

Non-vesicular transfer of membrane proteins from nanoparticles to lipid bilayers

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Discoidal lipoproteins are a novel class of nanoparticles for studying membrane proteins (MPs) in a soluble, native lipid environment, using assays that have not been traditionally applied to transmembrane proteins. Here, we report the successful delivery of an ion channel from these particles, called nanoscale apolipoprotein-bound bilayers (NABBs), to a distinct, continuous lipid bilayer that will allow both ensemble assays, made possible by the soluble NABB platform, and single-molecule assays, to be performed from the same biochemical preparation. We optimized the incorporation and verified the homogeneity of NABBs containing a prototypical potassium channel, KcsA. We also evaluated the transfer of KcsA from the NABBs to lipid bilayers using single-channel electrophysiology and found that the functional properties of the channel remained intact. NABBs containing KcsA were stable, homogeneous, and able to spontaneously deliver the channel to black lipid membranes without measurably affecting the electrical properties of the bilayer. Our results are the first to demonstrate the transfer of a MP from NABBs to a different lipid bilayer without involving vesicle fusion.

INTRODUCTION

The study of membrane proteins (MPs) remains a major challenge in protein biochemistry mainly because of problems of protein aggregation and thermal instability in nonnative preparations of MPs in detergents, or native-like but insoluble preparations in liposomes. As a result, nonconventional surfactants with properties intermediate to lipids and detergents are becoming increasingly popular as alternative platforms for MPs (Bayburt and Sligar, 2002; Boldog et al., 2006; Park et al., 2007; Dalal et al., 2009; Popot, 2010). Discoidal lipoproteins, which include reconstituted high-density lipoprotein particles, nanodiscs, and nanoscale apolipoprotein-bound bilayers (NABBs), constitute a type of nonconventional platform that has enabled novel assays on MPs sequestered in a well-defined, soluble native-like lipid environment, such as single-particle imaging, surface plasmon resonance, nuclear magnetic resonance, and surface-enhanced infrared absorption spectroscopy (Banerjee et al., 2008; Das et al., 2009; Shnkarev et al., 2010; Zaitseva et al., 2010). However, it is not known whether MPs incorporated in these lipid nanoparticles retain the range of key functional properties, as established with other lipid preparations like liposomes and planar lipid bilayers, which are generally used for single-molecule measurements of the movement of ions and molecules through ion channels and transporters.

Traditional biophysical assays of purified ion channels in lipid bilayers are performed via reconstitution of detergent-solubilized channels into liposomes, followed by fusion of these channel-containing proteoliposomes with the target bilayer. The precise molecular mechanism of this type of vesicle fusion process remains hypothetical, although it is generally accepted to involve a close approach of the two bilayers, followed by minimization of solvent-exposed nonpolar surfaces, local perturbation of bilayer structure, merger of proximal monolayers, stalk formation and expansion, bending of the elongated stalk, and finally, pore formation for vesicle integration (Jahn and Grubmüller, 2002; Kozlovsky and Kozlov, 2002). The proteoliposome fusion process commonly envisages extended bilayers, with fusion accelerated by divalent metal ions, membrane curvature-inducing lipids or fusogenic proteins (Cohen et al., 1980; Chernomordik and Kozlov, 2003), resulting in the vesicle lipids and proteins getting incorporated into the “expanded” target bilayer. Techniques that do not involve liposomal fusion include channels transferred directly from cells (Holden et al., 2006) or solid supports (Holden and Bayley, 2005; Hirano et al., 2009) and monitored electrophysiologically using black lipid membranes (BLMs).

We investigated whether ion channels could be transferred from NABBs, which are planar, small bilayers

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Abbreviations used in this paper: apo A-I, apolipoprotein A-I; BLM, black lipid membrane; EM, electron microscopy; MP, membrane protein; NABB, nanoscale apolipoprotein-bound bilayer.

