METHODS AND APPROACHES

Reconstitution and functional characterization of ion channels from nanodiscs in lipid bilayers

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Recent studies have shown that membrane proteins can be efficiently synthesized in vitro before spontaneously inserting into soluble nanoscale lipid bilayers called nanodiscs (NDs). In this paper, we present experimental details that allow a combination of in vitro translation of ion channels into commercially available NDs followed by their direct reconstitution from these nanobilayers into standard bilayer setups for electrophysiological characterization. We present data showing that two model K+ channels, Kcv and KcsA, as well as a recently discovered dual-topology F+ channel, Fluc, can be reliably reconstituted from different types of NDs into bilayers without contamination from the in vitro translation cocktail. The functional properties of Kcv and KcsA were characterized electrophysiologically and exhibited sensitivity to the lipid composition of the target DPhPC bilayer, suggesting that the channel proteins were fully exposed to the target membrane and were no longer surrounded by the lipid/protein scaffold. The single-channel properties of the three tested channels are compatible with studies from recordings of the same proteins in other expression systems. Altogether, the data show that synthesis of ion channels into NDs and their subsequent reconstitution into conventional bilayers provide a fast and reliable method for functional analysis of ion channels.

Introduction

Ion channels are protein tunnels, which conduct a regulated and selective transport of ions across cell membranes (Hille, 2001). Because this function is essential for many aspects of cellular life, structure/function correlates of channel proteins are intensively studied (Kurachi and North, 2004; Catterall et al., 2017). For the same reason, channels are also considered major targets of drugs (Terstappen et al., 2010; Yu et al., 2016). The planar lipid bilayer (PLB) technique is one of the many methods that are currently used for studying channel activity and their sensitivity to the lipid environment and to drugs (Zakharian, 2013). This technique has the advantage over other electrophysiological approaches in that it allows recording of channel activity on a single-molecule level under very defined conditions (e.g., phospholipid composition and electrolyte concentrations). In recent years, many attempts were made to improve the classical method of PLBs (Montal and Mueller, 1972). This resulted, among others, in a greater mechanical stability (Tien et al., 1991; Schuster et al., 1999; Khan et al., 2016), miniaturization of the recording device (Mach et al., 2008), and the ability of using asymmetrical bilayer (Syeda et al., 2008; Iwamoto and Oiki, 2015). Bilayer recordings and their variants have also been adapted for lab-on-a-chip technologies (Kongsphol et al., 2013), which further paved the way for a scaling up for medium and high throughput screening of channel drugs.

In all the major bilayer methods, the proteins of interest are either isolated directly from cells (Nelson et al., 1980; Hirano-Iwata et al., 2016) or expressed in heterologous systems (Tapper and George, 2003). The most frequently used heterologous system is Escherichia coli. In the case that a protein fails to express in bacteria, it may require an alternative expression system such as the yeast Pichia pastoris (Pagliuca et al., 2007). After standard purification procedures, which are, depending on the expression system, more (P. pastoris) or less demanding and time consuming (E. coli), the proteins can be reconstituted into PLBs for single-channel analysis. All these procedures are well established and have been extensively used in the past decades to study structure/function correlates of many channels. One shortcoming of this procedure is that it takes, depending on the channel, 1 wk or more from the expression in bacteria to the isolation of the channel for reconstitution in the bilayer. Another potential problem, which is occasionally encountered, concerns the purity of the protein of interest. This is particularly relevant in cases in which the yield of the channel of interest is low relative to the total amount of membrane proteins in the expression system. It

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has been reported that the protein of interest may contain contaminants, including channel proteins from the expression system (e.g., from *E. coli*), even after purification (Andersen et al., 1986; Accardi et al., 2004).

An alternative strategy to a heterologous expression is provided by recent progress in cell-free translation of proteins (Rosenberg and East, 1992; Berrier et al., 2004; Katzen et al., 2005; Syeda et al., 2008; Junge et al., 2011). A channel protein is, for this purpose, translated in vitro and reconstituted in PLBs after purification. This approach has been shown to work properly in several experiments (Rosenberg and East, 1992; Shim et al., 2007; Kovácsová et al., 2015). However, in our experience, the protein of interest is often contaminated by channels from the lysate in which the protein was synthesized, even after purification.

