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Porous membranes for reconstitution of ion channels

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Abstract

Functional biological synthetic composite (BSC) membranes were made using phospholipids, biological membrane proteins and permeable synthetic supports or membranes. Lipid bilayers were formed on porous polycarbonate (PC), polyethylene terephthalate (PETE) and poly (L-lactic acid) (PLLA) membranes and in 10–100 μm laser-drilled pores in a 96-well plastic plate as measured by increased resistance or decreased currents. Bilayers in 50 μm and smaller pores were stable for up to 4 h as measured by resistance changes or a current after gramicidin D reconstitution. Biological membrane transport reconstitution was then carried out. Using vesicles containing Kv1.5 K^+ channels, K^+ currents and decreased resistance were measured across bilayers in 50 μm pores in the plastic plate and PLLA membranes, respectively, which were inhibited by compound B, a Kv1.5 K^+ channel inhibitor. Functional reconstitution of Kv1.5 K^+ channels was successful. Incorporation of membrane proteins in functional form in stable permeable membrane-supported lipid bilayers is a simple technology to create BSC membranes that mimic biological function which is readily adaptable for high throughput screening.

Keywords: Polycarbonate; PETE; PLLA membrane; Lipid bilayer

1. Introduction

The goal of the present study was to make functional biological synthetic composite (BSC) membranes using phospholipids, membrane proteins isolated from cells, and permeable synthetic supports or membranes. Stabilization of phospholipid bilayers was achieved on a variety of substrates including solid supports [1], porous alumina attached to a solid support [2], micro porous membranes and macro porous scaffolds [3]. Different micro porous membranes studied include filters composed of glass fibers, polytetrafluoroethylene (PTFE) and polycarbonate (PC) [3]. Triacetyl cellulose filters were excellent supports for bilayer formation [4]. Electrical measurements across porous or permeable membrane-supported lipid bilayers have been made suggesting that micro-lipid bilayer formation occurred in the pores of the membrane [5,6] and were stable for long times. Some membrane proteins have been successfully reconstituted on solid supported membranes in functional form such as the Na/K ATPase [7,8] and sarcoplasmic reticulum Ca ATPase [9] as well as Kv1.5 K^+ channels [10] and maxi K^+ channels [11]. Procedures to make solid supported membranes are complex and lengthy and except for one study [10] they are usually not multi-array in design, limiting the number of assays that can be performed at one time. An additional goal of the present studies was to devise multi-array BSC membranes that are simple to make and use. Thus, they would be very adaptable for high throughput screening technologies.

In the present study, commercially available Millipore micro porous PC membranes of various pore size (0.4, 3, 5 and 8 μm), commercially available PETE membranes with pore size of 3 μm, laboratory synthesized porous PLLA membranes with various shaped pores of 3–15 μm and a multi-well plastic plate with 10–100 μm laser drilled holes (1/well) were used for lipid bilayer formation. Gramicidin D, a linear polypeptide antibiotic which forms cation-selective channels in lipid bilayers [12] and Kv1.5 K^+ channels [10] were incorporated into these synthetic membrane-supported bilayers to create BSC membranes,

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which were then tested for biological function. Resistance measurements across the membranes were used to monitor bilayer formation, which was also verified by examining the effect of gramicidin D on the resistance. Successful reconstitution of gramicidin D in the lipid bilayer results in a decrease in resistance in medium containing a cation which is permeable through gramicidin D. Functional Kv1.5 K^+ channel incorporation was monitored by measuring K^+ currents across the bilayer after addition of membrane vesicles containing Kv1.5 K^+ channels, which were blocked by compound B (2-(3,4-dimethylphenyl)-3-[2-(4-methoxyphenyl)ethyl]-thiazolidin-4-one), an inhibitor of Kv1.5 K^+ channels. Functional Kv1.5 K^+ channel incorporation into PLLA membrane-supported bilayers was also examined by resistance measurements.