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made with POPC and zebrafish apolipoprotein A-I (apo A-I), to BLMs comprising of POPE/POPG (PE/PG) lipids. The main advantage of an efficient transfer of channels between platforms is that the same monodisperse NABB system can then be used for both ensemble biochemical assays and single-molecule biophysics. The flexibility of using the same preparation for multiple distinct types of assays is unique to NABBs and not possible in vesicles because vesicles divide the environment of the channel into two biochemically distinct compartments. Although there have been reports of NABBs preserving stability and conformational flexibility of MPs (Banerjee et al., 2008; Zaitseva et al., 2010; Zou and McHaourab, 2010), there have not been any reports to confirm whether the NABB-embedded proteins can be safely transferred to other bilayers—a result that could extend the use of these particles further, as targeted delivery vehicles for MPs. Here, we demonstrate for the first time, a nonvesicular, spontaneous transfer of ion channels from NABBs to lipid bilayers using a well-characterized ion channel, KcsA, incorporated into NABBs. We used a non-inactivating KcsA mutant, KcsA E71A, with a recognizable electrophysiological footprint (Cordero-Morales et al., 2006; Thompson et al., 2008), which was replicated upon transfer to lipid bilayers from NABBs, ascertaining that the reconstitution into NABBs and the subsequent transfer process to bilayers did not negatively impact the protein function.

MATERIALS AND METHODS

Protein expression and purification

KcsA E71A was expressed using the pASK90 vector (Skerra, 1994) in JM83 *Escherichia coli* and purified in *n*-decyl- β -D-maltopyranoside (DM) detergent using Ni²⁺ affinity and gel-filtration chromatography, as described previously (Thompson et al., 2008). The concentration of KcsA E71A was determined spectrophotometrically by absorbance at 280 nm, using $\epsilon_{280} = 34,950 \text{ M}^{-1}\text{cm}^{-1}$.

NABBs were made using zebrafish, *Danio rerio*, apo A-I (the pE28-Zap1 plasmid was provided by T. Sakmar, The Rockefeller University, New York, NY), which has a distinct amino acid sequence from the human apo A-I, providing it with different patterns of hydrophobicity and salt bridging (Segrest et al., 1999), which are beneficial in forming lipoprotein discs that are both homogeneous in diameter and stable to dilution (Banerjee et al., 2008). Zap1 and Zap1Q26C proteins were expressed in BL21 (DE3) *Rosetta 2 E. coli*, purified, and labeled site specifically (Zap1Q26C) using the fluorophore tetramethyl rhodamine-5-maleimide, as described previously (Banerjee et al., 2008), to form TMR-Zap1. The concentration of Zap1 was determined spectrophotometrically by absorbance at 280 nm, using $\epsilon_{280} = 16,640 \text{ M}^{-1}\text{cm}^{-1}$, and the labeling ratio was determined in TMR-Zap1 using $\epsilon_{550} = 95,000 \text{ M}^{-1}\text{cm}^{-1}$ for tetramethyl rhodamine. The labeling ratio (TMR:Zap1) obtained was typically 0.7–0.9.

Preparation of phospholipids

Lipids were purchased from Avanti Polar Lipids, Inc. A chloroform solution of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was dried under N₂ gas and mixed with solid [(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS)

detergent and buffer (20 mM HEPES, pH 7.5, and 100 mM KCl) to obtain 100 mg/ml POPC and 100 mg/ml CHAPS (10% POPC-CHAPS). The mixture was subjected to several freeze-thaw cycles until the solution turned clear. To make the fluorescent lipid stock, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) salt (NBD-DOPE) powder was dissolved in CHAPS and buffer to obtain a concentration of 10 mg/ml NBD-DOPE in 10 mg/ml CHAPS, followed by sonication. The two lipid stocks were mixed to obtain a final mixture of 1 mg/ml NBD-DOPE and 90 mg/ml POPC in 91 mg/ml CHAPS (9.1% doped lipid stock). For lipid bilayer recordings, the bilayer was prepared from chloroform-solubilized POPE/POPG (combined in 3:1 mass ratio), as described previously (Thompson et al., 2009).

