably monitored using the capacitively coupled system of the SSM if the limitations and specific properties of the equivalent circuit are kept in mind. A complete reconstruction of the pump current may be obtained by numerical processing the measured current on the basis of circuit analysis [19,20,22].

### 2.4.2. Charging of the vesicles

In general, the peak value of the measured transient current is used to quantify the transport activity of the investigated protein. Ideally, this current should be measured under short circuit conditions. This is not necessarily the case in the capacitively coupled system because the transport activity of the protein leads to charging of the membrane. However, the peak current is an initial current amplitude recorded just when the substrate reaches the binding site and transport starts. At this point the membrane has not yet build up a significant voltage to affect the kinetic properties of the enzyme. Experimental support for this conclusion comes from a comparison of proteoliposomes with a lipid to protein ratio of 500 and 10. Even at extremely high pump densities the kinetic properties of the transporter were found to be unaltered [5]. Therefore, the peak current recorded on the SSM is a reliable measure for the short circuit kinetic properties of the transport protein.

### 2.4.3. Time resolution

The time resolution of the SSM system is limited by the solution exchange at the surface of the SSM. Because of unstirred layers a fast solution exchange is difficult. To measure the rise time of the substrate concentration advantage is taken of the strong interaction of ions with the lipid headgroups of the SSM [23]. The displaced charge can be used as a relative measure of the time dependence of the local ion concentration at the surface of the SSM [23]. Here we have determined the concentration risetime for the configuration shown in Fig. 2A by performing a 100 mM NaCl/NaClO4 solution exchange at a flow rate of 1.6 ml/s (Fig. 7). The generated transient current is numerically integrated and the displaced charge is fitted using the empirical function shown in the figure. In this configuration, the concentration rise time is 16 ms. Using a modified flow configuration and a higher flow rate rise times as low as 2 ms may be obtained [15].

### 2.4.4. Orientation

An important parameter for the interpretation of the measured transport activities is the orientation of the protein in the adsorbed membrane vesicles, membrane fragments or proteoliposomes. In proteoliposomes the protein may be incorporated at random. But also native membranes may be randomly adsorbed to the surface of the SSM. If both populations of transporters are then analyzed on the SSM, the kinetic properties determined in the experiments represent an average of the characteristics of both transport direc-
Oriented incorporation of proteins in proteoliposomes can be achieved with special reconstitution procedures because some proteins seem to have a natural tendency for unidirectional incorporation. Interestingly, right-side-out [5] as well as inside-out proteins seem to have a natural tendency for unidirectional incorporation. But also natural membranes can be prepared to yield preferentially inside-out or right-side-out vesicles [26]. For some transport proteins side selective activation is possible. For example cytochrome oxidase is reduced by electron donors only from its extracellular side [27] and NaK-ATPase binds ATP only at its intracellular side [28]. Therefore, even if randomly incorporated in proteoliposomes or randomly adsorbed to the SSM, only one population of enzymes is active in these preparations and can be selectively analyzed.

3. Concluding remarks

SSM-based electrophysiology has been proven to be a valuable complement to the electrophysiological toolbox. In contrast to conventional electrophysiology, the SSM technique avoids complications involved with the interaction of the transport protein with intracellular components or, in the case of purified preparations, other membrane proteins. Its particular value becomes apparent when membrane proteins from bacteria and intracellular compartments, like the sarcoplasmic reticulum or vesicles from parietal cells, have to be studied. Because of their small size, these structures are in general not accessible for standard electrophysiology, like the two electrode voltage clamp or patch clamp techniques.

The compound membrane is a well suited substrate for the investigated transport protein providing an aqueous space for the protein on both sides of the membrane. A further advantage of adsorbing membrane fragments or proteoliposomes to a planar membrane is the much simpler and more effective procedure compared to direct incorporation of the protein, leading to a superior signal to noise ratio and time resolution of the electrical measurement.

SSM-based electrophysiology has been employed for a direct measurement of charge movements of a variety of electrogenic transporters. Using the high time resolution of the technique rate constants of transport steps can be determined and electrogenic partial reactions can be identified. Besides its use in basic research, the rugged SSM sensor combined with robotized instrumentation contains potential for industrial applications. This is expected to become a promising platform technology for drug screening and development [9,10].

Acknowledgments

We thank Lina Hatahet for excellent technical assistance and the mechanical workshop of the MPI für Biophysik for the construction of the cuvette and the faraday cage. Jürgen Reichtert and Robin Krause are acknowledged for contributing to Figs. 1 and 6 and Carola Hunte for providing the Helicobacter pylori NhaA protein sample.

Appendix A. Supplementary data


References


Fig. 7. Normalized integrated current = translocated charge obtained by a NaCl/NaClO4 concentration jump at a flow rate of 1.6 ml/s. The charge is fitted using the equation given in the figure and the concentration rise time t is determined.