Reconstitution of Ryanodine Receptor/Ca^{2+} Release Channels in S-layer Supported Lipid Membranes

Vanessa-Dominique Larisch, Angelika Schrems,
Uwe B. Sleytr, Bernhard Schuster
Department of NanoBiotechnology
University of Natural Resources and Life Sciences, Vienna
Vienna, Austria
{bernhard.schuster; uwe.sleytr}@boku.ac.at

Martin Hohenegger
Institute of Pharmacology
Medical University Vienna
Vienna, Austria
martin.hohenegger@meduniwien.ac.at

***Abstract*—A great challenge nowadays is the systematic study on medicinal relevant functional (trans)membrane proteins reconstituted in tunable biomimetic model lipid membranes. Ryanodine receptor/Ca^{2+} release channels (RyRs), integral membrane proteins present in various mammalian tissues, are of great importance for muscle contraction. For the present study, the RyR isoform 1 (RyR1) was isolated from the skeletal muscle of rabbits. Here, we show the reconstitution of RyR1 into an artificial model lipid system, the so-called S-layer supported lipid membrane (SsLM). Bacterial crystalline surface layer (S-layer) proteins render the possibility to generate solid supported membranes of high longevity on various supports in order to enable investigations by a great variety of surface-sensitive techniques. According to preliminary results, RyR1 was successfully reconstituted into the SsLM as proven by quartz crystal microbalance with dissipation monitoring. Moreover, control experiments revealed that RyR1 did not stick to an S-layer lattice without an attached lipid membrane. Hence, evidence is provided that the SsLM as multifunctional interface might constitute a pharmaceutically interesting platform to investigate, e.g., the effect of a broad range of drugs on reconstituted RyR1 or other membrane proteins in general.**

Keywords-biomimetics; supported lipid membrane; S-layer protein; functional reconstitution; ryanodine receptor/Ca^{2+} release channel.

I. INTRODUCTION

Membrane proteins are essential for life and offer various functions by serving as key components in inter- and intracellular communication. Therefore, they represent very attractive drug targets, but at the same time, studying membrane proteins and their pharmacological applications is a highly challenging task [1]. In an effort to develop synthetic membrane mimics of the natural cell membrane in both, structure and function, fabrication of a supported lipid bilayer on a solid substrate is receiving growing attention [2]. Solid supported lipid membranes are a well-known class of model membranes, extremely useful for studying biophysical and biochemical properties of biological membranes, their constituted lipids and in particular (trans)membrane proteins [2]-[4]. In order to design a synthetic lipid membrane that overcomes the challenges facing membrane-spanning protein reconstitution, a strategy based on surface (S-) layer proteins acting as biocompatible cushions separating the lipid bilayer from the solid support has been developed (Figure 1) [3][4]. The result of this approach based on the building principle of archaeal cell envelope structures are the so-called S-layer supported lipid membranes (SsLMs). The latter provide many advantages, e.g. reduces the non-specific binding of proteins to solid substrate, increase lipid membrane stability and longevity, provides a lubricant layer allowing the lipid bilayers to remain mobile, allows insertion of bulky transmembrane proteins into the lipid bilayer, and may assist self-healing of local defects in lipid bilayers deposited on large supports [3][4].

S-layer lattices are two-dimensional arrays of proteinaceous subunits which constitute the common surface structure of almost all archaea and most bacteria [3][5]. Bacterial S-layers have the unique property that, following disruption by chemical agents from cells, monomers of the protein can reassemble to their original lattice structure in suspension, on solid surfaces, at air–water interfaces, and on phospholipid mono- and bilayers. Most bacterial S-layer lattices are composed of a single protein or glycoprotein species having a center-to-center spacing of approximately 5–30 nm, are 5 to 10 nm thick and show a smooth outer and a more corrugated inner surface (Figure 1). The lattices with a surface porosity ranging between 30–70% are composed of identical pores with a diameter of 2 to 8 nm [3]-[5].