2. Materials and methods

2.1. Membrane preparation, characterization and attaching to the insert/well

Polycarbonate (PC) and polyethylene terephthalate (PETE) membranes were purchased (see Materials), but porous poly l-lactic acid (PLLA) membranes were synthesized using the solvent casting/particulate leaching technique [13]. Finely ground NaCl (0.1 g) was dispersed in PLLA (1 g) in chloroform (40 ml) with an ultrasonicator. The solution was cast in several glass Petri dishes and the chloroform was allowed to evaporate for 48 h to give PLLA/NaCl composite membranes. These membranes are unleached PLLA membranes. These unleached PLLA membranes were then washed in deionised water with vigorous shaking to dissolve the NaCl particles embedded in the membranes. When NaCl was leached out, a porous PLLA membrane was formed. The thickness of these membranes was between 10 and 15 μm.

Membrane pore size and surface morphology were examined using scanning electron microscopy. PC, PETE and PLLA membranes were immobilized on a sample holder using carbon tape (SPI supplies, West Chester, PA). Colloidal graphite (Ted Pella Inc, Redding, CA) was applied to the edges of the samples to ensure electron dissipation. Samples were sputtered with a Denton Vacuum, LLC and a HITACHI S-4000 scanning electron microscope was used to obtain the images. To monitor the leaching process the PLLA membranes (unleached and leached) were characterized using X-ray photoelectron spectroscopy (XPS). A Perkin Elmer 5300 Spectrometer [MgKα radiation (1254 eV)] was used to obtain the XPS data.

PC membranes at the base of polystyrene inserts or wells of Millipore Multiscreen-MIC filter plates were removed and replaced with PETE or PLLA membranes. The plastic surface of Millipore inserts was oxygen plasma etched for 30 s to promote the adhesion of the PETE or PLLA membranes to the base of the insert. A capacitively coupled, rf-powered plasma reactor operating at a frequency of 13.6 MHz was used for oxygen plasma etching. Oxygen plasma etching was carried out in a radio frequency reactor using 100 W power and 0.5 Torr pressure. The oxygen flow rate was maintained at 200 cm^3/min. PETE membranes were attached to the insert using a polyurethane adhesive. PLLA membranes were oxygen plasma etched for 5 s before attaching to inserts with two-part epoxy.

2.2. Phospholipid bilayer formation with and without gramicidin and resistance measurements

The experimental set-up is shown in Fig. 1A. Bilayer formation was studied using PC, PETE and PLLA membranes (area = 0.3 cm^2). Phospholipid bilayers were made with POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine]) and POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine). 10 μl of 3:1 POPS:POPE (40 mg/ml) in n-decane was added to the membranes and left for 30 min. 100 mM KCl/10 mM HEPES (pH 7.4) was then added to both sides of the membrane (150 μl inside insert or well; 200 μl outside solution) and left for 30 min. A multimeter and Ag/AgCl reference electrodes were used to measure the resistance across the phospholipid-coated membranes. When gramicidin D was used, it was mixed with the lipids to a final concentration of 1 μg/ml. 10 μl of the lipid/gramicidin mixture was added to the membranes. After 30 min, medium was added to both sides of the lipid-coated membrane (both inner and outer wells) and left for another 30 min. Resistances of the lipid-coated membranes were measured with 2 different media bathing the lipid bilayer on both sides: 100 mM KCl/10 mM HEPES (pH 7.4) and 100 mM *N*-methyl-D-glucamine chloride (NMDGCl)/10 mM HEPES (pH 7.4). Reconstitution of gramicidin D was also done using the 96-well plastic plate with laser drilled holes (Fig. 1B) and current was measured as described below.