NABB formation

Purified Zap1, fluorescently doped lipids (POPC doped with NBD-DOPE in CHAPS), and KcsA E71A (in DM) were mixed in different relative ratios in 0.2 ml in a detergent concentration of 1% (wt/vol) CHAPS and 0.25% (wt/vol) DM. The molar ratios of the components for the different samples were:

Empty NABBs, Zap1:Lipids:KcsA = 2:150:0

1K – NABBs, Zap1:Lipids:KcsA = 2:150:1

4K – NABBs, Zap1:Lipids:KcsA = 2:86:4

Zap1 concentration was 0.2 mM in each of the reactions. The components were mixed thoroughly, centrifuged, and incubated at room temperature for 30 min. Prepacked columns containing 2 ml of Detergent Removal Resin (Thermo Fisher Scientific) were equilibrated with ~10 ml of NABB formation buffer (10 mM HEPES, pH 7.6, and 100 mM KCl). The detergent-containing solution was layered on top of the column and allowed to enter by gravity flow. The NABBs were eluted under gravity flow by adding NABB formation buffer on top. The colored fractions were pooled.

Electron microscopy (EM) imaging

Negative staining of the sample was done using 2% uranyl acetate stain on a 200 mesh carbon-coated copper grid (Electron Microscopy Sciences). Concentration of particles on the EM grid was varied from ~10 to 100 ng/ml to obtain discrete single particles. Imaging was performed on an electron microscope (200 kV; Tecnai F20; FEI Company) at 2- μ m underfocus. Image analysis from micrographs was done using EMAN software suite (Lüdtke et al., 1999), using semiautomatic particle selection. Each class average used ~200 single particles.

Single-channel electrophysiology

Lipid bilayers (BLMs) were made of 10 mg/ml 3:1 (wt/wt) POPE/POPG lipid mixture in *n*-decane, which divided the cis (10 mM HEPES, pH 7) and trans (10 mM succinic acid, pH 4) recording chambers. Both sides had symmetric (100 mM) K⁺ concentrations made of 80 mM KCl and 20 mM KOH.

For 4K-NABBs, the concentration of KcsA (in NABBs) obtained after gel filtration was 0.7 mg/ml, with a calculated POPC concentration (based on stoichiometries used to make 4K-NABBs) of 0.6 mg/ml. This sample was diluted to 2 μ g/ml KcsA (1.7 μ g/ml POPC) in 10 mM HEPES pH 7, 80 mM KCl, 20 mM KOH 2 μ l of which was applied to the cis-chamber containing 500 μ l of buffer after the PE/PG bilayer was ‘painted’ over the partition hole by dragging an air bubble at the tip of a pipette. Channel incorporation was seen within a few minutes using these concentrations.

For 1K-NABBs, the concentration of KcsA after gel filtration was 0.17 mg/ml (1 mg/ml POPC). This sample was diluted to 2 μ g/ml KcsA (10 μ g/ml POPC). Channel incorporations were observed approximately within the same time frame as in the 4K-NABB sample. The 1K-NABB sample showed significantly lower propensity for multiple channel incorporation compared

with 4K-NABB. The KcsA concentration in both samples was similar to KcsA amounts (2 $\mu\text{g}/\text{ml}$) used in proteoliposomes made with PE/PG (2 mg/ml of lipids).

A voltage ranging from -250 to 250 mV was applied across the bilayer through Clampex 10.0 via an Axopatch 200 B (MDS Analytical Technologies). BaCl_2 dissolved in the pH 4.0 recording solution was perfused through the trans chamber. Single-channel amplitudes and open probabilities were calculated using Clampfit 10.0 (MDS Analytical Technologies), similar to the method described by Thompson et al. (2008). The data in Fig. 3 C was fit using Origin (OriginLab), and the Woodhull equation (Woodhull, 1973) is in the legend.