In this study, we show that the procedure of protein synthesis can be accelerated and that the problems of contaminations can be reduced by using a combination of in vitro translation and the recently developed nanodisc (ND) technology. These NDs are nanolipoproteins consisting of a pair of helical amphipathic scaffold proteins and a central core of lipids. In an alternative approach, the scaffold proteins are substituted by a polymer ring of styrene–maleic acid (Dörr et al., 2016). The nanoscale lipid bilayers have a well-defined diameter between ~9 and 12 nm, which provides a stable environment for membrane proteins also in detergent-free, aqueous solutions (Bayburt and Sligar, 2010). The ND technology became rapidly popular and enabled a growing number of biochemical and structural studies on membrane proteins, including ion channels. In a few recent publications, it was already reported that ion channels could be successfully incorporated from different types of NDs for electrophysiological recordings in PLBs (Banerjee and Nimigean, 2011; Braun et al., 2014a; Dörr et al., 2016). In this study, we present experimental details for a combination of in vitro translation of ion channels into commercially available NDs and their direct reconstitution from these nanoparticles into standard bilayers as a tool for rapid and contamination-free functional studies of ion channels.

**Materials and methods**

**Cloning and mutagenesis**

For in vitro protein expression, the coding sequences of KvNTS and KcsA were amplified by PCR and cloned into the pEXP5-CT/TOPO-vector with the pEXP5-CT/TOPO TA Expression kit (Invitrogen). To express the channel proteins in their native form, a stop codon was inserted directly upstream of the coding sequence of a 6xHis tag. The F– channel (Fluc-Ec2) coding sequence (Stockbridge et al., 2013) from *E. coli* virulence plasmid 2 was cloned into a pET-21a vector, amplified in DH5α cells, and purified via a Qiagen miniprep kit. The KcsA gene was provided by S. Keller (University of Kaiserslautern, Kaiserslautern, Germany). The well-characterized mutation E71A (Cordero-Morales et al., 2007) was introduced by site-directed mutagenesis (Pawpworth et al., 1996) into the KcsA sequence to abolish inactivation.

**Protein expression and purification**

In vitro expression was performed according to the manufacturer’s instructions of the MembraneMax (MM) HN Protein Expression kit (Invitrogen). The expression took place in the presence of different nanolipoproteins (NDs) on a shaker with 1,000 rpm at 37°C for a total of 1.5 h. For the experiments described below, we used the NDs provided with the MM expression kit (containing DMPC lipids) or MSP1D1-His discs from Cube Biotech. The latter were preassembled with DMPC, DMPG, or POPC lipids. The scaffold proteins of all used NDs were His tagged to allow the purification of channel–ND complexes via metal chelate affinity chromatography. The concentration of MSP1D1-His NDs in the reaction mixture was adjusted to 30 μM.

To purify the channel–ND complexes, the crude reaction mixture was adjusted to 400 μl with equilibration buffer (10 mM imidazole, 300 mM KCl, and 20 mM NaH$_2$PO$_4$, pH 7.4, with KOH) and subsequently loaded on an equilibrated, 0.2-ml HisPur nickel–nitrilotriacetic acid agarose spin column (Thermo Scientific). To allow the binding of the His-tagged NDs to the nickel–nitrilotriacetic acid agarose resin, the columns were incubated for 45 min at room temperature (RT) and 200 rpm on an orbital shaker. Afterward, the buffer was removed by centrifugation. To eliminate unspecific binders, the column was washed three times with 400 μl of a 20-μM imidazole solution. Finally, the His-tagged NDs were eluted in three fractions with 200 μl of a 250-μM imidazole solution. All centrifugation steps were performed at 700 g for 2 min. After purification, the elutions were stored at 4°C; in this way, channels remained functional for many months and even years.

**Vertical lipid bilayer experiments**

Vertical lipid bilayer experiments were performed on a setup from IonoVation, which is described in detail elsewhere (Bartsch et al., 2013). Two Teflon chambers, with a volume of 2.5 ml each, were separated by a 25-μm-thick Teflon foil with a central 100-μm large hole. Before the experiment, the rim of the hole was treated with ~1 μl of a 1% hexadecane in an n-hexane solution. Both chambers were subsequently filled up to the lower edge of the hole with electrolyte solution before adding 35 μl of 15 mg/ml phospholipid in n-pentane in each side of the chamber. Bilayers were formed by a folding technique (Montal and Mueller, 1972) in which opposing monolayers were generated over the hole in the septum by elevating the buffer level of each chamber. Alternatively, the lipid bilayers were formed by using the pseudo-painting/air bubble technique (Braun et al., 2014b). Bilayers generally had a capacitance of 90–110 pF.