2.3. Phospholipid bilayer formation, reconstitution of Kv1.5 K^+ channels, current and resistance measurements

To investigate reconstitution of ion channels plasma membrane vesicles containing Kv1.5, K^+ channels were prepared from an Ltk^- cell line (mouse fibroblast cells) stably overexpressing Kv1.5 K^+ channels under the control of a dexamethasone promoter as previously described [10]. Dexamethasone-specific induction of channel expression is totally specific for Kv1.5 channels. Plasma membrane vesicles were also prepared from Kv1.5 cDNA-transfected-but-uninduced Ltk^- cells which did not express any Kv1.5 K^+ channels. A 96-well plastic plate with laser drilled holes varying from 10 to 100 μm (1/well) was used. The well area was 0.3 cm^2. Fig. 1B shows a diagram of the experimental set-up showing a single well. The phospholipid bilayer was made by adding 5 μl of 40 mg/ml 3:1 POPS: POPE in n-decane to each well. After 30 min, 100 mM KCl/20 mM HEPES (pH 7.4) was added to both sides of the bilayer (both inner and outer wells). After a further 30 min, the solution from the inner well was removed and plasma membrane vesicles (5 μl) containing Kv1.5 K^+ channels were added directly to the lipid coated wells. After 30 min, 100 mM KCl/20 mM HEPES (pH 7.4) was returned to the well. Currents across the bilayer were measured using an Ag/AgCl reference electrode inside the well and a Dri-Ref™ reference electrode (DRIREF-5SH) in the outside solution. These were connected to an HS-2A headstage which was connected to a Gene Clamp 500 amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 50 Hz. Voltages ranging from −80 to +70 mV were applied in 10-mV increments for 200 ms and electrical currents were recorded using pCLAMP version 5.5. When indicated, compound B, an inhibitor of Kv1.5 K^+ channels [14] was added.

Reconstitution of Kv1.5 K^+ channels was also carried out using lipid coated PLLA membranes (area = 0.3 cm^2) and resistance was measured as described in the previous section. Effects of compound B were also tested.

2.4. Analysis

Statistical analysis was carried out using the Student's *t*-test. Curves were fitted using Origin 5. The number of experiments (n) and P values for statistical significance are indicated on graphs, in legends and in the text.

2.5. Materials

Multiscreen-MIC (10 μm thick) filter plates were obtained from Millipore Corporation (Bedford, MA.) and contained inserts with polycarbonate membranes with 3, 5 and 8 μm pores at the bottom. Micro porous 9 μm thick PETE membranes with 3 μm pores were obtained from GE Osmonics, Inc. (Minnetonka, MN). PLLA was obtained from Polysciences, Inc (Warrington, PA.). Polyurethane based adhesive 3M™ Scotch-Weld™ DP-605 NS was from 3M (St. Paul, MN). EPON® Resin 828, a 2-part epoxy, was used with EPICURE® 3140 curing agent, both purchased from Miller Stephenson Chemical Co Inc. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) were from Avanti Polar Lipids (Alabaster, Alabama) and dissolved in reagent grade n-decane. HEPES, NMDGCl, KCl, NaCl and gramicidin D were from Sigma (St. Louis, MO). Compound B (2-(3,4-dimethyphenyl)-3-[2-(4-methoxyphenyl)ethyl]-thiazolidin-4-one) was from Procter and Gamble Pharmaceuticals (Cincinnati, OH.) and was prepared as outlined in US patent

