Published in final edited form as:

Methods Mol Biol. 2020 ; 2127: 207–225. doi:10.1007/978-1-0716-0373-4_15.

Reconstitution of Proteoliposomes for Phospholipid Scrambling and Nonselective Channel Assays

Maria E. Falzone, Alessio Accardi

Abstract

Phospholipid scramblases catalyze the rapid trans-bilayer movement of lipids down their concentration gradients. This process is essential for numerous cellular signaling functions including cell fusion, blood coagulation, and apoptosis. The importance of scramblases is highlighted by the number of human diseases caused by mutations in these proteins. Because of their indispensable function, it is essential to understand and characterize the molecular function of phospholipid scramblases. Powerful tools to measure lipid transport in cells are available. However, these approaches provide limited mechanistic insights into the molecular bases of scrambling. Here we describe in detail an in vitro phospholipid scramblase assay and the accompanying analysis which allows for determination of the macroscopic rate constants associated with phospholipid scrambling. Notably, members of the TMEM16 family of scramblases also function as nonselective ion channels. To better understand the physiological relevance of this channel function as well as its relationship to the scrambling activity of the TMEM16s we also describe in detail an in vitro flux assay to measure nonselective channel activity. Together, these two assays can be used to investigate the dual activities of the TMEM16 scramblases/nonselective channels.

Keywords

Phospholipids; Scrambling; Scramblases; Lipid transport; TMEM16; Nonselective channel

1 Introduction

The eukaryotic cell membrane is asymmetric at rest with phosphatidylserine (PS) and phosphatidylethanolamine (PE) lipids sequestered to the inner leaflet of the bilayer while phosphatidylcholine (PC) and sphingolipids are exposed on the external leaflet. This asymmetry is generated and maintained by energy-dependent lipid flippases and floppases that transport lipids against their concentration gradients and is disrupted by phospholipid scramblases that transport lipids down their concentration gradients [1, 2]. Scramblase activation results in the exposure of PS on the extracellular surface, which is an essential signaling step in many processes including apoptosis, blood coagulation, and cell fusion [3, 4]. The indispensable role of scramblases in these signaling processes underscores their importance and mutations in many scramblases cause human diseases including disorders of blood coagulation, muscle repair, and bone formation [3, 4]. Thus far, members of two protein families have been identified as phospholipid scramblases, the TMEM16 Ca^{2+} -activated scramblases [5–11] and the caspase-activated XK-related (Xkr) proteins [12, 13]. Additionally, several G-protein-coupled receptors (GPCR's), including rhodopsin,

bacteriorhodopsin, and the β -adrenergic receptor, exhibit phospholipid scrambling activity in vitro [14–16]. Finally, several peptides mediate lipid scrambling in vitro [17, 18], and most recently a DNA nanostructure was shown to mediate rapid transbilayer lipid movement in synthetic vesicles and in cells [19], underscoring the diverse chemical natures and three-dimensional architectures of molecules that mediate lipid scrambling.

In cells, phospholipid scrambling can be measured using fluorescently labeled proteins, such as Annexin V and LactC2, that specifically recognize and bind to PS lipids [5, 20]. As the membranes of apoptotic cells lose integrity, these probes can access and bind PS on both membrane leaflets. Therefore, it is critical to use nuclear-staining probes such as propidium iodide or DAPI to separate apoptotic cells from healthy ones that externalize PS due to the activation of a scramblase. These assays are essential to determine if a protein is associated with scrambling in cells, and indeed enabled the identification of both TMEM16 and Xkr proteins as scramblases [5, 21]. However, these cell-based approaches provide only limited information about the activation mechanisms of the scramblases, on their transport kinetics and lipid specificity. Further, results from these assays leave a degree of ambiguity as to whether the identified proteins directly mediate lipid scrambling, or if they are indirect activators of other endogenous scramblases [22]. In vitro measurements of lipid scrambling using purified and reconstituted proteins circumvent these pitfalls and provide complementary information to that obtained from cell-based assays. The main advantages of in vitro assays are a near-complete control over the experimental system via the choice of membrane lipid composition, of the intra- and extra-liposomal solutions, and that—in some cases—they enable measurement of the kinetics of lipid transport [9, 23–25]. This allows a thorough characterization of the biophysical properties of the scramblases, which is essential to understand their function and their role in cellular signaling functions. The main limitations of the in vitro measurements are the requirement for purified protein, the lack of knowledge of the orientation of the reconstituted molecules in the liposomal membrane, and the use of non-native lipids due to the need for fluorescence to track their movement between leaflets. Recent advances in protein purification techniques have enabled the purification of several mammalian scramblases [9, 10], thus reducing the first hurdle, and some reconstitution approaches result in a preferential mode of protein incorporation in the membrane, diminishing the caveats associated with the second limitation [26]. Finally, it is important to carry out extensive controls to ensure that the position and nature of the chemically modified lipids does not affect the observations [7].

Several in vitro assays to measure scrambling have been developed, most of which use fluorescent nitrobenzoxadiazole (NBD)-labeled lipids [7, 14, 27]. These assays quantify the fluorescent lipids in the outer leaflet of the liposomes by adding a membrane-impermeant reagent that chemically reduces the fluorophore (i.e., dithionite) or a lipid-extracting agent that quenches the fluorescence (i.e., BSA). Alternative assays using radiolabeled lipids have been developed to monitor transbilayer movement of natural lipids [28–31]. However, these approaches are generally limited in time resolution, require synthesis of radiolabeled lipids and, in some cases, the use of phospholipases to cleave the externalized radiolabels from the membrane. The most frequently used assay, which will be described here, utilizes the irreversible reduction of NBD by membrane-impermeant sodium dithionite. This assay has

been used to characterize several TMEM16 scramblases as well as the secondary scramblase function of GPCRs [5–8, 12, 14–16, 21, 23].