One of the major advantages of planar supported lipid bilayer models is the wide range of surface-sensitive techniques, which can be applied to study the overall membrane characteristics and its constituents [2]. Quartz crystal microbalance with dissipation monitoring (QCM-D)

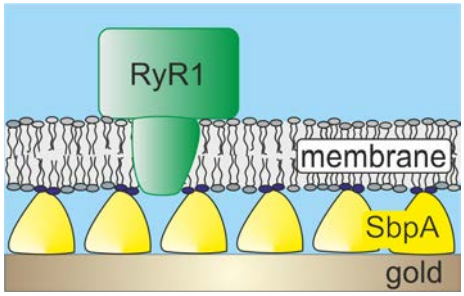

Figure 1. Schematic drawing of a ryanodine receptor/Ca2+ release channel (RyR1) incorporated in an S-layer (SbpA) supported lipid membrane.

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is one of these techniques, which allow real time quantitative measurements of the shift in frequency, which corresponds directly to the mass of bound material on the sensor surface including the associated liquid medium. Moreover, also the energy loss (dissipation) gives information related to changes of the viscoelastic properties of the deposited layer [6].

The present study is motivated by the high demand of studying responsive membrane proteins in a biomimetic environment. This intention is of paramount importance as the results of genome mapping showed that approximately one-third of all genes of an organism encode for membrane proteins, which are key factors in cell's metabolism and thus, in health and disease. Moreover, membrane proteins constitute preferred targets for pharmaceuticals (at present more than 60% of all consumed drugs) [1].

In the present study, RyR1 has been chosen to be reconstituted in SsLMs (Figure 1) since RyR1 is related to several severe human diseases, such as malignant hyperthermia, myocardial ischemia and central core disease [7][8]. The typical activator of the receptor is ryanodine, a plant alkaloid isolated from *Ryania speciosa.* It was soon discovered that ryanodine can cause muscles to contract or to reduce contractile force [8]. Experiments revealed that RyR1 activation can be reversed to inhibition of Ca^{2+} release after prolonged incubation with ryanodine in the micro-molar range. In general, RyRs are modulated by endogenous as well as pharmacological effectors. Ca^{2+} represents the most important endogenous factor, whereby its release from the sarcoplasmic reticulum (SR) is thought to be triggered by an action potential. As a result, the intracellular concentration of Ca^{2+} is increasing, which leads to the opening of other ion channels that are regulated by RyR1 and the subsequent release of Ca^{2+} from the SR [7].

To sum up, planar model lipid membranes, in particular SsLMs functionalized with pharmacologically relevant membrane proteins like the RyR1 may receive widespread recognition in drug discovery and protein–ligand screening. In future, the increased knowledge on membrane proteins will allow rebuilding sensory organs, e.g., an artificial nose, and are of high interest for the development of biosensors based on the function of membrane proteins [1]-[5].

In the following, Section II describes the S-layer protein isolation and modification, lipid membrane formation on the S-layer lattice, isolation of RyR1 and QCM-D measurements to provide evidence for the SsLM formation and RyR1 incorporation. Section III A deals with the assembly of the SsLM, whereas Sections III B and III C report on the reconstitution of RyR1 in the SsLM out from proteo-liposomes with and without CHAPS, respectively. Section III D shows that RyR1 cannot be attached to a plain S-layer lattice without any lipid membrane. Finally, Section IV gives a conclusion and outlook on the planned future work.

II. METHODS

Isolation of S-layer proteins The bacterial S-layer protein SbpA was isolated from *Lysinibacillus sphaericus* CCM 2177 as previously described [9]. The protein solution (0.1 mg/mL in recrystallization buffer: 0.5 mM Tris-HCl, 10 mM $CaCl_2$, pH 9) was self-assembled on gold substrates for 3 hours. A subsequent rinsing step with water was applied in order to remove excess protein.