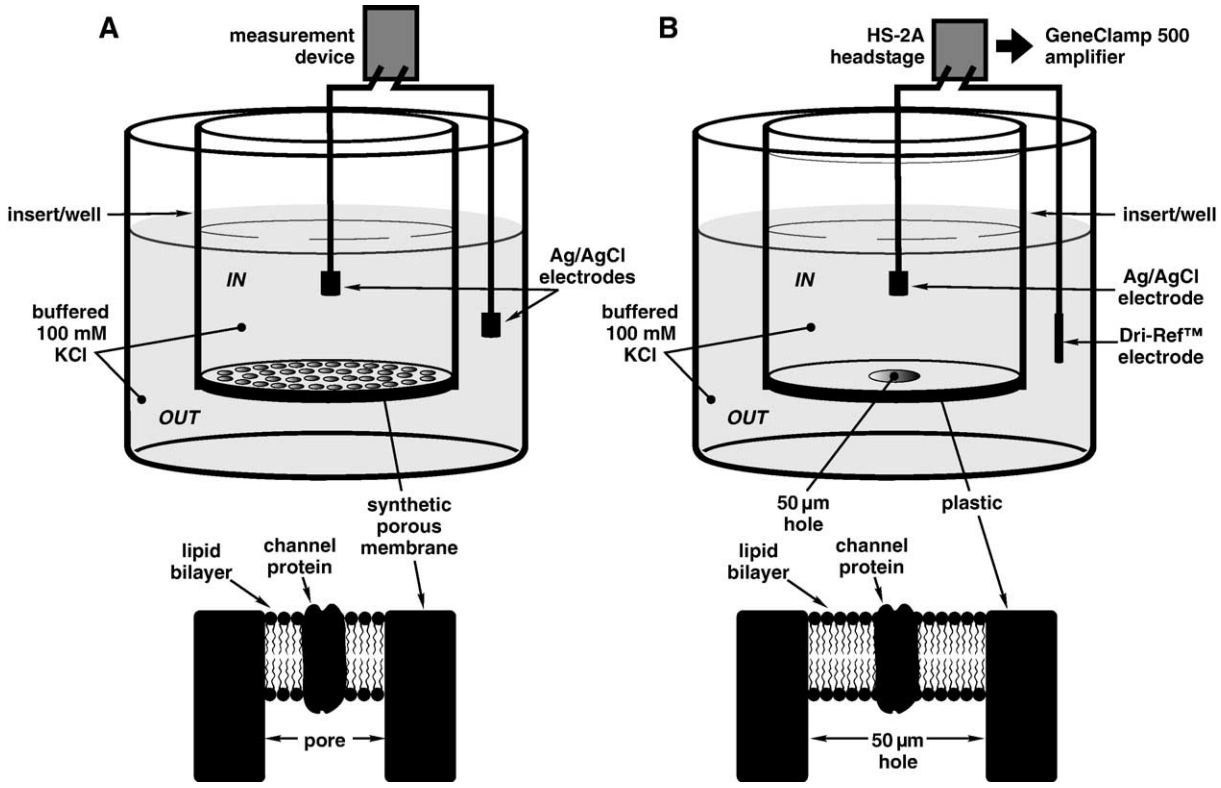

Fig. 1. (A) Schematic of experimental set-up used for resistance measurements (B) schematic of experimental set-up used for current measurements. Panel A shows one outer well of a multiscreen-MIC filter plate with a synthetic porous membrane at the bottom of the insert/well, forming an inner compartment (IN) and an outer compartment (OUT) which both contain 100 mM KCl, 10 mM HEPES pH 7.4. Ag/AgCl electrodes are positioned as shown and a multimeter were used to measure resistance. Panel B shows one insert/well of the 96-well plastic plate with a single laser-drilled 50 μm hole. There are both inner and outer compartments which contain 100 mM KCl, 20 mM HEPES pH 7.4. Currents were measured using an Ag/AgCl reference electrode inside the well and a Dri-Ref™ reference electrode in the outside solution. These were connected to an HS-2A headstage which was connected to a Gene Clamp 500 amplifier. In both A and B below the experimental set-up is shown a schematic diagram illustrating a lipid bilayer formed in the pore or hole in which a channel protein has been incorporated.

#6,174,908 [14]. Ag/AgCl electrodes and Dri-Ref electrodes were from World Precision Instruments, Inc (Sarasota, FL).

3. Results

3.1. Membrane characterization and assays of lipid bilayer formation

Scanning electron microscope studies of the microporous PC filters with 3, 5 and 8 μm pores were carried out and the micrographs are shown in Fig. 2A. A lipid bilayer was formed on the PC filters using 3:1 POPS:POPE (40 mg/ml) as described in the Methods. Bilayer formation was then assayed (Fig. 2B) by measuring resistance across the filters after adding phospholipids (PL) without or with gramicidin D (gramD). These assays were carried out in the presence of KCl (permeant cation) or NMDGCl (impermeant cation). The medium was 100 mM KCl or NMDGCl with 10 mM HEPES (pH 7.4). Gramicidin D allows ion conduction through the lipid bilayer only if the phospholipids are a single bilayer thick [15] and two gramicidin monomers align to form an ion channel [16] spanning the bilayer. This is schematically shown in Fig. 1C. Similar resistances of 30.66 ± 5.29 ($n=6$), 31.02 ± 4.92 ($n=6$) and 26.21 ± 6.25 ($n=6$) MΩ were measured after bilayer formation in the presence of KCl using PC filters with 3, 5 and 8 μm pores, respectively. Irrespective of pore size gramicidin D reduced the resistance significantly ($P<0.005$) to virtually zero in the presence of K^+, a permeant cation, but not in the presence of $NMDG^+$, an impermeant cation. These findings indicate that phospholipid bilayers were successfully formed on PC filters containing 3, 5 and 8 μm pores and gramicidin D channels were successfully incorporated into the bilayer.