In the absence of a scramblase protein, fluorescently labeled lipids distribute nearly evenly between liposomal membrane leaflets (Fig. 1a, top panel). Upon addition of an extra-liposomal reducing agent a ~50% decrease of fluorescence is expected [7, 14]. In the presence of an active scramblase, there is rapid and bidirectional movement of lipids between leaflets (Fig. 1a, lower panel). Thus, under the assumption that fluorescently labeled lipids are transported as well as unlabeled lipids, a ~100% loss in fluorescence is expected as all fluorophore-conjugated lipids are reduced (Fig. 1a, lower panel). The time constant associated with the chemical reduction of free NBD in solution by dithionite is fast, <1 s [7, 23]. However, the proximity of the fluorophore to the lipid membrane slows the reaction to ~10–30 s, depending on the lipid used and on whether the NBD label is conjugated to the head or the tail of the lipid. This enables the extraction of kinetic information on scrambling processes that occur on time scales comparable to or slower than the time constant of the chemical reduction [7, 23]. It is important to note that the fluorescence decay after dithionite in proteoliposomes containing a scramblase does not generally reach 0, but rather plateaus at a finite value. This reflects the fact that a fraction of the vesicles is refractory to protein reconstitution and/or are multilamellar, and therefore not accessible to the dithionite [7, 14, 23, 24, 32].

The TMEM16 scramblases also function as nonselective ion channels [7, 32] [20] [11], and some TMEM16 homologs function as bona fide Ca^{2+} -activated, Cl^- selective channels [33–35]. The physiological role of the nonselective ion channel activity of the TMEM16 scramblases is unknown. Initially, it was hypothesized that nonspecific ion transport was a by-product of scrambling, as ions and lipids might traverse the membrane through a proteolipidic pore [36, 37]. However, the identification of mutations that interconvert TMEM16 proteins between scramblases and Cl^- channels [20, 37], as well as scramblase mutants that affect only lipid scrambling but not ion transport, and vice versa [24], suggested this might not be the case. Indeed, recent structural work on the TMEM16A Cl^- channel and on the nhTMEM16 scramblase shows that these proteins form a protein-delimited pore [38–41]. In cells, the ion channel activity of the TMEM16 scramblases can be readily assayed using the patch clamp technique [10, 20, 37, 42]. However, in liposomes the nonselective nature of the TMEM16 scramblase/channels has only been measured using an end-point ion flux assay [7, 23, 24, 32]. In this assay, proteoliposomes are buffer exchanged from high external KCl to low KCl and the trapped internal Cl^- is measured using a Cl^- electrode following disruption of the liposomes with detergent. Only liposomes containing at least one active non-selective channel will lose their chloride content during the buffer exchange step. Thus, the ratio of the Cl^- trapped in TMEM16 proteoliposomes to that trapped in protein-free vesicles reflects the fraction of vesicles containing at least one active channel. The assay is robust and sensitive enough to determine whether the reconstituted protein has nonselective channel activity. However, as an endpoint measurement, it provides no kinetic information. Thus, it can underestimate the effects of various manipulations (i.e. mutations and/or changes in ligand concentration) on the ion transport activity of the scramblase [7, 24]. Together, the scrambling and flux assays have been used to extensively characterize the TMEM16 scramblases contributing significantly to our understanding of both functions of

these proteins [7, 23–25, 32]. Here, we provide a step-by-step description of scrambling and flux assays, including the details of liposome preparation, and of how to analyze the resulting data.

2 Materials

Prepare all solutions using deionized ultrapure water.

2.1 Liposome Reconstitution

1. 10 M KOH (50 mL).
2. 1 M HEPES pH 7.4 (1 L).
3. 2 M KCl (1 L).
4. Reconstitution buffer, 50 mM HEPES pH 7.4, 300 mM KCl, 50 mL: Add 2.5 mL of 1 M HEPES pH 7.4 stock and 7.5 mL of 2 M KCl to graduated cylinder. Bring volume up to 50 mL. Mix and store at room temperature.
5. 20 mg/mL phospholipid stocks in chloroform: adjust concentration of purchased stock to 20 mg/mL using chloroform or dissolved powdered lipids in chloroform.
6. 1 mg/mL NBD-labeled lipid stock in chloroform: adjust concentration of purchased stock to 1 mg/mL using chloroform or dissolved powdered lipids in chloroform.
7. Bio-Beads™ SM-2 Resin.
8. (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (CHAPS) powder.
9. Water-bath sonicator.
10. 4× Laemmli Sample Buffer (Bio-Rad).

2.2 Scrambling Assay

1. 1 M $\text{Ca}(\text{NO}_3)_2$ (50 mL).
2. 0.5 mL ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) pH 7.4 (50 mL): Add 25 mL H_2O to a beaker with a magnetic stirbar. Add 9.5 g of EGTA and mix on magnetic stirplate. Using a pH meter, slowly add in 10 M KOH to facilitate the dissolving until the final pH is 7.4. When the solid is dissolved and the pH is at 7.4. Bring volume up to 50 mL with H_2O . Filter and store at room temperature.
3. Assay Buffer + Ca^{2+} , 50 mM HEPES pH 7.4, 300 mM KCl, 0.5 mM $\text{Ca}(\text{NO}_3)_2$, 50 mL: Add 2.5 mL of 1 M HEPES pH 7.4 stock, 7.5 mL of 2 M KCl, and 25 μL of 1 M $\text{Ca}(\text{NO}_3)_2$ stock to graduated cylinder. Bring volume up to 50 mL with H_2O . Mix and store at room temperature.
4. Assay Buffer +EGTA, 50 mM HEPES pH 7.4, 300 mM KCl, 2 mM EGTA, 50 mL: Add 2.5 mL of 1 M HEPES pH 7.4 stock, 7.5 mL of 2 M KCl, and 200 μL

EGTA from 0.5 M stock to graduated cylinder. Bring volume up to 50 mL with H₂O. Mix and store at room temperature.

5. 1 M Tris-HCl pH 10 (50 mL)
6. Sodium dithionite aliquots: Weigh a 10 mg aliquot of sodium dithionite into a 1.5 mL Eppendorf tube for every experiment that will be completed. Store on ice. This should be done the day of the experiment.
7. Liposome Extruder and 400 nm membrane.
8. Fluorescence spectrophotometer and a computer with an appropriate acquisition software.
9. Magnetic stirplate for fluorescence spectrophotometer.
10. Fluorescence cuvettes.
11. Magnetic stirbar that fits inside fluorescence cuvette.