Chemical activation of SbpA The S-layer protein was modified with 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide hydrochloride (EDC, Sigma-Aldrich; 15 mg/mL, pH 4.5). Subsequently, vesicles containing 1,2-dimyristoyl-*sn*-glycero-3-phospho-ethanolamine (DMPE) were bound via EDC coupling onto the activated SbpA lattice [10].

S-layer supported lipid membrane formation Phosphatidylcholine isolated from egg yolk (Egg PC) and DMPE were purchased from Sigma-Aldrich. The β-diketone ligand has been synthesized as described elsewhere [10]. Phospholipids with 1% β-diketone ligand were dissolved in chloroform and dried under vacuum for at least 3 hours at 45°C. The lipid film was subsequently rehydrated and extruded 21 times through a polycarbonate membrane (Whatman, UK) by using a Mini Extruder (Avanti Polar Lipid Inc, USA) to form a suspension of large unilamellar vesicles (LUVs). Before use, LUVs were diluted in sucrose/ glucose (1/2) to give a final lipid concentration of 1 mg/mL.

Isolation of the ryanodine receptor/Ca^{2+} release channel RyR1 was isolated from heavy SR of rabbit skeletal muscle as described elsewhere [11]. RyR1 was provided in unilamellar proteoliposomes composed of α-phosphatidyl-choline and approx. 0.1% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate hydrate (CHAPS). The stock RyR1 concentration was 1.38 mg/mL, which was diluted 1:50, 1:100 and 1:500 as indicated in each experiment.

Quartz crystal microbalance with dissipation monitoring (QCM-D) The QCM-D E4 device (Q-Sense AB, Sweden) was operated with gold-coated quartz crystal sensors (QSX 301, Q-Sense AB) to measure the shift in frequency, Δf, and dissipation, ΔD, as a function of time [6]. QCM-D experiments were measured at 25 ± 0.02°C. All given data were measured at the 5th overtone. The flow rate was 25 µL/min for all fluid injections and rinsing steps.

III. RESULTS AND DISCUSSION

A. Assembly of S-layer supported lipid membranes

QCM-D was not only applied to follow SsLM formation, (i.e., recrystallization of S-layer proteins on gold sensor and successive formation of a lipid bilayer by binding and subsequent fusion of LUVs) but was also used to investigate the incorporation features of RyR1. Representative measurements of Δf and ΔD in the course of the formation of the SsLM and interaction of the latter with RyR1 as a function of time are shown in Figure 2. In a first step, the assembly of S-layer proteins from solution on gold sensors was determined and showed a rapid decrease of Δf to ~-75 Hz and a slight increase of ΔD ($<1\times10^{-6}$) (Figure 2; Table 1). Both values recorded by QCM-D for S-layer proteins recrystallization on the gold sensor are in good agreement with previously published work [2][10][12].

Two obstacles face bilayer formation onto S-layer protein lattices; vesicle attachment and successive spontaneous vesicle fusion. The S-layer lattice has a so-called antifouling characteristic, meaning that nothing sticks to it and hence,

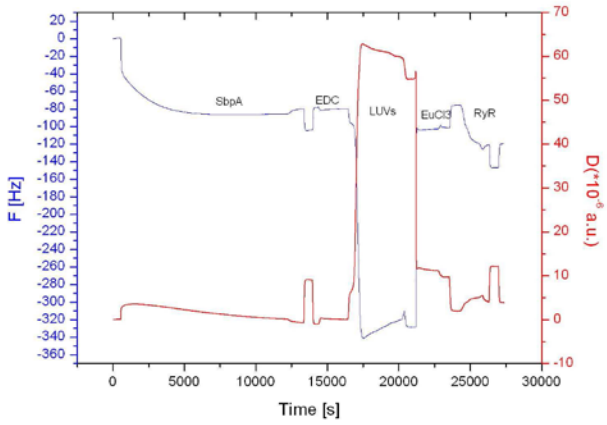

Figure 2. QCM-D measurement showing the SsLM formation process and the subsequent incorporation of RyR1 out from dialyzed proteoliposomes.