RESULTS

KcsA-NABB formation

NABBs are formed by apo A-I wrapping circumferentially around a lipid bilayer in a “double-belt” orientation (Segrest et al., 1992; Gu et al., 2010). NABBs differ from nanodiscs in their use of an engineered apo A-I derived from zebrafish (Zap1) instead of variants of human apo A-I to form homogeneous, monodisperse discoidal lipid nanoparticles (Banerjee et al., 2008). We optimized the molar ratios of KcsA, POPC, and Zap1 to obtain a high efficiency of KcsA reconstitution into NABBs.

We tested a range of ratios of KcsA with respect to Zap1 and POPC to determine homogeneity of the KcsA-NABB preparation. The concentration of KcsA incorporated into NABBs after detergent removal and gel filtration was determined spectrophotometrically by using site-specifically labeled Zap1 with the fluorescent

dye tetramethyl rhodamine (absorbance maximum = 550 nm) for NABB formation and calculating the excess protein absorption over Zap1 and lipid. We found that $>90\%$ of the KcsA was incorporated into NABBs, even in samples with high KcsA/NABB concentrations (4K-NABBs). There was no change in the gel-filtration elution peak position between empty NABBs and KcsA-NABBs with a stoichiometry of 1 KcsA monomer per NABB (1K-NABB) (Fig. 1 A, blue and red curves). When the initial KcsA/NABB stoichiometry was four KcsA monomers per NABB, we obtained a homogeneous peak, shifted toward slightly higher elution volumes (Fig. 1 A, gray and red curves), by lowering the relative amount of POPC. We saw an increase in protein absorption and a concomitant decrease in lipid absorption in 1K-NABBs compared with empty NABBs. Because the only difference in the two samples was the presence of KcsA in 1K-NABBs, we concluded that lipids were displaced by KcsA during incorporation into NABBs. For 4K-NABBs, the increased KcsA amount translated into a larger increase in protein absorption and a corresponding decrease in lipid absorption relative to empty NABBs (even after correcting for the lower lipid amount used for 4K-NABBs), further suggesting that KcsA incorporates into NABBs at the expense of lipids. KcsA tetramers were detected in both 4K-NABB and 1K-NABB samples by SDS-PAGE analysis of the peak fraction (used for electrophysiology), unless the gel samples were heated, which resulted in monomers (Fig. 1 A, inset).