2.3 Ion Flux Assay

1. Extracellular Solution +Ca²⁺, 1 mM KCl, 50 mM HEPES pH 7.4, 300 mM Na-glutamate, 0.5 mM Ca(NO₃)₂, 100 mL: Add 60 mL H₂O to a beaker with a magnetic stirbar. Add 5 mL of 1 M HEPES pH 7.5 stock, 50 µL of 2 M KCl stock, and 50 µL of 1 M Ca(NO₃)₂ stock. Add 5.6 g of Na-glutamate monohydrate and mix on magnetic stirplate. When solid is dissolved adjust pH to 7.4 using KOH. Use graduated cylinder to bring volume to 100 mL with H₂O. Store at room temperature.
2. Extracellular Solution +EGTA, 1 mM KCl, 50 mM HEPES pH 7.4, 300 mM Na-glutamate, 2 mM EGTA, 100 mL: Add 60 mL H₂O to a beaker with a magnetic stirbar. Add 5 mL of 1 M HEPES pH 7.5 stock, 50 µL of 2 M KCl stock, and 400 µL of 0.5 M EGTA stock. Add 5.6 g of Na-glutamate monohydrate and mix on magnetic stirplate. When solid is dissolved adjust pH to 7.4 using KOH. Use graduated cylinder to bring volume to 100 mL with H₂O. Store at room temperature.
3. G50 beads swelled with extracellular solutions: Weigh 0.6 mg of G50 for every 10 mL of resin desired and place in a 50 mL falcon tube. Add extracellular solution up to desired volume. Rotate overnight (at least 4 h) at room temperature.
4. 100 mM KCl stock (50 mL).
5. 10 mM KCl stock (50 mL).
6. Agar bridges (100 mM KCl and 2% Agarose) stored in 100 mL KCl.
7. 1.5 M Octyl-beta-Glucoside (5 mL): Add 2.2 g of octyl-beta-glucoside to a 15 mL falcon tube with 2 mL of H₂O. Incubate at 37 °C for 10 min. When solid is dissolved, ensure the volume is 5 mL. The solution will be very viscous.
8. Thermo IEC Centra CL2 centrifuge.

9. Saturated solution of FeCl_3 or bleach.
10. Two chamber recording setup with flat-bottomed cylinders, ~3–4 mL volume secured on a magnetic stirplate.
11. A magnetic stirbar that fits inside the sample chambers.
12. AgCl electrode.
13. pH meter with an analog or digital electrical output.
14. A digitizer.
15. A computer with an appropriate acquisition software.

3 Methods

3.1 Preparation of Bio-Beads for Both Assays

1. Use 150 mg/mL Bio-Beads™ SM-2 Resin for each condition for each incubation. For lipid compositions with melting temperatures near room temperature complete four incubations. For lipid compositions with melting temperatures around or below 4 °C complete five incubations.
2. Weigh the desired amount of biobeads and add to a new 50 mL falcon tube.
3. Add 30 mL of methanol and rotate at room temperature for 20 min.
4. Let the biobeads settle and pour off the methanol, add 50 mL of H_2O , and rotate at room temperature for 20 min.
5. Let the biobeads settle and pour off the water, add another 50 mL of H_2O , and rotate at room temperature for 20 min.
6. Let the biobeads settle and pour off the H_2O , add 30 mL of reconstitution buffer, and rotate at room temperature for 20 min.
7. Let the biobeads settle, pour off the buffer and use a pipette to remove additional liquid.
8. Weigh the necessary amount of biobeads for each incubation into 2 mL Eppendorf tubes.

3.2 Lipid Preparation

For the scrambling assay, prepare a starting volume of 200 μL of 20 mg/mL lipids per condition (i.e., protein-free, with protein +/- ligand). For the flux assay, prepare a starting volume of 500 μL of 20 mg/mL lipids per condition. For both assays, always include a protein-free condition for each reconstitution.

1. Mix the desired chloroform-dissolved lipids together in glass tubes and dry under a stream of compressed nitrogen. For the scrambling assay, add 1% w/w of the desired NBD-labeled lipid (see Note 1).

2. Add an equal volume of pentane to the dried lipid film, gently resuspend the lipids and dry again under a stream of compressed nitrogen while rotating the tube to form a uniform lipid film on the bottom of the tube.
3. When all pentane has evaporated, add solid CHAPS to the dried lipid film to a concentration of 35 mM according to the final desired volume.
4. Add the desired reconstitution buffer volume (no more than 500 μ L) taking care to wash down all the CHAPS on the sides of the tube (*see* Note 2).
5. Incubate for 20 min at room temperature to dissolve CHAPS.
6. Sonicate using bath sonicator until lipids are completely solubilized producing a clear and transparent solution (*see* Note 3).
7. When solution is clear, bring the volume up to final desired volume for 20 mg/mL lipids using reconstitution buffer.
8. Separate the lipids for each condition (i.e., protein-free and protein-containing).
9. Add the protein at desired ratio (for TMEM16's we use 5 μ g protein/mg lipid).
10. Incubate at room temperature for 20 min.
11. Add lipids to 2 mL Eppendorf tubes with pre-weighed bio-beads. Place individual tubes into a 50 mL falcon tube and incubate on a rotator for 2 h at 4 °C.
12. After 2 h, briefly spin down the individual tubes and transfer the lipids into the next tube of pre-weighed biobeads. Remove 15 μ L of liposomes for SDS-PAGE analysis. Repeat 2-h incubation at 4 °C.
13. Change biobeads again after 2 h and rotate overnight (12–14 h) at 4 °C (*see* Note 4).
14. After the overnight incubation, change the biobeads one or two more times depending on the lipid composition (*see* bio-bead preparation section).
15. After the final incubation, remove the liposomes from the biobeads and transfer into glass tubes. Solution should be slightly cloudy. Remove 15 μ L of liposomes for SDS-PAGE analysis.
16. Freeze at –80 °C if not using immediately.

3.3 Analysis of Reconstitution Efficiency Using SDS-PAGE

1. Remove 15 μ L of liposomes from reconstitution as described above. Store at –80 °C without SDS loading dye until running the gel.
2. Thaw samples before use.
3. Add 5 μ L of 4 $\times$ SDS loading dye.
4. Run the SDS-PAGE gel as any other gel (*see* Note 5).

3.4 Freeze and Thaw Cycles to Introduce Ligand to the Inside of the Liposomes

1. Thaw liposomes if they are frozen.
2. Measure the volume of each condition. Loss of 10–30% of the starting volume is normal.
3. Add the desired amount of ligand according to the measured volume (i.e., Ca^{2+} or EGTA).
4. Sonicate liposomes for 2–3 s.
5. Flash freeze in liquid nitrogen.
6. Leave at room temperature to thaw.
7. Repeat 4–6.
8. Flash freeze (without sonicating) in liquid nitrogen for a third time.
9. Store at $-80\text{ }^{\circ}\text{C}$ until use and then thaw on ice.