All measurements in this study were performed at RT (20–25°C) in pure 1,2-diphytanyloxy-sn-glycero-3-phosphocholine (DPhPC; Avanti Polar Lipids) membranes or 1–2 mol% phosphatidylinositol 4,5-bisphosphate (PIP$_2$) containing DPhPC lipids or MSP1D1-His discs from Cube Biotech. The latter were preassembled with DMPC, DMPG, or POPC lipids and with symmetrical KCl solutions (100 mM KCl and 10 mM HEPES, pH 7.0, or 90 mM KCl and 10 mM potassium acetate, pH 4). For incorporation of channel proteins into the lipid bilayer, a small amount (~2 μl) of the purified channel–ND conjugates was added directly below the bilayer in the trans-compartment with a bent 25-μl Hamilton syringe. Multiple channel recordings were performed with the undiluted first elution fraction. For reconstitution of single-channel proteins, the first elution fraction was diluted with a pure 250-mM imidazole solution by a factor of 10$^3$–10$^6$. After channel incorporation
in the lipid bilayer, constant voltages between 160 and \(-160\) mV were applied. Both compartments of the bilayer chamber were connected with Ag/AgCl electrodes to the headstage of an L/M-EPC-7 patch-clamp amplifier (List-Medical). Currents were filtered with a 1-kHz, four-pole Bessel filter and digitized with a sampling frequency of 5 kHz by a 16-bit LIH 1600 A/D converter (HEKA Elektronik).

**Horizontal lipid bilayer experiments**

Fluc-Ec2 and monobody (Mb) S9 expression and purification and Fluc-Ec2 reconstitution into *E. coli* polar lipid extract (EPL) liposomes were completed as previously reported (Stockbridge et al., 2014; Turman and Stockbridge, 2017). The horizontal planar bilayer system (Heginbotham et al., 1999) consisted of two aqueous chambers separated by an 80-µm-thick partition crafted from a planar DMPC bilayer. After in vitro translation and purification, the globular channel KcvNTS (Braun et al., 2014b) was expressed in vitro into MM NDs or NDs mixed with DMPC, DMPG, or POPC membranes and recorded in PLBs. Their activity was measured via Ag/AgCl electrodes connected with Ag/AgCl electrodes to the headstage of an L/M-EPC-7 patch-clamp amplifier (List-Medical). The open probability for Kcv and KcsA was calculated with KielPatch (version 3.20 ZBM/2011) and Patchmaster (HEKA Elektronik). The open probability for KcvNTS channel translated in vitro into MM NDs or NDs with DMPC, DMPG, or POPC membrane and recorded in PLBs.

**Liposome preparation**

To produce liposomes, 15 mg DPhPC or DPhPC + PIP2 (Avanti Polar Lipids) were solubilized in 1 ml chloroform (AppliChem), dried in a glass vial, and finally resuspended in 10 ml of measuring solution consisting of 50 µg/ml BSA with the trans-chamber defined as electrical ground. For all recordings, the solution was frozen in 50-µl aliquots and thawed for further use.

**Ion channel recordings, data analysis, and statistics**

After insertion of an active channel into the bilayer, the membrane was routinely clamped for periods between 5 s and 1 min to a range of positive and negative voltages (usually from 160 to \(-160\) mV in 20-mV steps). Data analysis was performed with KielPatch (version 3.20 ZBM/2011) and Patchmaster (HEKA Elektronik). The open probability for KcvNTS function that are not depicted in Fig. 1. Fig. S1 shows mean I/V relation and P_o/V relation of KcvNTS Channel translated in vitro into MM NDs or NDs with DMPC, DMPG, or POPC membrane and recorded in PLBs.