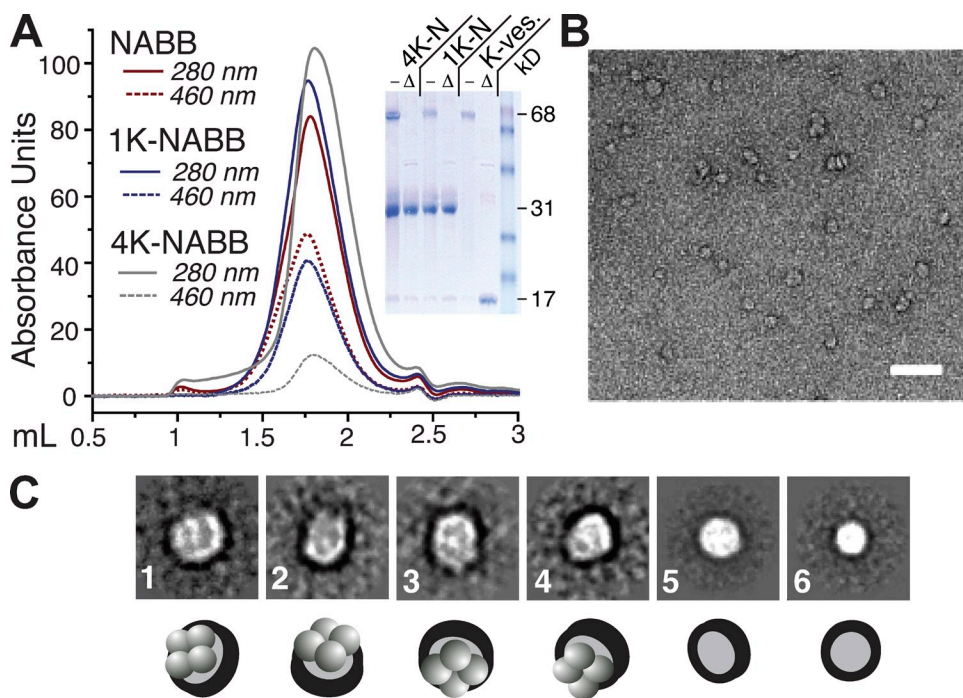


Figure 1. Tetrameric KcsA incorporates efficiently into NABBs. (A) Gel filtration of empty NABBs and 1K-NABB and 4K-NABB samples. Solid (280-nm) and dashed (460-nm) curves represent protein and NBD lipid absorbance, respectively. (Inset) Coomassie-stained SDS-PAGE of gel filtration-purified samples of 4K-NABB, 1K-NABB, and KcsA in PE/PG vesicles, with (Δ) or without ($-$) heating at 95°C before loading. The bands represent KcsA tetramer (68 kD), monomer (17 kD), and Zap1 (31 kD). (B) Negative-stain EM image of 4K-NABBs. Some particles are seen characteristically “stacking” together, oriented with the plane of the disc and perpendicular to EM grid. Bar, 100 nm. (C) Class averages of single particles picked from EM micrographs of 4K-NABBs (panels 1–4) show density within the discs oriented at different angles

relative to the plane of the disc. Cartoon representations below each class average show the likely orientation of the densities emerging from the discs. Class averages of empty NABBs (panels 5 and 6) lack these densities. Each class average was computed from ~ 200 isolated single particles. The side of each panel is 30 nm.

To confirm the presence of the ion channel in NABBs, KcsA-NABBs were imaged by negative-stain EM (Fig. 1 B). Notably, we found no evidence of vesicle formation in our images, indicating stable NABBs even at high KcsA ratios and at particle dilutions of ~ 50 – 100 ng/ml on the EM grid. The diameters of the particles imaged were 10–12 nm. The EM micrographs were analyzed by single-particle averaging from 4K-NABB sample micrographs (Fig. 1 C). Class averages constructed from $\sim 1,200$ individual particles show tetrameric density emerging from the discoidal density of the NABBs (Fig. 1 C, panels 1–4), likely representing the extended KcsA cytoplasmic domain (Uysal et al., 2009). In comparison, empty NABBs show a featureless, discoidal density upon class averaging (Fig. 1 C, panels 5 and 6). Regardless of the KcsA to NABB ratio, we detected the presence of a tetrameric density in the NABBs by EM, indicating that KcsA forms strong tetramers that cannot be disrupted by the NABB formation conditions, a conclusion also supported by the presence of tetrameric KcsA in the samples detected by SDS-PAGE electrophoresis (Fig. 1 A, inset). The 1K-NABB sample, therefore, contains one KcsA channel per four NABBs (three empty NABBs for each KcsA containing NABB), whereas the 4K-NABB sample is more likely to have one channel per NABB.

KcsA transfer from NABBs to lipid bilayers

To assay transfer of the protein from the NABBs to a planar lipid bilayer, we added gel filtration-purified KcsA E71A-NABBs to the cis chamber of a horizontal bilayer setup after setting up a BLM with a 3:1 (wt/vol) POPE/POPG lipid mixture (Fig. 2 A) (Nimigeon and Miller, 2002; Thompson et al., 2009). KcsA is known to open at acidic intracellular pH (Thompson et al., 2008); thus, the cis chamber contained the buffer at pH 7.0

and the trans chamber at pH 4.0 to selectively record from channels oriented with their intracellular side in the trans chamber. We observed KcsA channel-like activity within a few minutes of application of KcsA-NABB samples, evidence that the channels spontaneously transferred from NABBs to bilayers (Fig. 2 B). We observed that for the same concentration of KcsA in the 4K-NABB and 1K-NABB samples (2–5 ng/ml), 4K-NABBs resulted in more channels transferring to the BLM compared with 1K-NABBs (Fig. 2 B, gray and blue traces), likely a result of the higher density of channel-containing NABBs than empty NABBs in the 4K-NABB sample. Importantly, the application of empty NABBs into the bilayer chamber did not affect the electrical properties of the bilayer (Fig. 2 B, red trace). These experiments also indicate that detergent removal in the samples using our method was sufficient not to cause any noticeable perturbations in the bilayer.