3.5 Phospholipid Scrambling Assay

1. Set up the fluorometer to collect a 1200 s time-course with excitation at 470 nm and emission at 530 nm.
2. Thaw liposomes on ice if they are frozen.
3. Extrude the liposomes 21 times using 400 nm membrane (*see* Note 6 and 7).
4. Store the extruded liposomes at room temperature.
5. Add 1.98 mL of assay buffer to the cuvette with a stirbar.
6. Add 20 μL of extruded liposomes to the cuvette.
7. Start recording the fluorescence signal.
8. Add 60 μL of the 1 M Tris pH 10 stock to one aliquot of solid sodium dithionite and dissolve by vortexing.
9. At $t = 100\text{ s}$ of the fluorescence recording, add 40 μL of the dithionite solution into the cuvette while still recording (*see* Note 8).
10. Record the fluorescence decay until the signal plateaus or for $\sim 20\text{ min}$.
11. After each experiment wash the cuvette and stirbar with water.

3.6 Quantification of Scrambling Activity

The scrambling assay described above can be utilized to extract detailed mechanistic information on the activity of the reconstituted protein. This information is contained in the steady-state level at which the fluorescence decay plateaus after dithionite addition and in the kinetics of the decay itself. Two analysis approaches have been used to extract mechanistic information: monitoring changes in the fluorescence at a fixed time point or analysis of the full-time course of fluorescence decay.

3.6.1 Approach 1: Fixed Time Fluorescence—When scrambling is fast compared to the rate of chemical reduction of the fluorophores, the kinetics of scrambling cannot be separated from those of the chemical reduction [7, 14, 23]. Therefore, the plateau value of the fluorescence decay is the only functional readout of the scrambling activity of the reconstituted proteins [7, 14, 15] as it directly reflects the fraction of liposomes containing at least one active scramblase. Indeed, when determined at various protein reconstitution densities, it can be used to determine the stoichiometry of the active scramblase complex [7, 15, 43]. However, the dynamic range of the signal associated with these changes is small; in ideal conditions, the maximal reduction is 50% of the total fluorescence, but in practice the range is ~30–40% due to the presence of liposomes lacking protein (Fig. 2a). This, together with the intrinsic variability of the plateau level, reduces the available range of the signal, potentially obfuscating the effects of changes in ligand concentrations or mutations that do not cause complete loss-of-function. To obviate—in part—these limitations, it is useful to consider the drop in fluorescence at a fixed time point when all lipids on the external leaflet have been reduced, which is $t = 3\tau$, where τ is the time constant of the decay in protein-free liposomes. This also allows to partially capture the effects of manipulations that reduce the rate of scrambling but not the final plateau [7, 10, 32, 44].

1. Normalize the trace to the fluorescence value at $t = 90\text{--}95$ s (right before the addition of dithionite).
2. Fit the normalized fluorescence decay of the protein-free liposomes to a single exponential function and determine the time constant.
3. Determine the normalized fluorescence value at $t = 3\tau$ from the protein-containing traces and from the protein-free liposomes.
4. The relative change in fluorescence, F , can be determined as the ratio of the change in a given condition, $F^* = F_{\text{PF}}(t = 3\tau) - F^*(t = 3\tau)$, to that measured in conditions of maximal activity, $F_{\text{Max}} = F_{\text{PF}}(t = 3\tau) - F_{\text{Max}}(t = 3\tau)$, where $F_{\text{PF}}(t = 3\tau)$ is the fluorescence value at $t = 3\tau$ measured in protein-free liposomes, and $F^*(t = 3\tau)$ and $F_{\text{Max}}(t = 3\tau)$, respectively, are the fluorescence values measured at $t = 3\tau$ for the experimental condition

$$\Delta F = \frac{100 \cdot \Delta F(t = 3\tau)}{\Delta F_{\text{Max}}(t = 3\tau)} = \frac{100 \cdot (F_{\text{PF}}(t = 3\tau) - F^*(t = 3\tau))}{F_{\text{PF}}(t = 3\tau) - F_{\text{Max}}(t = 3\tau)} \quad (1)$$

3.6.2 Approach 2: Analysis of the Kinetics of Fluorescence Decay—In conditions where the kinetics of scrambling are distinguishable from those of the NBD chemical reduction, the time course of fluorescence decay can be analyzed to determine the macroscopic rates of scrambling. Below we detail the analysis procedure as well as the main assumptions and limitations of the approach [23].

The time course of fluorescence decay after dithionite addition to a population of liposomes containing a scramblase is described by a three-state Markov model (Fig. 1a)

where L_i are fluorescent lipids in the inner leaflet, L_o are fluorescent lipids in the outer leaflet, L^* are the fluorescent lipids that were irreversibly reduced by sodium dithionite, α and β are the forward and reverse macroscopic rate constants of scrambling, and γ is the rate constant of dithionite reduction of NBD. It is important to note that α and β are macroscopic rate constants, reflecting the rate of lipid scrambling of a liposome. Therefore, α and β are the product of the unitary rates of lipid transport (i.e., the conductance), of the number of active scramblases, and of the probability of the scramblase to be in a conductive conformation. This value depends on the number of active scramblase proteins reconstituted and on the number of liposomes. Since a fraction, f_0 , of liposomes is refractory to protein incorporation, the time course of fluorescence decay is described by the sum of three exponentials

$$F_{\text{tot}}(t) = f_0(L_i^{PF}(0) + (1 - L_i^{PF}(0))e^{-\gamma t}) + \frac{(1 - f_0)}{D(\alpha + \beta)} \times \left\{ \alpha(\lambda_2 + \gamma)(\lambda_1 + \alpha + \beta)e^{\lambda_1 t} + \lambda_1\beta(\lambda_2 + \alpha + \beta + \gamma)e^{\lambda_2 t} \right\} \quad (2)$$

where

$$\lambda_1 = -\frac{(\alpha + \beta + \gamma) - \sqrt{(\alpha + \beta + \gamma)^2 - 4\alpha\gamma}}{2}$$

$$\lambda_2 = -\frac{(\alpha + \beta + \gamma) + \sqrt{(\alpha + \beta + \gamma)^2 - 4\alpha\gamma}}{2}$$

and

$$D = (\lambda_1 + \alpha)(\lambda_2 + \beta + \gamma) - \alpha\beta$$

where L_i^{PF} is the fraction of fluorescent lipids on the extracellular leaflet in protein-free liposomes. Importantly, L_i^{PF} and γ are experimentally determined by fitting the fluorescence decay of protein-free liposomes to:

$$F_{PF}(t) = L_i^{PF}(0) + (1 - L_i^{PF}(0))e^{-\gamma t} \quad (3)$$

1. Measure scrambling in protein-free liposomes and in proteoliposomes prepared in the desired conditions.
2. Normalize the trace to the fluorescence value at $t = 90\text{--}95$ s (right before the addition of dithionite).