**Contact bubble lipid bilayer experiments**

Single-channel currents were alternatively measured with the contact bubble bilayer (CBB) technique (Iwamoto and Oiki, 2015), a method for single-channel recordings in very small bilayers. Two borosilicate glass pipettes (melting point, 1.5–1.8 × 100 mm; Kimble Chase) with tip diameters of \(\leq 30\) µm were pulled with a PP-830 micropipette puller (Narishige) and filled with measuring solution (100 mM KCl and 10 mM HEPES, pH 7.0, or 90 mM KCl and 10 mM potassium acetate, pH 4). The solution in both pipettes also contained channel proteins in different NDs (MM, DMPC, DMPG, and POPC), as well as liposomes of DPhPC or DPhPC + PIP2. We typically added 2–8 µl of a 1:10 dilution from the first elution fraction to 25 µl of the pipette solution. A low concentration of the channel–ND preparation increases the chance of single-channel recordings. The glass pipettes were mounted on micromanipulators, and their tips were immersed in a hexadecane bath. Upon applying pressure to both pipettes, small bubbles (>50 µm diameter) formed at their tips in the oil–water interface. By maneuvering the glass pipettes, the two bubbles were brought in close contact, which resulted in the formation of a small bilayer at the contact zone with a capacitance of 2–5 pF in <1 min. After bilayer formation, channel proteins from the pipette solution inserted spontaneously into the target membrane. Their activity was measured via Ag/AgCl electrodes in the pipettes with a 3900A Integrating Patch Clamp amplifier (Dagan). Currents were filtered at 1 kHz and digitized with a sampling frequency of 10 kHz by an Instrutech LIH 8+8 data acquisition system A/D converter (HEKA Elektronik).

**Results and discussion**

We tested the combination of in vitro translation of channel proteins into NDs and their functional reconstitution from the NDs into two different types of bilayers. For this purpose, the small viral channel KcvNTS (Braun et al., 2014b) was expressed in vitro into four different NDs. Three of these NDs differed in the lipids (DMPC, DMPG, and POPC), which make up the discs (source membrane). The fourth type is a frequently used commercial ND with an unknown His-tagged protein scaffold that encloses a planar DMPC bilayer. After in vitro translation and purification, which took no more than a few hours, the NDs were added directly to a lipid bilayer (target membrane) for single-channel recordings. Channels were recorded with a conventional PLB setup and alternatively in so-called CBBs. In the latter case, the
NDs were added to the pipette solution, which was used to form CBBS. The exemplary current traces recorded at 100 mV in Fig. 1 show that the same kind of channel fluctuations were measured with both bilayer methods and from all ND preparations. None of the >100 experiments were ever contaminated with activity from another channel. Representative for all the different conditions, Fig. 1 (B and C) shows the mean I/V relations and the P_o/V relations for the channel in NDs with DMPC as a source membrane. The overall characteristics of the I/V and P_o/V relations are similar in the two recording conditions. A closer scrutiny of the data only shows that the unitary conductance of KcvNTS is independent of the type of ND and consistently smaller in CBB compared with PLB recordings (Fig. 1, B and D). Overall, the I/V and P_o/V relations obtained under the variable conditions are also similar to those from previous studies in which the same channel was expressed and purified from P. pastoris and reconstituted in PLBs (Braun et al., 2014a). The larger voltage window, which was tested here, only highlights the pronounced negative slope conductance at hyperpolarizing voltages, which is typical for this type of channel (Gazzarrini et al., 2009).

Comparison of the current traces (Fig. 1 A) and a detailed analysis of its activity confirm that the channel exhibits the same functional features independent of the type of ND into which it was produced. This includes the unitary conductance (Fig. 1 D), open probability (Fig. 1 E), and the overall shape of the I/V and P_o/V relations (Fig. S1). The results of these experiments confirm that NDs with channel proteins can be added directly to bilayers and that the channel proteins insert spontaneously in the final target membrane. The functional properties of the channel are largely unaffected by the type of lipid that is used for making the ND source membranes and the type of method with which channel activity is recorded (Fig. 1).