Fig. 3A and B show scanning electron micrographs of 9 μm thick PETE membranes containing 3 μm pores with an average pore density of 2×10^6 pores/cm^2. Fig. 3C shows that PETE membranes with phospholipids had a high resistance of 39.25 ± 0.75 ($n=6$) MΩ, which decreased significantly ($P<0.001$) to 5.41 ± 1.11 ($n=5$) MΩ with gramicidin D and KCl, but did not change with gramicidin D and NMDGCl. As with PC membranes, phospholipid bilayers and gramicidin D channels were also successfully formed on PETE membranes.

In the next experiments PLLA membranes synthesized as described in the Methods were used. X-ray photoelectron spectroscopic (XPS) scans and scanning electron micrographs (as insets) are shown in Fig. 4A. High resolution XPS scans were obtained at 35.75 eV pass energy and were corrected for charge by assigning a value of 284.6 eV to the C (1s) peak. The unleached PLLA membrane showed peaks at 284.6 eV and 531 eV indicating the presence of carbon [C(1s)] and oxygen

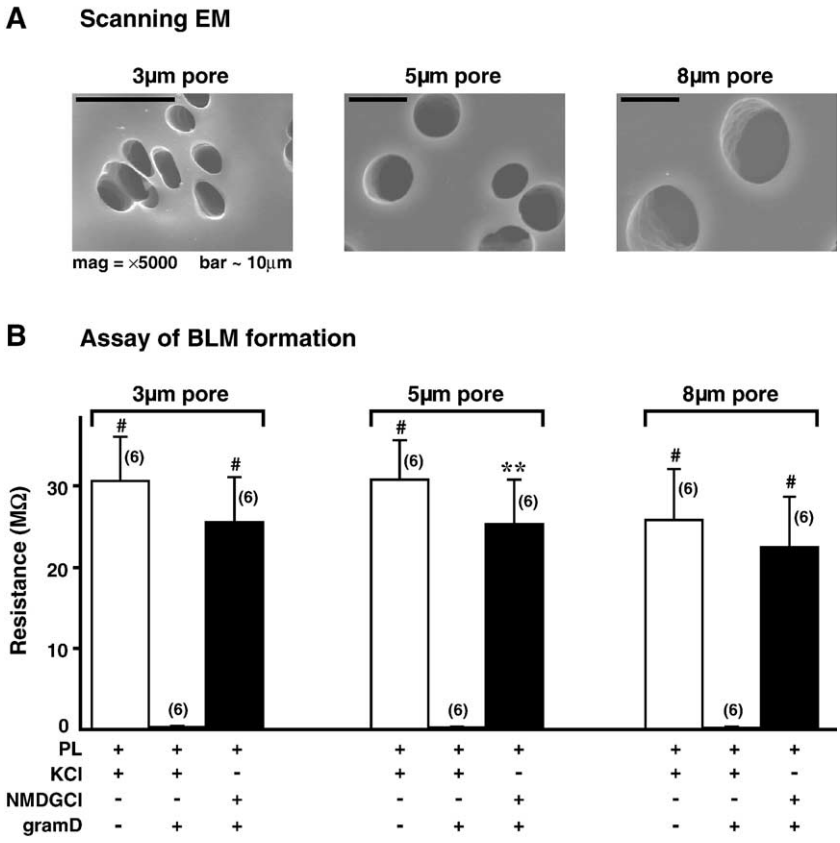

Fig. 2. (A) Scanning electron micrographs and (B) assay of lipid bilayer formation on polycarbonate (PC) filters with 3 different pore sizes. Microporous PC filters (area 0.3 cm^2) with 3, 5 and 8 μm pore sizes were used. Panel A shows the scanning electron micrographs of the filters at 5000× magnification. The bar indicates 10 μm. (B) Assay of bilayer formation is shown as summarized resistance measurements across the PC filters after adding phospholipids (PL) without or with gramicidin D (gramD) in the presence of KCl (permeant cation) or NMDGCl (impermeant cation) medium. Medium is 100 mM KCl or NMDGCl with 10 mM HEPES (pH 7.4). PL used were 3:1 POPS:POPE (40 mg/ml). Data are plotted as mean ± S.E. Number of experiments is indicated in brackets. $^{\#}P<0.005$, $^{**}P<0.01$ compared with PL + KCl + gramD.