3. Fit the protein-free fluorescence decay to Eq. 3 to determine L_i^{PF} and γ .
4. Determine α , β , and f_0 by fitting the fluorescence decay of proteoliposomes to Eq. 1, with L_i^{PF} and γ constrained to the values determined above.

The value of f_0 corresponds to the fraction of protein-free liposomes or inaccessible to dithionite. This parameter is affected by the variability of the reconstitution efficiency of the protein into liposomes, and changes in f_0 affect the estimates of α , β . Thus, to compare the effects of different manipulations on the protein (i.e., +/- ligand or WT vs. mutant) on α , β it is necessary to ensure that the efficiency of reconstitutions across the different conditions is comparable, as evaluated by the gels (see above). If this is the case, then the best procedure is to first determine f_0 for the WT protein in conditions of maximal activity, $f_0(WT)$, and fix the f_0 values of the other conditions to $f_0(WT)$. For example, for the Ca^{2+} -activated TMEM16 scramblases, the positive standard is the $+Ca^{2+}$ condition. To determine the rates in the absence of Ca^{2+} or of a mutant, the f_0 is constrained to the value of the WT protein in $+Ca^{2+}$ from the same reconstitution.

3.7 Flux Assay

3.7.1 Preparation of the Set-up

1. Strip the previous AgCl coating of the silver electrode with a razor blade.
2. Incubate the electrode in the saturated ferric chloride solution for 1 min.
3. Rinse with H_2O .
4. Place the electrode into the holder above the sample chamber and position the reference and sample electrodes into the appropriate chambers.
5. Add 100 mM KCl to the reference chamber, with enough to cover the end of the reference electrode.
6. Add 1.8 mL of extracellular solution to the sample chamber with a stirbar.
7. Connect the two chambers with the agar bridge by placing one end in each chamber. Ensure that the tip of the electrode and the ends of the agar bridges are inside the solution.
8. Start recording the voltage from the electrode.
9. To calibrate, add 18 μ L of 10 mM KCl to the sample chamber and note the change in voltage.
10. Complete two more additions and 18 μ L of 10 mM KCl noting the voltage each time.
11. Then complete three additions of 18 μ L of 100 mM KCl noting the voltage each time.
12. Save the calibrations to the experimental record.

3.7.2 Preparation of the G-50 columns

13. Prepare one G50 column for each sample by adding 3 mL of swelled G-50 resin to a 5 mL column with filter.
14. Remove excess buffer by centrifuging for 5 s at 5000 rpm using a Thermo IEC Centra CL2 centrifuge 2–3 times.

3.7.3 Preparation of liposomes

15. Thaw liposomes on ice if they are frozen.
16. Extrude the liposomes 21 times using 400 nm membrane (see Note 6 and 7).
17. Store the extruded liposomes at room temperature.

3.7.4 Flux measurement

18. Add 100 μ L of liposomes to a prepared G50 column and spin for 1 min at 2000 rpm using a Thermo IEC Centra CL2 centrifuge.
19. While the sample is spinning, start the recording and add 18 μ L of 10 mM KCl to the sample chamber for calibration. Note the voltage change.
20. Add 200 μ L of the flow-through from the G50 column into the sample chamber.
21. Note the remaining volume in the tube to determine how dilute the final sample was.
22. After ~30s, add 40 μ L of 1.5 M octyl-beta-glucoside to disrupt the liposomes and release the trapped Cl^- . Note the voltage change.

3.8 Flux Assay Analysis

1. Export the trace file from an ASCII or text format and import it to the analysis program of choice.
2. Measure the voltage at the following points (Fig. 3):
 - At the beginning of the recording (V_0);
 - After addition of the calibrating pulse (V_{cal});
 - After addition of the liposomes (V_{lipo});
 - After addition of detergent (V_{tot});
3. Use the Nernst equation to determine the experimental value of $\alpha = \frac{RT}{zF}$ (see Note 9) as

$$\alpha = \frac{\Delta V_{\text{cal}}}{\ln\left(1 + \frac{\text{Vol}_{\text{Cal}}}{1800} \cdot \frac{[\text{Cl}]_{\text{cal}}}{[\text{Cl}]_{\text{in}}}\right)} \quad (4)$$

where Vol_{cal} is the volume of the calibration pulse in μL , 1800 is the chamber volume at the beginning of the experiment expressed in μL , $[Cl]_{cal,in}$ are the Cl^- concentrations of the calibration pulse and of the external buffer in mM and $V_{cal} = V_{cal} - V_0$ expressed in mV.

4. Calculate the Cl^- concentrations from the measured voltages as follows:

$$[Cl]_{lipo} = \left([Cl]_{in} + \frac{9}{1000} [Cl]_{cal} \right) e^{(V_{lipo} - V_{cal})/\alpha} \quad (5)$$

$$[Cl]_{tot} = \Delta Cl_{lipo} e^{(V_{tot} - V_{lipo})/\alpha} \quad (6)$$

so that the Cl^- content of the liposomes is

$$\Delta Cl = [Cl]_{tot} - [Cl]_{prot} \quad (7)$$

The factor 9/1000 comes from the dilution of the 18 μL calibration pulse into the 2000 μL final volume.

5. Calculate the channel activity of the sample, A, as the percentage of liposomes containing at least one active nonselective channel using the following equation:

$$A = \left(1 - \frac{\Delta Cl_{prot}}{\Delta Cl_{PF}} \right) 100 \quad (8)$$

where $Cl_{prot,PF}$ are, respectively, the chloride content of the proteoliposomes and of the protein-free vesicles.