In the next experiment, we addressed the question of whether only the channel protein becomes exposed to the target bilayer or whether the ND remains intact in the target bilayer. In the first case, the channel property should be sensitive to the bilayer of the target membrane, whereas it should be insensitive in the latter case. To answer this question, we produced KcvNTS in four different NDs and reconstituted the channel in a planar DPhPC lipid bilayer with or without 1 mol% PIP_2. The exemplary current traces at 160 mV in Fig. 2 A show that the presence of PIP_2 resulted in a strong reduction of the unitary conductance. This effect, which is not further analyzed here, was voltage dependent and increased with positive voltages (Fig. 2 B). The results of these experiments show that the channel function depends on the bilayer composition of the target membrane. Hence, the channel must be exposed to the target lipid. It is reasonable to speculate that the ND at some point disintegrates and that the lipids, which surround the channel, are no longer dominated by those from the ND.

In the next step, we tested whether the combination of protein synthesis and reconstitution of the channel protein into the target membrane is affected by the flavor of the lipids in the source membrane. For this purpose, we used the same preparations that were used in Fig. 1. The respective NDs were added at a high concentration (2 µl of the undiluted elution) to a vertical DPhPC bilayer, and the insertion of the channel in the target bilayer was continuously monitored. Fig. 3 shows an example in which NDs were added at time 0 to a bilayer kept at 60 mV. After a few seconds, the first channel activity could be registered. To compare different experiments, we measured the time it took before 10 channels (= 50 pA) were active in the bilayer. In the present example, it took <1 min before this threshold was achieved. A comparison between different NDs shows that the channels inserted quickly into the target membrane when they were in NDs with DMPC or DMPG. When the channels were in NDs with a POPC bilayer, the insertion was less efficient. The results of these experiments suggest that NDs with DMPC membrane are the
most suitable for functional reconstitution of the KcvNTS channel in PLBs. At this point, we cannot answer the question of whether the lipid of the source ND supports protein synthesis into the ND or the transfer from the ND to the target membrane.

In the course of the experiments, we realized that the efficiency of functional channel reconstitution progressively decreased after storing the channel-containing NDs in measuring buffer. To examine this phenomenon systematically, we collected the first fraction from an in vitro synthesis/purification of KcvNTS into MM NDs and diluted it 1:100 in 250 mM imidazole or 1:100 in measuring buffer (100 mM KCl and 10 mM HEPES, pH 7). 1 d later, the reconstitution efficiencies of both preparations were estimated. Fig. 4 A shows two representative examples in which the ND–channel stored in imidazole or in buffer were added to the bilayer. In both cases, the channels inserted into the bilayer. However, the efficiency of insertion, which was estimated from the current amplitude 1 min after the first channel had inserted into the bilayer, was on average ~10 times lower for protein stored in measuring buffer.

To further examine this negative effect of the saline buffer on the channel, we performed experiments in which the channel was, during the course of the purification, exposed to different wash and elution buffers (Fig. 4 B). The first elution fraction was then added directly to the bilayer to examine the reconstitution efficiency. The data show that the highest efficiency was obtained when the protein was washed and eluted in a pure imidazole solution. The presence of PBS (300 mM KCl and 20 mM NaH2PO4, pH 7.4) in the wash buffer decreased the reconstitution efficiency. A reasonable explanation for this result is that salts in the buffer may remove NDs from the column and hence decrease the protein yield in the final preparation. The data also show that the presence of PBS in the elution buffer had no appreciable effect on the reconstitution efficiency (Fig. 4 B). The results of these experiments suggest that ions in the elution buffer have no immediate negative effect on channel insertion and/or function.

During storage in an ionic buffer, the NDs must undergo some sort of denaturation or aggregation of NDs. This effect is more augmented by the absence of imidazole than by the presence of ions. Imidazole occurs to have a stabilizing effect on the isolated ND–channel preparation. The negative effect of ions seems to become relevant already during the course of an experiment. We routinely found that we had to progressively increase the amount of NDs, which were required for a successful recording of channel activity, over a period of 3–4 h.