We compared the efficiency of incorporation of KcsA into BLMs from either NABBs or liposomes by monitoring the appearance of channel-like electrical activity within 15 min of sample application to the bilayer. We performed the experiment using different BLMs over a 48-h period with the same BLM lipid preparation to ensure conditions as similar as possible between the different sample applications. Out of 12 sample applications from each preparation, 8 from 1K-NABBs, 9 from 4K-NABBs, and 8 from PE/PG vesicles resulted in channel incorporations, suggesting little difference between the efficiency of BLM incorporations from NABBs and liposomes. Interestingly, the time required for the first channel to incorporate in the BLM from 1K-NABB, and 4K-NABB samples was shorter than from liposomes, with most of the BLMs showing channel incorporations within the first minute after KcsA-NABB sample application.

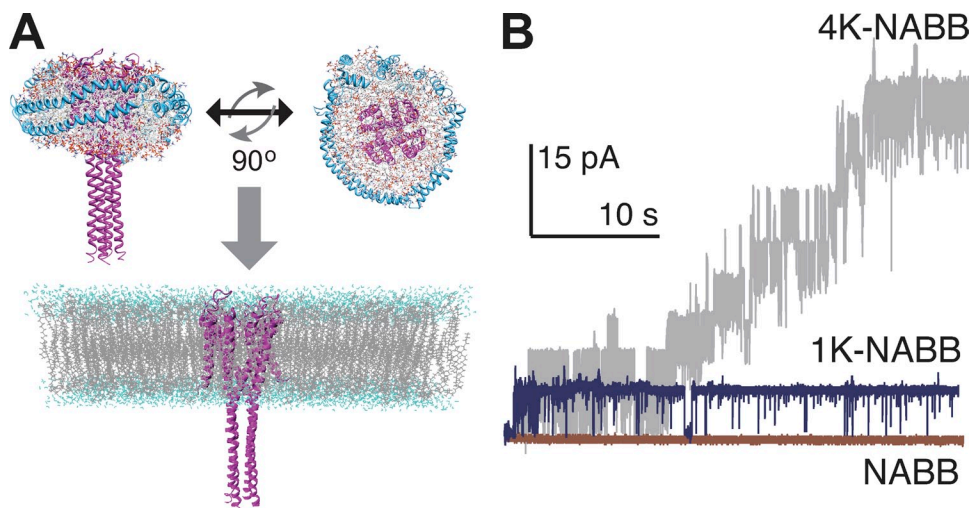


Figure 2. KcsA E71A channels transfer from NABBs to lipid bilayers. (A) Cartoon representation of KcsA transfer from NABBs into lipid bilayers. The NABB model was produced using the University of California, San Francisco, Chimera package using HDL particle coordinates provided by J.P. Segrest [University of Alabama at Birmingham, Birmingham, AL] and KcsA coordinates deposited in the Protein Data Bank (accession no. 3EFF). The BLM was constructed using Visual Molecular Dynamics software. (B) Spontaneous transfer of channels as a function of time from NABBs after adding NABBs with or without KcsA E71A to BLMs. The same amount of KcsA, from a 2- μ g/ml stock, was added in each case.

Initial incorporations of KcsA from PE/PG liposomes were uniformly distributed throughout the 15 min.