4 Notes

1. It is helpful for sonication and lipid resuspension to separate lipids into tubes with no more than 2 mL of lipids. For example, if you need 4 mL of lipids, it is easier to prepare 2 tubes with 2 mL each rather than one tube with 4 mL.
2. Sonication is more effective with smaller volumes. It is best to resuspend the lipids and CHAPS in no more than 500 μL of buffer and bring the lipids to the final volume after they are resuspended. Of course, if your desired volume is less than 500 μL , then add the entire desired volume.
3. If lipids do not become clear with sonication alone, cycles of sonication and freezing and thawing can be helpful.
4. For lipid compositions with melting temperatures around room temperature (particularly POPE:POPG mixtures), it is very important to be strict with the incubation times. Leaving the liposomes longer (i.e., 15 h) results in a loss of protein from the liposomes.

5. To monitor the reconstitution efficiency and incorporation of the protein of interest, we use SDS-PAGE analysis of samples taken throughout the reconstitution (after the first incubation with biobeads and from the final sample before extrusion, see steps 12 and 15 above). This confirms that the protein was not lost during the reconstitution and is important for situations where a mutation or a ligand might result in reduced activity. Knowing the reconstitution efficiency is also important for the rate analysis as discussed later. In general, it is advisable to compare the intensity of the bands using densitometry [15]. This is of critical importance for samples with low protein-to-lipid ratios, as changes in the reconstitution efficiency can lead to large changes in the number of proteins inserted in each vesicle and in the total fraction of protein-free liposomes. At high protein-to-lipid ratios each liposome contains multiple copies of the reconstituted protein; a qualitative estimate of reconstitution efficiency can suffice, as small changes in the number of proteins per liposomes will not significantly affect the estimates of the kinetic parameters [39]. Adding SDS-loading dye to liposomes will make the solution viscous; ensure that care is taken when handling the sample and loading the gel.
6. For liposomes with melting temperatures around room temperature, they are likely to be in a gel phase after thawing on ice. Before extruding warm them by vortexing briefly or using your hands. This ensures that the gel material is not left inside the tube.
7. The number of times extruded must be odd so that you collect the extruded liposomes in the opposite syringe from which you loaded the sample.
8. It is important to have a fluorometer where solutions can be added without opening the lid to ensure recording of the initial kinetic information of the fluorescence decay after dithionite addition.
9. The empirical determination of α serves three purposes: it ensures that the system is responding properly, it offers a measure of the consistency of the instrument's response between experiments, and it is a check that no mistakes were made in the solution making.

Acknowledgement

The authors thank members of the Accardi lab for helpful discussions. This work was supported by NIH Grant R01GM106717 (to A.A.). M.E.F. is the recipient of a Weill Cornell Medicine Margaret & Herman Sokol Fellowship.

References

1. Pomorski T, Menon AK (2006) Lipid flippases and their biological functions. *Cell Mol Life Sci* 63(24):2908–2921 [PubMed: 17103115]
2. Bevers EM, Williamson PL (2016) Getting to the outer leaflet: physiology of Phosphatidylserine exposure at the plasma membrane. *Physiol Rev* 96(2):605–645. 10.1152/physrev.00020.2015 [PubMed: 26936867]
3. Kodigepalli KM, Bowers K, Sharp A, Nanjundan M (2015) Roles and regulation of phospholipid scramblases. *FEBS Lett* 589(1):3–14. 10.1016/j.febslet.2014.11.036 [PubMed: 25479087]

4. Nagata S, Suzuki J, Segawa K, Fujii T (2016) Exposure of phosphatidylserine on the cell surface. *Cell Death Differ* 23(6):952–961. 10.1038/cdd.2016.7 [PubMed: 26891692]
5. Suzuki J, Umeda M, Sims PJ, Nagata S (2010) Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* 468:834–838 [PubMed: 21107324]
6. Suzuki J, Fujii T, Imao T, Ishihara K, Kuba H, Nagata S (2013) Calcium-dependent phospholipid scramblase activity of TMEM16 protein family members. *J Biol Chem* 288 (19):13305–13316. 10.1074/jbc.M113.457937 [PubMed: 23532839]
7. Malvezzi M, Chalal M, Janjusevic R, Picollo A, Terashima H, Menon AK, Accardi A (2013) Ca^{2+} -dependent phospholipid scrambling by a reconstituted TMEM16 ion channel. *Nat Commun* 4 10.1038/ncomms3367
8. Brunner JD, Lim NK, Schenck S, Duerst A, Dutzler R (2014) X-ray structure of a calcium-activated TMEM16 lipid scramblase. *Nature* 516(7530):207–212 [PubMed: 25383531]
9. Bushell SR, Pike ACW, Falzone ME, Rorsman NJG, Ta CM, Corey RA, Newport TD, Shintre CA, Tessitore A, Chu A, Wang Q, Shrestha L, Mukhopadhyay S, Love JD, Burgess-Brown NA, Sitsapesan R, Stansfeld PJ, Huiskonen JT, Tommaro P, Accardi A, Carpenter EP (2019) The structural basis of lipid scrambling and inactivation in the endoplasmic reticulum scramblase TMEM16K. *Nat Commun* 10:3956 10.1038/s41467-019-11753-1 [PubMed: 31477691]
10. Alvadia C, Lim NK, Clerico Mosina V, Oostergetel GT, Dutzler R, Paulino C (2019) Cryo-EM structures and functional characterization of the murine lipid scramblase TMEM16F. *Elife* 8:e44365 10.7554/eLife.44365 [PubMed: 30785399]
11. Di Zanni E, Gradogna A, Scholz-Starke J, Boccaccio A (2017) Gain of function of TMEM16E/ANO5 scrambling activity caused by a mutation associated with gnathodiaphy-seal dysplasia. *Cell Mol Life Sci* 75:1–14. 10.1007/s00018-017-2704-9 [PubMed: 29080091]
12. Suzuki J, Denning DP, Imanishi E, Horvitz HR, Nagata S (2013) Xk-related protein 8 and CED-8 promote phosphatidylserine exposure in apoptotic cells. *Science* 341 (6144):403–406 [PubMed: 23845944]
13. Suzuki J, Imanishi E, Nagata S (2016) Xkr8 phospholipid scrambling complex in apoptotic phosphatidylserine exposure. *Proc Natl Acad Sci U S A* 113(34):9509–9514 [PubMed: 27503893]
14. Menon I, Huber T, Sanyal S, Banerjee S, Barré P, Canis S, Warren JD, Hwa J, Sakmar TP, Menon AK (2011) Opsin is a phospholipid flippase. *Curr Biol* 21(2):149–153. 10.1016/j.cub.2010.12.031 [PubMed: 21236677]
15. Goren MA, Morizumi T, Menon I, Joseph JS, Dittman JS, Cherezov V, Stevens RC, Ernst OP, Menon AK (2014) Constitutive phospholipid scramblase activity of a G protein-coupled receptor. *Nat Commun* 5:5115 10.1038/ncomms6115 [PubMed: 25296113]
16. Verchère A, Ou W-L, Ploier B, Morizumi T, Goren MA, Bütikofer P, Ernst OP, Khelashvili G, Menon AK (2017) Light-independent phospholipid scramblase activity of bacteriorhodopsin from *Halobacterium salinarum*. *Sci Rep* 7(1):9522–9522. 10.1038/s41598-017-09835-5 [PubMed: 28842688]
17. Kol MA, Van Laak ANC, Rijkers DTS, Antoinette Killian J, De Kroon AIPM, De Kruijff B (2003) Phospholipid flop induced by trans-membrane peptides in model membranes is modulated by lipid composition. *Biochemistry* 42(1):231–237. 10.1021/bi0268403 [PubMed: 12515559]
18. Mihajlovic M, Lazaridis T (2010) Antimicrobial peptides in Toroidal and cylindrical pores. *Biochim Biophys Acta* 1798(8):1485–1493. 10.1016/j.bbamem.2010.04.004 [PubMed: 20403332]
19. Ohmann A, Li C-Y, Maffeo C, Al Nahas K, Baumann KN, Göpflich K, Yoo J, Keyser UF, Aksimentiev A (2018) A synthetic enzyme built from DNA flips 10(7) lipids per second in biological membranes. *Nat Commun* 9 (1):2426–2426. 10.1038/s41467-018-04821-5 [PubMed: 29930243]
20. Yu K, Whitlock JM, Lee K, Ea O, Yuan Cui Y, Hartzell HC (2015) Identification of a lipid scrambling domain in ANO6/TMEM16F. *Elife* 4:1–23. 10.7554/eLife.06901
21. Suzuki J, Imanishi E, Nagata S (2014) Exposure of phosphatidylserine by Xk-related protein family members during apoptosis. *J Biol Chem* 289(44):30257–30267 [PubMed: 25231987]
22. Yang H, Kim A, David T, Palmer D, Jin T, Tien J, Huang F, Cheng T, Coughlin SR, Jan YN, Jan LY (2012) TMEM16F forms a Ca^{2+} -activated Cation Channel required for lipid scrambling in platelets during blood coagulation. *Cell* 151(1):111–122 [PubMed: 23021219]