To obtain more information on storing ND–channel preparations, we synthesized the KcvNTS channel as in Fig. 1. The isolated NDs were then split into two batches, of which one was frozen at −20°C and the other stored at 4°C. 4 d later, both preparations were used on the same day for reconstitution in a DPhPC bilayer. The representative recordings from three experiments in Fig. 4 C show that the batch, which had been frozen for storage, failed to generate any channel activity in the bilayer. However, the second aliquot, which was stored in the refrigerator, generated reliable channel fluctuations. In other experiments, we found that a preparation kept for 3 yr at 4°C was still generating channel activity in these assays. The results of these experiments imply that ND–channel preparations are very stable when stored at 4°C but may lose activity as a result of freezing. Similar results were obtained with the Fluc-Ec2 channel (see below). Also, this protein could be stored after synthesis into NDs at 4°C but not at −80°C without compromising activity. Collectively, these data suggest that ND–channel preparations are best stored at 4°C even though freezing may not be detrimental for all types of ND–channel preparations. It has been reported that the integrity of the KcsA channel in NDs is neither compromised by freezing/thawing nor by lyophillization (Dörr et al., 2016).

The experiments in Fig. 4 (A and B) indicate that it is possible to measure very large currents after adding high amounts of active channels to the bilayer. This is remarkable because
host bilayers generally tend to become unstable and burst when large numbers of channel proteins are reconstituted by fusion with liposomes. Encouraged by these large currents, we tested the possibility of measuring the macroscopic I/V properties of the KcvNTS channel in a DPhPC bilayer. The protein was synthesized as in Fig. 1 into MM NDs and added at high concentration (1:100 dilution of the first elution) to a DPhPC bilayer. In these types of experiments, we were able to measure macroscopic currents in the nanoampere range (Fig. 5 A). Worth noting is that the amplitudes of the currents were more limited by the amplifier than by the stability of the bilayer. The normalized steady-state I/V curve of the exemplary experiment in Fig. 5 A is plotted in Fig. 5 B. Note that the shape of the I/V curve with the pronounced negative slope at negative clamp voltages is the same as that of the unitary conductance multiplied with the open probability of the channel from data in Fig. 1 C. Worth noting in our experiments is that the macroscopic currents, including the negative slope conductance, which we recorded in the bilayer (Fig. 5, A and B), are similar to those recorded in Xenopus laevis oocytes, which express a closely related channel (Gazzarrini et al., 2009). This underscores that these channels are inserting with a strong directional bias into the host bilayer. The orientation of the channel in the bilayer must be the same as in a cell; the side that faces the cytosol of the cell is facing the cis-chamber in the bilayer.

Because of the high open probability of the channel at positive voltages, we can estimate that a macroscopic current of 1.25 nA at 100 mV is, in the example of Fig. 5 A, equivalent to 180 active channels in the bilayer. In similar measurements, we could record at 100 mV in a solution with 10 mM KCl currents as large as 6 nA (mean of 3.4 ± 0.5 nA) translating into ~5,000 (2,800 ± 400) active channels in the bilayer. From these measurements, we predict that this method is also suitable for a functional reconstitution of ion channels with a small unitary conductance or even from transport proteins like pumps or anti-/symporters with a low turnover rate.

The experiments have so far shown that the small viral K+ channels can be successfully produced in NDs and directly incorporated into bilayers for functional testing. To examine whether the method is also suitable for other channels, we performed similar experiments with a mutant of the KcsA channel (KcsA271A)
and Fluc-Ec2 channels. KcsA is a small bacterial model channel, which provided the first crystal structure of a K+ channel (Doyle et al., 1998). The Kcv and KcsA channels share a similar architecture with two transmembrane helixes, but KcsA has much larger cytosolic domains. The wealth of structural information has made KcsA an important model system for studying structure function relations in K+ channels. The final channel, Fluc-Ec2, belongs to a family of dual-topology, F–-specific ion channels that protect bacteria from F– toxicity (Ji et al., 2014). The channel is a small, 15-kD, four-pass transmembrane homodimer with antiparallel topology and two pores that are interwoven between subunits (Stockbridge et al., 2015).

The exemplary current trace in Fig. 6 A shows that KcsA E71A could also be successfully reconstituted from NDs into bilayers made from a 3:1 mix of DPhPC and 1,2-diphytanoyl-sn-glycero-3-phospho-L-serine (DPhPS). This noninactivating KcsA mutant (Cordero-Morales et al., 2006) shows its typical gating in which the channel exhibits long periods of high open probability (Fig. 6 A). The same behavior has been reported in previous experiments in which KcsA E71A was expressed and purified from E. coli and reconstituted via liposomes (Cordero-Morales et al., 2006) or nanoscaled apolipoprotein-bound bilayers (Banerjee and Nimigean, 2011) into planar bilayers. Under the experimental conditions, in all recordings the channel exhibited a slope conductance of $\sim 100$ pS at 0 mV (Fig. 6 C) and an asymmetric I/V relation in which the inward conductance is $\sim 1.4$ times higher than the outward conductance. The asymmetry of the I/V relation also suggests that KcsA E71A inserts into the membrane with a strong directional bias. A comparison shows that the functional parameters like unitary currents and $P_0$ values from the present recordings are within the experimental variability identical to those reported from other studies in which the same channel