vesicles would simply roll over the proteinaceous lattice [3]. This intrinsic feature may be a biological function of the S-layer lattice covering many prokaryotic cells. Therefore, EDC was used to activate the carboxyl groups of the S-layer protein forming highly reactive *O*-acylisourea intermediates. Subsequently a solution of vesicles (LUVs) comprising of Egg PC, DMPE and β-diketone ligand was passed over the S-layer lattice [10]. This composition was chosen for several reasons: The Egg PC is the bulk component forming the spherical vesicle, DMPE as linker molecule is covalently bound with its terminal amino group to the activated S-layer protein, and the amphiphilic β-diketone ligand molecules incorporated in the LUVs master their fusion by the inter-vesicular complex formation of two β-diketone ligands with one Eu^{3+}-ion. The accompanied dramatic structural change of the lipid layer can nicely be investigated by QCM-D. Initially, the layer composed of bound vesicles gave rise to a huge decrease in Δf (~-340 Hz) and increase in ΔD (~65 × 10^{-6}), corresponding to a very soft, highly water–containing vesicular layer. After Eu^{3+}-ion triggered vesicle fusion, Δf increased instantly to ~-100 Hz and ΔD decreased to ~10×10^{-6} (Figure 2). The pronounced shift in Δf and ΔD provide clear evidence for the change of the attached lipid structure. The obtained difference in Δf between the S-layer lattice with and without an attached lipid membrane is approximately 22 Hz (Table 1). This value is in good agreement with the previously reported QCM-D response for bilayer lipid membrane formation [10][13]. In addition, the same ΔD was previously reported for SsLMs [10]. Subsequently the lipid membranes were rinsed with recrystallization buffer to remove unbound excess material.

TABLE 1: QCM-D DATA FOR SSLM FORMATION AND RYR1 RECONSTITUTION USING DIALYZED (A) AND UNDIALYZED (B) PROTEOLIPOSOMES.

	Data calculated from QCM-D measurements; n = 8		
	Frequency [Hz]	***Dissipation×10^{-6}***	***Mass [ng]***
SbpA	74.5 ± 5.4	1.01 ± 0.05	1317.7 ± 95.0
SsLM	21.8 ± 1.3	8.69 ± 0.02	385.4 ± 22.1
RyR1$^{(A)}$	33.0 ± 22.9	0.91 ± 2.71	584.3 ± 386.3
RyR1$^{(B)}$	15.6 ± 4.9	-6.07 ± 0.42	275.2 ± 86.1

B. Reconstitution of RyR1 via undialyzed proteoliposomes

Addition of RyR1 proteoliposomes (1:100 dilution; 13.8 µg/mL RyR1) causes a significant shift in Δf indicating the attachment of RyR1 to the lipid membrane. Due to the moderate shift in Δf and ΔD, an attachment of intact proteoliposomes can be excluded. The incorporation of RyR1 is evidenced by the fact that Δf does not decrease after rinsing. The difference in Δf in respect to the SsLM was determined to be ~33 Hz. This value is corresponding to a mass of 584.3 ng. Mean values of Δf, ΔD, and changes in mass of repeated measurements are summarized in Table 1. Since proteoliposomes hosting RyR1 still contained CHAPS, it is not possible to determine the most probably small influence of the detergent on the bound mass.

To investigate the influence of the RyR1 concentration on its incorporation into the SsLM, the experiment was repeated with a lower (1:500 dilution – 2.76 µg/mL) and a higher concentration (1:50 dilution – 27.6 µg/mL) of RyR1. For the 1:500 dilution Δf was determined to be -19.5 Hz. Hence, the decrease in Δf was slightly more than half of the value observed for the 1:100 dilution. Looking at the mass change, the attached mass was slightly higher (344.6 ng) than half of the mass determined for the 1:100 dilution. In contrast, Δf was -30.1 Hz for the 1:50 dilution. Although the concentration of RyR1 was the double value than for the 1:100 dilution, the decrease in Δf was approximately 3 Hz lower. The calculated mass of incorporated RyR1 was even slightly lower (532.1 ng) despite the higher concentration. However, as RyR1 is very bulky (29 nm × 29 nm per RyR1 complex [14]), there is a limitation in the number of incorporated RyR1 simply by the available membrane area, which is theoretically reached at the 1:100 dilution.