23. Malvezzi M, Andra KK, Pandey K, Lee B-C, Brown A, Iqbal R, Menon AK, Accardi A (2018) Out of the groove transport of lipids by TMEM16 and GPCR scramblases PNAS. doi:10.1073/pnas.1806721115
24. Lee B-C, Khelashvili G, Falzone M, Menon AK, Weinstein H, Accardi A (2018) Gating mechanism of the extracellular entry to the lipid pathway in a TMEM16 scramblase. Nat Commun 9(1):3251 10.1038/s41467-018-05724-1 [PubMed: 30108217]
25. Falzone ME, Rheinberger J, Lee B-C, Peyear T, Sasset L, Raczkowski AM, Eng ET, Di Lorenzo A, Andersen OS, Nimigeon CM, Accardi A (2019) Structural basis of Ca²⁺-dependent activation and lipid transport by a TMEM16 scramblase. Elife 8:e43229. doi: 10.7554/eLife.43229 [PubMed: 30648972]
26. Andra KK, Dorsey S, Royer C, Menon AK (2018) Structural mapping of fluorescently-tagged, functional nhTMEM16 scramblase in a lipid bilayer. J Biol Chem 293:12248 10.1074/jbc.RA118.003648 [PubMed: 29903908]
27. Marx U, Lassmann G, Holzthutter HG, Wustner D, Muller P, Hohlig A, Kubelt J, Herrmann A (2000) Rapid flip-flop of phospholipids in endoplasmic reticulum membranes studied by a stopped-flow approach. Biophys J 78(5):2628–2640. 10.1016/S0006-3495(00)76807-X [PubMed: 10777759]
28. Sanyal S, Frank CG, Menon AK (2008) Distinct flippases translocate glycerophospholipids and oligosaccharide diphosphate dolichols across the endoplasmic reticulum. Biochemistry 47:7937–7946 [PubMed: 18597486]
29. Sanyal S, Menon AK (2009) Specific transbilayer translocation of dolichol-linked oligosaccharides by an endoplasmic reticulum flippase. Proc Natl Acad Sci U S A 106(3):767–772 [PubMed: 19129492]
30. Sanyal S, Menon AK (2010) Stereoselective transbilayer translocation of mannosyl phosphoryl dolichol by an endoplasmic reticulum flippase. Proc Natl Acad Sci U S A 107:11289–11294 [PubMed: 20534553]
31. Wang L, Iwasaki Y, Andra KK, Pandey K, Menon AK, Bütikofer P (2018) Scrambling of natural and fluorescently tagged phosphatidylinositol by reconstituted G protein-coupled receptor and TMEM16 scramblases. J Biol Chem 293(47):18318–18327. 10.1074/jbc.RA118.004213 [PubMed: 30287690]
32. Lee B-C, Menon AK, Accardi A (2016) The nhTMEM16 Scramblase is also a nonselective Ion Channel. Biophys J 111(9):1919–1924. 10.1016/j.bpj.2016.09.032 [PubMed: 27806273]
33. Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, Pfeffer U, Ravazzolo R, Zegar-Moran O, Galletta LJV (2008) TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. Science 322(5901):590–594. 10.1126/science.1163518 [PubMed: 18772398]
34. Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim W-S, Park SP, Lee J, Lee B, Kim B-M, Raouf R, Shin YK, Oh U (2008) TMEM16A confers receptor-activated calcium-dependent chloride conductance. Nature 455 (7217):1210–1215. 10.1038/nature07313 [PubMed: 18724360]
35. Schroeder BC, Cheng T, Jan YN, Jan LY (2008) Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. Cell 134(6):11
36. Whitlock JM, Hartzell HC (2016) A pore idea: the ion conduction pathway of TMEM16/ANO proteins is composed partly of lipid. Pflugers Arch 468(3):455–473. 10.1007/s00424-015-1777-2 [PubMed: 26739711]
37. Jiang T, Yu K, Hartzell HC, Tajkhorshid E (2017) Lipids and ions traverse the membrane by the same physical pathway in the. Elife 16:1–26. 10.7554/eLife.28671
38. Paulino C, Neldner Y, Lam AKM, Kalienkova V, Brunner JD, Schenck S, Dutzler R (2017) Structural basis for anion conduction in the calcium-activated chloride channel TMEM16A. Elife 6:1–23. 10.7554/eLife.26232
39. Paulino C, Kalienkova V, Lam AKM, Neldner Y, Dutzler R (2017) Activation mechanism of the calcium-activated chloride channel TMEM16A revealed by cryo-EM. Nature 552 (7685):421–425. 10.1038/nature24652 [PubMed: 29236691]
40. Dang S, Feng S, Tien J, Peters CJ, Bulkley D, Lolicato M, Zhao J, Zuberbühler K, Ye W, Qi L, Chen T, Craik CS, Nung Jan Y, Minor DL Jr, Cheng Y, Yeh Jan L (2017) Cryo-EM structures of