### Table 1. Comparison of unitary channel currents and $P_0$ values at selected voltages of KcsA E71A channel from this study with data from the literature

| Source                        | Mean current | Open probability |
|-------------------------------|--------------|------------------|
|                               | $-100$ mV    | $100$ mV         | $-100$ mV | $100$ mV | $40$ mV |
|                               | $P_0$        | $P_0$            | $P_0$    | $P_0$   | $P_0$   |
| This study                    | $-9.3 \pm 1.3$ | $6.5 \pm 1.1$   | $0.84 \pm 0.1$ | $0.91 \pm 0.13$ | $0.86 \pm 0.11$ |
| Banerjee and Nimigean, 2011   | $-12 \pm 1.5$ | $7.2 \pm 1$     | $0.97 \pm 0.01$ | $0.97 \pm 0.02$ | n.a.    |
| Hirano et al., 2011           | n.a.         | n.a.             | n.a.     | n.a.    | $0.85 \pm 0.2$ |
| Zhao et al., 2015             | n.a.         | $6.5$            | n.a.     | $0.98 \pm 0.01$ | n.a.    |

KcsA E71A was produced in E. coli (Banerjee and Nimigean, 2011; Hirano et al., 2011; Zhao et al., 2015) and reconstituted in PLBs from NDs (Banerjee and Nimigean, 2011) or via microsomes (Hirano et al., 2011; Zhao et al., 2015). In all cases, channel activity was recorded in bilayers containing anionic lipids in buffers with 100 mM K+, pH 4.0 (Banerjee and Nimigean, 2011; Zhao et al., 2015), or 200 mM K+, pH 4.0 (Hirano et al., 2011). Literature data were estimated from figures in respective publications. Data from this study are from Fig. 6. Information on current amplitudes or open probabilities, which is not available from the literature, is indicated by n.a. in the table.
was synthesized and/or reconstituted in PLBs by different methods (Table 1).

To further test whether the KcsA protein also exhibits its characteristic functional features after reconstitution from NDs, we examined its sensitivity to Ba\(^{2+}\). In classical bilayer studies, it has been shown that the KcsA wild-type channel (KcsA\(_{wt}\)) is blocked in a nonsymmetrical manner by Ba\(^{2+}\) (Piasta et al., 2011). Fig. 6 B shows recordings from an experiment in which Ba\(^{2+}\) was first added at 500 \(\mu\)M to the trans-, and in a second step, to the cis-chamber. The current traces and I/V data (Fig. 6, B and C) are in agreement with previous measurements with KcsA\(_{E71A}\) (Banerjee and Nimigean, 2011) that the channel exhibits in the presence of Ba\(^{2+}\) still full openings. The lifetime of these events is, however, dramatically shortened in the presence of Ba\(^{2+}\) compared with the control. It is also evident that the Ba\(^{2+}\) effect is stronger when the blocker is added to the cis- rather than to the trans-side. On average, 500 \(\mu\)M Ba\(^{2+}\) reduced in three experiments \(P_0\), at \(-100\) mV and \(100\) mV to 23\% and 1\% when added to the trans- and cis-side, respectively (Fig. 6 D). In all these functional aspects (e.g., Ba\(^{2+}\)-induced reduction of the open lifetime and nonsymmetrical sensitivity to the blocker), the KcsA\(_{E71A}\) channel reconstituted from NDs behaves in the same manner as reported from previous experiments in which KcsA\(_{wt}\) was recorded in classical bilayer experiments.