[O(1s)], respectively, and 2 further oxygen Auger peaks at 743.50 eV [O(2s)] and 993.50 eV [O93s)] were also evident. Sodium (1074 eV) and chlorine (200 eV) were present only in the unleached membrane due to NaCl particles. After leaching the membrane with water, sodium and chlorine disappeared from the XPS scan indicating that NaCl particles were dissolved away, leaving a pore. This was supported by the images obtained with scanning electron microscopy before and after leaching NaCl particles out of the PLLA membrane (Fig. 4A insets). The unleached PLLA membrane appears to contain a NaCl particle in it, while the leached PLLA membrane shows pores in place of the NaCl particles. The pore shapes varied from being triangular and rectangular to being nearly circular. The resistance across the unleached and leached PLLA membranes in the absence of phospholipids and in KCl medium was then measured and is shown in Fig. 4B. The resistance of leached PLLA membranes (0.19 ± 0.004, $n=3$) MΩ was significantly lower ($P<0.02$) than that of unleached PLLA membranes (0.95 ± 0.16, $n=3$) MΩ, indicating that pores had been formed. The assay of bilayer formation was performed using leached porous PLLA membranes (Fig. 4C). Resistance of the PLLA membrane increased after adding phospholipids to 11.38 ± 1.65 ($n=6$) MΩ. On addition of gramicidin D, the resistance decreased significantly ($P<0.002$) to 0.23 ± 0.15 ($n=6$) MΩ in the presence of K^+ and was unaffected in the presence of $NMDG^+$. These results indicate that phospholipid bilayers were formed on PLLA membranes and channel forming gramicidin D was successfully incorporated into the lipid bilayer. Resistance of phospholipid-coated PLLA membranes was significantly lower than that of phospholipid-coated PC with 3, 5 and 8 μm pores ($P<0.01$, $P<0.01$, $P<0.05$) and PETE ($P<0.001$) membranes. This perhaps was due to the irregular shape and size of the pores in the PLLA membranes. Commercially available PC and PETE membranes had pores that were regular in size and had uniform structure.

3.2. Reconstitution of functional Kv1.5 K^+ channels in phospholipid bilayers formed in 50 μm holes in a plastic plate and pores in PLLA membranes

The ability to reconstitute ion channels in phospholipid-coated 10–100 μm holes which were laser drilled into the wells of a 96-well plastic plate, one hole/well was then investigated. The range of hole/pore sizes was examined and only 50 μm and smaller pores were able to support POPS: POPE phospholipid bilayers that were stable up to 4 h. 50 μm holes were therefore used in these experiments. The exper-

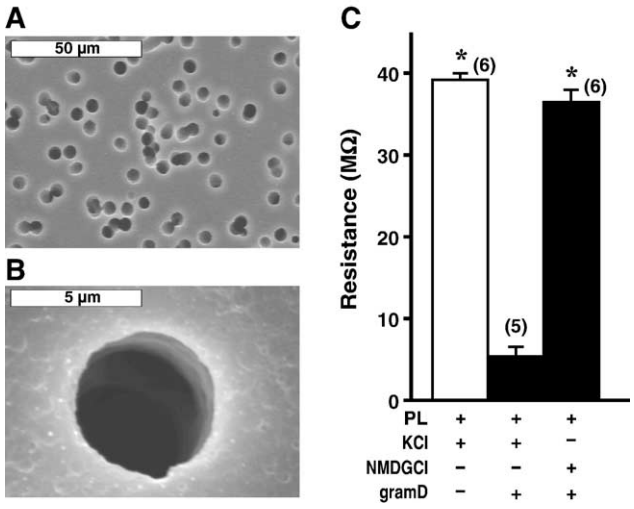

Fig. 3. (A, B) Scanning electron micrographs and (C) assay of lipid bilayer formation on PETE membranes. Scanning electron micrographs of PETE membranes at 2000× (A) and 20,000× (B) magnification are shown. Bars indicate 50 and 5 µm, respectively. (C) Assay of bilayer formation is shown as summarized resistance measurements across the PETE membranes (area 0.3 cm^2) after adding phospholipids (PL) without or with gramicidin D (gramD) in the presence of KCl (permeant cation) or NMDGCl (impermeant cation) medium. Medium is 100 mM KCl or NMDGCl with 10 mM HEPES (pH 7.4). PL used were 3:1 POPS:POPE (40 mg/ml). Data are plotted as mean±S.E. Number of experiments is indicated in brackets. *$P<0.001$ compared with PL+KCl+gramD.