C. Reconstitution of RyR1 via dialyzed proteoliposomes

To investigate the influence of CHAPS on the incorporation of RyR1 into the SsLM, the experiment were repeated with the 1:100 dilution of dialyzed RyR1 proteoliposomes (Figure 2). The shifts in Δf after formation of an S-layer lattice and closed bilayer were equal to those observed in previous experiments. Table 1 shows that Δf as well as the calculated mass for incorporated RyR1 is much lower for dialysis treated proteoliposomes than for the previous set of experiments without dialysis (275 ng vs. 584 ng, respectively). This finding could be explained by the absence of CHAPS, which was still present in the undialyzed proteoliposomes.

Another interesting result is the decrease in dissipation: In the experiment with undialyzed RyR1, ΔD between SsLM and incorporated RyR1 was determined to $+0.91\times10^{-6}$. In contrast to this value, the difference for dialyzed RyR1 has a negative value (-6×10^{-6}; Table1). Hence, it is conceivable that the lipid membrane containing a detergent, which may induce defects in the lipid bilayer structure, behaves more

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viscoelastic than the membrane purely comprised of phospholipid molecules.

All experiments both, with dialyzed and undialyzed RyR1 proteoliposomes evidenced the incorporation of RyR1 into solid SsLMs. The use of proteoliposomes lacking CHAPS gave a much lower value for mass change indicating that the presence of CHAPS facilitate the incorporation of RyR1 via proteoliposomes into the SsLM. Another explanation for the different results could be that the protein content was higher in the undialyzed assay, thereby resulting in a higher mass attached to the sensor (Table1). Finally, one cannot exclude that a certain part of the total mass determined by QCM-D has to be referred to the detergent CHAPS.

D. Negative control

All previously performed experiments provide evidence for the incorporation of RyR1 into SsLMs. To confirm that the receptor is not attached due to unspecific adsorption to the naked SbpA lattice, control experiments without previous formation of a lipid membrane were conducted. The attachment of RyR1 (13.8 µg/mL) directly on an S-layer lattice is clearly visible by the decrease in Δf (data not shown). However, flushing of the system causes an increase in Δf until the initial value for the SbpA lattice was reached. Thus, the whole mass assigned to RyR1 attached to the S-layer lattice was washed away. This result proves that RyR1 can successfully be reconstituted into an SsLM but it does not stick to a plain S-layer lattice on a sustained basis.

IV. CONCLUSION AND FUTURE WORK

The preliminary data provide evidence, that the SsLM constitute a promising platform for studying transmembrane proteins in particular as RyR1 is a huge 2.3 MDa homo-tetrameric, membrane-protruding complex [14]. Moreover, SsLMs do not get disintegrated by the presence of detergents like CHAPS. The future work will elucidate the orientation of the reconstituted RyR1 in the SsLM by binding of, e.g., fluorescently labelled ryanodine. Also replacement of the latter by natural ryanodine will give information on the correct folding of RyR1. Electrophysiological studies on the modulation of (single) ryanodine receptor/Ca^{2+} release channel by effectors and drugs are the ultimate goal of this ongoing work. Finally, other receptor proteins, in particular the important class of G-protein coupled receptors will be functionally reconstituted and studied in the multifunctional interface of this robust platform. In this context it is interesting to note that recently a rapid technique for the sampling of membrane-associated proteins from the surface of a living cell and their subsequent deposition into SsLM has been achieved [15]. The future aims are the rebuilding of sensory organs like, e.g., artificial noses or tongues and the design of highly sensitive and specific biosensors based on arrays of responsive membrane proteins [16].

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