It has been reported that the KcsA\(_{wt}\) channel requires anionic phospholipids in the bilayer for function (Valiyaveetil et al., 2002). This dependency provides another test for the question of whether the channel is, after insertion, fully exposed to the phospholipids of the target bilayer or remains under the influence of lipids from the channel protein is, after insertion, fully exposed to the phospholipids of the target bilayer. Finally, anionic phospholipids, which may remain associated with the channel protein (Valiyaveetil et al., 2002), e.g., from the ND lipid, have no obvious influence on channel gating and conductance. Collectively, the data suggest that the dependency of KcsA activity on anionic lipids may be more related to the insertion of the protein into liposomes or the transfer from the liposomes into the target membrane.

The Fluc-Ec2 ion channel is a topologically unusual system for in vitro translation and reconstitution into NDs. This topology requires that each monomer of the homodimer inserts in the opposite direction relative to its neighbor within the ND during in vitro translation. As Fig. 8 attests, using single-channel PLB recordings as a functional indicator, channels from an in vitro synthesis reconstituted into MM NDs are indistinguishable from channels expressed in E. coli and reconstituted in a traditional EPL proteoliposome system. A general overview of the channels in Fig. 8 A (top) shows the functional characteristics of Fluc-Ec2 single channels, such as brief, millisecond closings and short-lived subconducting states that are the same in both reconstitution methods (Stockbridge et al., 2013). Fig. 8 A (bottom) shows discrete, seconds-long, full-blocking events from a highly specific Mb blocker, S9, confirming that the channels from the MM ND reconstitution are properly folded and have the specific epitope required for Mb block available (Stockbridge et al., 2015; Turman et al., 2015; Turman and Stockbridge, 2017). Moreover, the open probabilities (Fig. 8 B) of the unblocked channels are 0.99 ± 0.002 (n = 3) as previously noted (Stockbridge et al., 2013). In the presence of 250 nM Mb S9, the open probabilities are 0.29

Figure 7. Functional reconstitution of KcsA\(_{E71A}\) channel in PLBs in pure DPhPC membrane. (A) Exemplary current traces of KcsA\(_{E71A}\) channel activity at 100 mV in symmetrical KCI (100 mM, pH 4). The protein was produced into MM NDs or NDs with DMPG, DMPG, or POPC membranes and reconstituted in a pure DPhPC membrane. (B) Mean I/V relations from KcsA\(_{E71A}\) channel produced in DMPC NDs and reconstituted in pure DPhPC bilayers (orange circles) or in bilayers made of a 3:1 mix of DPhPC and DPhPS (data from Fig. 6). (C) Mean slope conductance (±SD, n given as number of experiments in parentheses) between ±40 mV for KcsA\(_{E71A}\) channel reconstituted from different NDs into pure DPhPC membranes in vertical PLBs from pure DPhPC. (D) \(P_0/V\) relation of KcsA\(_{E71A}\) synthesized into NDs with different lipids and reconstituted into pure DPhPC membranes in vertical PLBs. Color of symbols in C cross-references with data in D.
functional reconstitution of the ion channel in bilayers. The absence of contaminations from other methods. This combination of reliable and reproducible channel properties is sensitive to the lipid composition of the target bilayer, suggesting that the proteins are fully immersed into the target membrane. The basic functional properties of the tested channels are similar to those measured from single-channel insertion events denoted by asterisks from MM NDs (open bars) and EPL liposomes (closed bars). (C) Left: Single-channel insertion events denoted by asterisks from MM NDs (top) and EPL liposomes (bottom). Right: Single Fluc-Ec2 channel conductance measured from insertion events.

Conclusion
These experiments show that different types of K+ channels and an F− channel can be easily synthesized in vitro into NDs. After purification via a His tag on the scaffold protein, which surrounds the lipid ND, the channels can then be efficiently reconstituted directly from the NDs into conventional bilayers for functional characterization. The entire procedure takes no longer than a few hours. The efficiency of channel reconstitution depends on the lipids in the NDs and on the buffer in which the preparations are stored. This suggests that the method can be optimized for each channel of interest. The successful reconstitution of the reconstituted channels are sensitive to the lipid composition of the target bilayer, suggesting that the proteins are fully immersed into the target membrane. The basic functional properties of the tested channels are similar to those reported from measurements of the same channels with other methods. This combination of reliable and reproducible channel function with the short time for channel synthesis and purification, as well as the absence of contaminations from other channels, makes this procedure a very versatile method for a functional reconstitution of the ion channel in bilayers.

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