imental set-up is shown in Fig. 1B. Plasma membrane vesicles from Lkt^- cells transfected with Kv1.5 cDNA and induced with dexamethasone were used. Lipid bilayers were first formed in the 50-µm holes/pores and then vesicles containing Kv1.5 K^+ channels were added to the lipid coated wells as described in Materials and methods. Currents across the bilayers were measured at different holding potentials (+70 mV to −80 mV) with 100 mM KCl/20 mM HEPES (pH 7.4) on both sides of the bilayer. Typical current recordings are shown in Fig. 5A together with current–voltage ($I-V$) curves and a summary of data obtained from a number of experiments. Before bilayer formation there were large, measurable currents which were virtually eliminated upon formation of the phospholipid bilayer. After addition of the vesicles containing Kv1.5 K^+ channels, a large current was measured which was inhibited by 1 µM compound B, a Kv1.5 K^+ channel inhibitor [14]. The large currents measured before bilayer formation and after Kv1.5 K^+ channel-containing vesicles were added are highly significantly ($P<0.001$) different from the very low currents measured with PL alone and with PL+Kv1.5 vesicles+1 µM compound B. Fig. 5B shows control experiments using plasma membrane vesicles isolated from Lkt^- cells transfected with Kv1.5 cDNA, but not induced with dexamethasone and therefore without Kv1.5 K^+ channels (−Kv1.5 vesicles); boiled membrane vesicles containing Kv1.5 K^+ channels (from dexamethasone-induced Lkt^- cells) and gramicidin D (gramD) reconstituted into the lipid bilayer. Typical currents obtained are shown as well as a summary of current measurements at +70 mV from a number of experiments. When Kv1.5 K^+ channels were not present in the membrane vesicles, no currents were evident as was also the case when boiled vesicles containing Kv1.5 K^+ channels were used. Large currents (about 30 nA) were measured with gramicidin D in KCl medium as expected. The findings of Fig 5A and B indicate that the measured K^+ currents were mediated by Kv1.5 channels and therefore functional Kv1.5 K^+ channels were successfully reconstituted into bilayers formed in 50 µm laser-drilled holes in a 96-well plastic plate.

In order to further investigate the reconstitution of Kv1.5 K^+ channels in functional form, phospholipid-coated porous PLLA membranes were used (Fig. 5C). With phospholipids alone, the resistance was 7.39±0.63 ($n=8$) MΩ and following addition of membrane vesicles containing Kv1.5 K^+ channels, the resistance decreased significantly ($P<0.001$) to 0.55±0.15 ($n=8$) MΩ in the presence of KCl. 1 µM compound B significantly ($P<0.001$) partially inhibited this decrease resulting in a resistance of 4.13±0.52 ($n=8$) MΩ. These findings indicate that Kv1.5 K^+ channels were incorporated into the bilayer formed in porous PLLA membranes and were functional resulting in compound B-inhibitable K^+ currents.

3.3. Effect of varying inhibitor compound B on the reconstituted Kv1.5-mediated K^+ current

To further support the view that Kv1.5 K^+ channels were reconstituted in functional form into bilayers formed in 50 µm laser-drilled holes in a 96-well plastic plate, the effect of increasing concentrations of compound B on Kv1.5 K^+ currents was measured. Fig. 6A shows the effect of 200 nM and 500 nM compound B on the $I-V$ curve. The control $I-V$ curve in the absence of compound B was linear. 200 nM and 500 nM compound B inhibited Kv1.5 K^+ channel currents such that the $I-V$ curve appeared to be rectified at both positive and negative holding potentials as previously observed [10]. One possible explanation of the observed rectification is that the membrane vesicles are a mixture of both inside-out and outside-in oriented Kv1.5 channels. The resultant channel current would be the sum of the channel activities in both orientations, which could result in rectification at both positive and negative potentials. In Fig. 6B, the effect of compound B is shown plotted as a dose–response curve with Kv1.5-mediated K^+ currents expressed as ΔI at 70 mV as % maximum. The data were fit by a sigmoidal plot with a concentration that results in 50% inhibition (IC_{50}) of 170±40 nM ($n=6$) and (chi) $\chi^2=35$, $P<0.001$. This IC_{50} for compound B was similar to that measured in whole cell patch clamp experiments [10], indicating not only that the reconstituted Kv1.5 K^+ channels are functional, but that they have maintained their sensitivity to compound B.