the TMEM16A calcium-activated chloride channel. *Nature* 552:426 10.1038/nature25024 [PubMed: 29236684]

41. Khelashvili G, Falzone ME, Cheng X, Lee B-C, Accardi A, Weinstein H (2019) Dynamic modulation of the lipid translocation groove generates a conductive ion channel in Ca²⁺-bound nhTMEM16. *Nat Commun* 10(1):4972 10.1038/s41467-019-12865-4 [PubMed: 31672969]
42. Scudieri P, Caci E, Venturini A, Sondo E, Pianigiani G, Marchetti C, Ravazzolo R, Pagani F, Galletta LJV (2015) Ion channel and lipid scramblase activity associated with expression of TMEM16F/ANO6 isoforms. *J Physiol* 593(593):3829–3848. 10.1113/JP270691
43. Ploier B, Caro LN, Morizumi T, Pandey K, Pearing JN, Goren MA, Finnemann SC, Graumann J, Arshavsky VY, Dittman JS, Ernst OP, Menon AK (2016) Dimerization deficiency of enigmatic retinitis pigmentosa-linked rhodopsin mutants. *Nat Commun* 7:12832 10.1038/ncomms12832 [PubMed: 27694816]
44. Kalienkova V, Clerico Mosina V, Bryner L, Oostergetel GT, Dutzler R, Paulino C (2019) Stepwise activation mechanism of the scramblase nhTMEM16 revealed by cryo-EM. *Elife* 8:e44364 10.7554/eLife.44364 [PubMed: 30785398]

Fig. 1.

Schematic of phospholipid scrambling and ion flux assays. **(a)** Schematic of the in vitro scramblase assay. Liposomes are reconstituted with NBD-labeled phospholipids (orange) that distribute equally in the two leaflets. Addition of extraliposomal sodium dithionite reduces the NBD fluorophore (black), causing 50% fluorescence loss in protein-free vesicles (top panel). When a scramblase is present (bottom panel), all NBD-phospholipids become exposed to dithionite, resulting in complete loss of fluorescence [7]. **(b)** Schematic of in vitro flux assay. Liposomes with high internal KCl concentration (300 mM) are buffer exchanged into low (1 mM) external KCl. In the absence of a scramblase (top panels), the KCl gradient is maintained after buffer exchange. The addition of dithionite (*D) disrupts the liposomes and releases the remaining KCl which can be measured with a AgCl electrode. In the presence of an active nonselective channel (bottom panels), the KCl gradient is lost following buffer exchange due to ion flux. The addition of dithionite (*D) disrupts the liposomes and the remaining KCl is released which is measured with a AgCl electrode

Fig. 2.

Methods to analyze scrambling assay traces. **(a)** Normalized fluorescence decay from protein-free liposomes (green) and scramblase proteoliposomes (red, +Ca²⁺ and black, 0 Ca²⁺). * indicates the addition of dithionite to the extracellular solution. **(b)** Fixed point method of analyzing scrambling assay traces. The dashed line indicates $t = 3\tau$ and arrows point to the fluorescence values (F_{PF} , $F_{0Ca^{2+}}$ and F_{max}) used for the analysis (Eq. 1). For the traces in this example, $F_{PF} = 0.53 F_{0Ca^{2+}} = 0.4$ and $F_{max} = 0.14$ so $F = 33\%$, indicating that removal of Ca²⁺ causes a 66% decrease in scrambling activity. **(c)** Normalized fluorescence decays fit (blue) to Eqs. 2 and 3 for scramblase proteoliposomes and protein-free vesicles, respectively. The fit of the fluorescence decay of protein-free liposomes determines L_i^{PF} and γ while that of the proteoliposomes determines f_0 , α , and β . Arrows below the red trace denote f_0 . Note the gain in dynamical range with the rate constant analysis. For the traces in this example, $f_0(+Ca^{2+}) = 0.2$, $\alpha(+Ca^{2+}) \sim \beta(+Ca^{2+}) \sim 0.1 \text{ s}^{-1}$, while $\alpha(0 \text{ Ca}^{2+}) \sim \beta(0 \text{ Ca}^{2+}) \sim 0.001 \text{ s}^{-1}$ and $f_0(0 \text{ Ca}^{2+})$ was constrained to be equal to the value in +Ca²⁺, after verifying that the reconstitution efficiency is the same in the two conditions. Note that by fitting the complete trace to Eq. 2 the decrease in activity is ~ 100 -fold, which reflects the dramatic slowdown of the scrambling process visible in the raw data

Fig. 3. Flux assay analysis. Raw trace for flux assay of protein-free liposomes (black) and proteoliposomes (red). Points needed for analysis are marked with arrows: V_0 is the beginning of the trace and measures the buffer before calibration; V_{cal} is measured after the addition of 18 μ L of 10 mM KCl to calibrate the system (indicated by #); V_{lipo} is measured after the addition of liposomes (indicated by ^) and V_{tot} is the signal after the addition of detergent (indicated by *). This is the measure of the trapped Cl^- from inside the liposomes. For the traces in this example, $Cl(\text{Protein-free})$ 0.49 mM, and $Cl(\text{Proteoliposomes})$ = 0.16 mM, so that $A = 0.67$, indicating that ~67% of the channel liposomes contain at least one active nonselective channel