To investigate whether the incorporation of KcsA into NABBs (see Figs. 1 and 2) and its subsequent transfer to the bilayer affected channel function, we analyzed the single-channel conductance and the gating of KcsA E71A in the BLM at different trans-bilayer voltages in the presence or absence of Ba²⁺, a well-known blocker of K⁺ channels (Neyton and Miller, 1988). In the absence of Ba²⁺, the electrophysiological trace showed long channel openings with very brief closures (at 100-mV trans-bilayer voltage), characteristic of non-inactivating KcsA E71A. The channel was almost always open at pH 4.0 (open probability [Po], ~0.98) and closed at pH 7, expected from the E71A variant (Cordero-Morales et al., 2007), and was blocked by micromolar concentrations of intracellular (trans side of the partition) Ba²⁺ (Fig. 3 A). The single-channel conductance recorded over a range of voltages closely resembled that previously reported for E71A KcsA (Cordero-Morales et al., 2007; Thompson et al., 2008) and was not affected by Ba²⁺ (Fig. 3 B). However, the channel Po decreased in a voltage-dependent way in the presence of intracellular Ba²⁺ as a result of an increase in the residence time of Ba²⁺ on its binding site in the electric field of the membrane (Fig. 3 C), consistent with Ba²⁺ acting as a slow voltage-dependent blocker of KcsA (Piasta and Miller, 2009). Assuming that the blocker dissociates back toward the intracellular solution, a Woodhull (Woodhull, 1973) equilibrium block fit of the decrease in Po with voltage in the presence of intracellular Ba²⁺ yields an apparent affinity of block of ~48 μM, which is consistent with the Ba²⁺ block affinity found for BK channels (~36 μM at 100 mM K⁺) (Vergara and Latorre, 1983). Thus, the KcsA E71A channels studied here display similar functional properties when delivered from NABBs to lipid bilayers compared with delivery from channel-containing liposomes.

DISCUSSION

The method generally used to incorporate MPs into a lipid bilayer for single-channel electrophysiology was via vesicular fusion with the target bilayers (Cohen et al., 1980). We show here that it is possible to directly transfer ion channels from NABBs to planar lipid bilayers, while keeping their properties intact, in a manner that does not involve an intermediate vesicular fusion step. Although we cannot fully exclude the possibility that the NABBs disintegrate to form vesicles at the extreme dilutions required to monitor single channels in the bilayers, it seems unlikely considering that vesicles were not observed in EM micrographs where the sample was similarly diluted. Furthermore, from our observations, the efficiency of KcsA incorporation into BLMs from NABBs (where the main lipid is POPC) was similar to that observed using PE/PG liposomes, with the channels

incorporating earlier when they transfer from NABBs. This would not be expected if the incorporations from the NABB sample resulted via POPC vesicle formation, because POPC vesicles are known to be less fusogenic than PE/PG vesicles (Chernomordik and Kozlov, 2003).

