

Materials and Methods

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol) DOPG and 1,2-di-O-(9Z-octadecenyl)-*sn*-glycero-3-phosphocholine (Diether PC) were obtained from Avanti Polar Lipids Inc. (USA). Detergents n-Dodecyl- β -D-Maltopyranoside (DDM), n-Octyl- β -D-Glucopyranoside (OG), Octaethylene glycol monododecyl ether (C₁₂E₈) and 2 (trimethylammonium) ethyl methane thiosulphonate bromide (MTSET) were purchased from Affymetrix (USA). TALON® Superflow™ Metal Affinity Resin was purchased from Qiagen. All other compounds were purchased from Sigma-Aldrich (UK) unless otherwise stated.

Expression and purification - *E. coli* BL21 (ED3) cells carrying the pET28a (Novagen) vector with chemically gated MscL gene were grown in Luria broth (30 μ g/ml kanamycin) at 37°C to a ~ maximum of the exponential phase (OD₆₀₀ = 1) and then induced for 90 min in the presence of isopropyl β -D-thiogalactopyranoside (IPTG) (0.5 mM). MscL was purified using a modified protocol of Perozo et al., (2001)²⁶. Briefly, cells were collected by centrifugation at 16,000g and disrupted by passage through a cell disruptor (Constant Systems Cell Disruptor Model T5) at 25kpsi. Membranes were isolated by centrifugation at 100,000g for 1hr at 4°C and solubilised in solubilisation buffer (2% w/v DDM, 100mM KCl, EDTA free protease inhibitors and 20mM HEPES pH 7.2) for a minimum of 2 hr at 4°C. Insoluble material was removed by centrifugation (35,000g, 30 min and 4°C) and DDM-solubilised protein was incubated with 1.5 ml of copper chelated resin for 90 min at 4°C. Non-specifically bound protein was removed by washing with 0.13% DDM (w/v), 100 mM KCl, 0.1 mM phenylmethanesulfonylfluoride (PMSF), 6 mM imidazole and 20 mM HEPES pH 7.2. Chemically gated MscL was eluted in 0.13% DDM (w/v), 100 mM KCl, 0.1 mM PMSF, 120 mM imidazole and 20 mM HEPES pH 7.2 and concentrated to 1-2 mM in an Amicon Ultra 100,000 MWCO centrifugal concentrator (Millipore) to 1-2 mM as determined by A₂₈₀ (A₂₈₀ of 1 mgml⁻¹ = 0.433), snap frozen in liquid nitrogen and stored at -80°C till further use.

Reconstitution - 12.6 mM (10 mg) of 1:1 DOPC:DOPG (w/w) were dried down in a glass vial and resuspended in 1 ml of buffer (50 mM calcein, 40 mM OG, 100 mM KCl and 20 mM HEPES pH 7.2) and extruded to 100 nm in diameter liposomes. Liposomes were added to MscL at a molar ratio of 1:1000 MscL pentamer:lipid. Detergent was removed by addition of 200 mg

of SM2 Bio-Beads (mesh size 20–50; Bio-Rad) and a subsequent 140-fold dilution in buffer (100 mM KCl, 20 mM HEPES pH 7.2) which also removed unencapsulated dye. Proteoliposomes were collected by centrifugation at 125,000g for ~ 2 hr at 4°C. Proteoliposomes were resuspended in sucrose buffer (0.5 M sucrose, 100 mM KCl and 20 mM HEPES, pH 7.2) and stored at 4°C until further use. Control samples (protein free liposomes) were treated as described above with the exception of replacing the MscL sample with the same volume of 0.13% DDM.

Calcein Flux Assay²⁷ – Proteoliposomes and control samples, liposomes without reconstituted channels, were diluted in sucrose buffer at a 1:50 ratio in a 96-well plate. Baseline recordings were initiated for 10 min prior to channel activation. PLA₂ was added at concentrations ranging between ~0.15 nM – 10 nM and change in fluorescence at 517 nm was recorded for 1 hr (figure 3). 10 nM PLA₂ was used in all other experiments (figures 2 and 4). Mechanical and chemical MscL gating through the addition of PLA₂ and MTSET respectively was measured sequentially and in parallel experiments. Control experiments to measure calcein leakage in liposomes without reconstituted MscL were undertaken in the presence of PLA₂ and MTSET following the same procedures. To calculate the percentage increase in calcein fluorescence 200 µM of C₁₂E₈ was added to all samples and the change in intensity was recorded for 10 minutes. This determined the maximum fluorescence following complete calcein release. Fluorescence emission was measured at 517 nm with excitation at 494 nm on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, USA). To show that no significant change in fluorescence is observed prior to channel activation data in figure 2 were normalised to the data point at time = 0. For clarity subsequent data in figures 3 and 4 are normalised to the first data point following channel activation at time = 10 minutes.