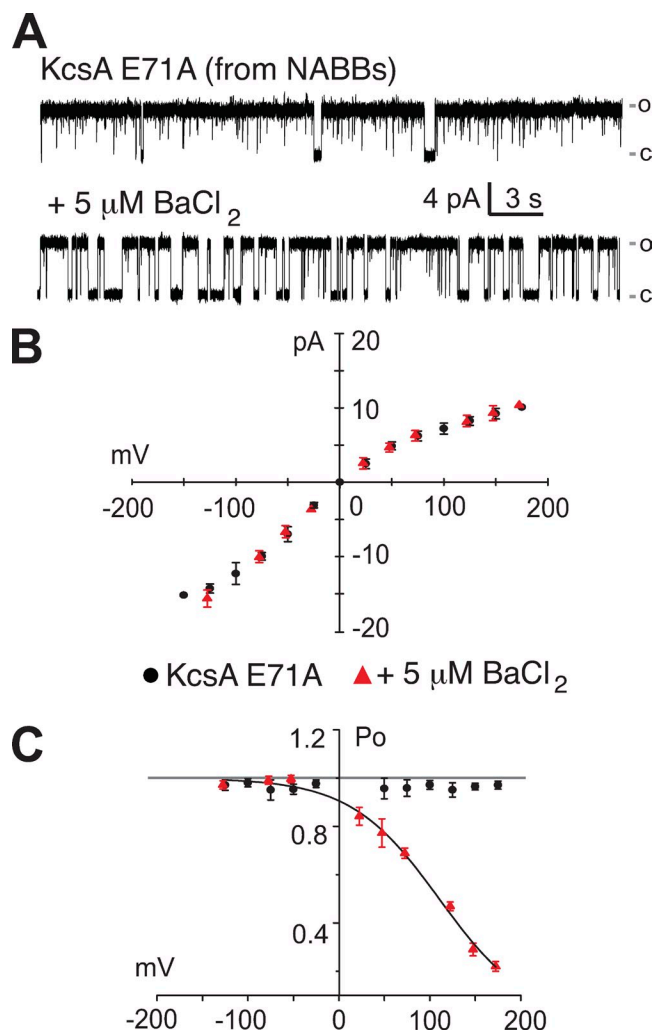


Figure 3. KcsA E71A functional properties are preserved after transfer from NABBs to BLMs. (A) Representative single-channel traces of KcsA E71A transferred from 1K-NABBs to BLMs in the absence (top) or presence (bottom) of 5 μM BaCl₂ added to the trans chamber. The electrophysiology recording conditions were 100 mM of symmetric K⁺ across the BLM ($V_m = +100$ mV; low-pass filter at 1 kHz). (B) Single-channel I-V plot of KcsA E71A transferred from NABBs. (C) Open probability (Po) of the transferred channel as a function of voltage. The change in Po in the case of Ba²⁺ block was fit to the equation:

$$P_o(V) = \frac{1}{1 + \frac{B}{K_0 \cdot e^{-\frac{zFV}{RT}}}}$$

where B is the blocker concentration (5 μM), K₀ is the apparent blocker affinity at 0 mV, and z is the voltage dependence. The fit values for K₀ and z were 48.2 ± 5.6 μM and 0.54 ± 0.02, respectively. Symbols represent averages ± SD from three separate experiments.

Thus, vesicle fusion does not seem to be the transfer mechanism here. These results open up the intriguing possibility that the transfer of MPs from vesicles to planar lipid bilayers may also occur via a similar direct transfer mechanism in addition to, or instead of, vesicle fusion.

What could be the mechanism of a direct bilayer-to-bilayer protein transfer from NABBs to BLMs? It is possible that when they come in close proximity, some lipids in the NABBs transfer to the BLM along with the protein cargo, followed by a remodeling of the lipid-poor apo A-I (Li et al., 2004; Catte et al., 2006). Lipid transfer between reconstituted high-density lipoprotein and vesicles has been reported (Tricerri et al., 2005), and it has been hypothesized to occur as a result of lipid desorption from nanodiscs, which is entropically favorable because of the closer packing of lipids in a disc compared with other membranes (Nakano et al., 2009). It is likely that presence of the MP further constrains the lipid chains in the NABB. The molecular mechanism of this form of transfer via nonvesicular fusion is as yet unknown. Other nonconventional surfactants have also been used for MP delivery to lipid membranes, but with limited success. Amphipols caused a perturbation of the target bilayer by dissolving into the bilayer (Pocanschi et al., 2006), whereas hemifluorinated surfactants reportedly resulted in denaturation of the MP during transfer (Popot, 2010). We have not encountered either of these problems using NABBs.

In conclusion, NABBs provide a stable, soluble lipid environment for functional reconstitution of ion channels. The incorporated channels may be transferred directly to other model membranes for biophysical assays, and the channels retain their functional properties as measured with sensitive single-molecule assays. Further characterization of the transfer of MPs, including in vitro–translated (Katzen et al., 2008) channels, from NABBs to other bilayer systems will enhance the study of MPs and extend the versatility of discoidal lipoproteins as an ideal platform for MPs outside of the cellular environment.

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