4. Discussion

The goals of the present studies were to develop simple procedures for making functional biological synthetic composite (BSC) membranes using phospholipids, membrane proteins isolated from cells, and permeable synthetic supports or

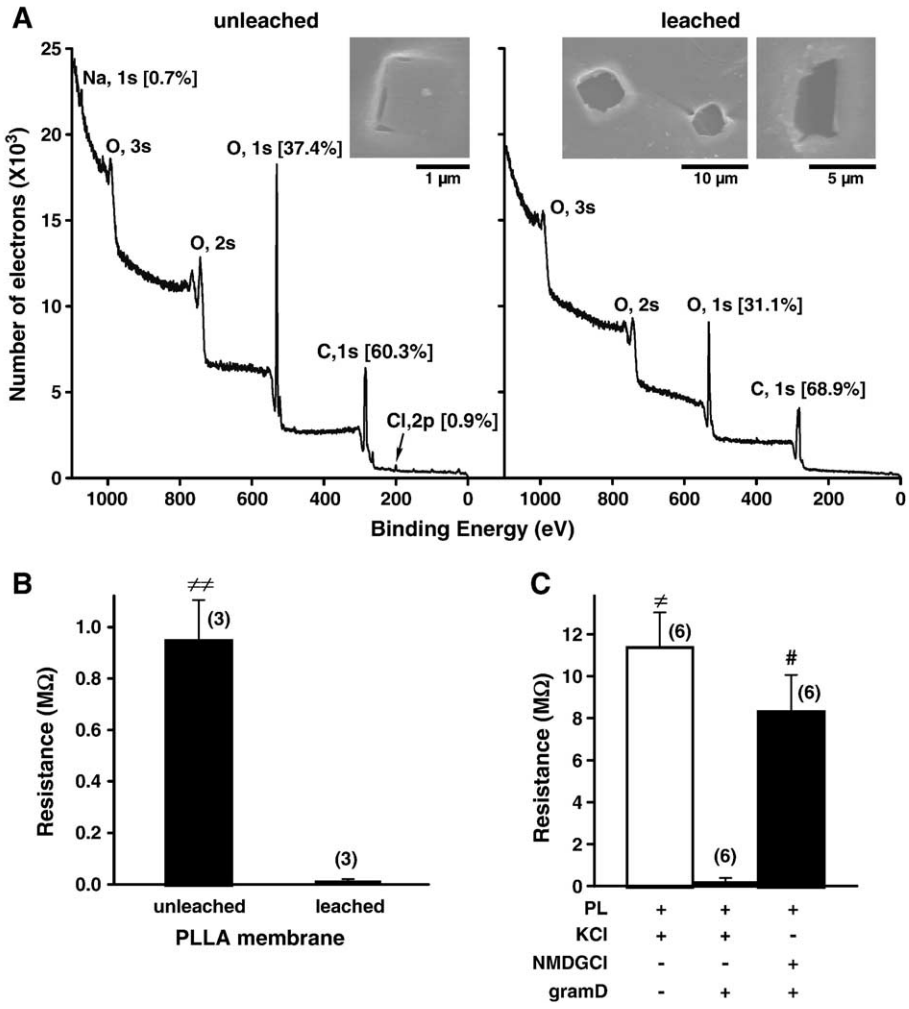

Fig. 4. (A) X-ray photoelectron spectra and scanning electron micrographs (insets), (B) resistance measurements of unleached and leached PLLA membranes (C) assay of lipid bilayer formation using leached PLLA membranes. (A) X-ray photoelectron spectra and scanning electron micrographs (insets) of unleached and leached PLLA membranes. Elements are indicated at the peaks and atomic concentrations are indicated in brackets. The scanning electron micrographs were obtained at 5000×. Panel B shows resistance measurements of unleached and leached PLLA membranes in KCl medium without phospholipids present. Data are plotted as mean ± S.E. Number of experiments is indicated in brackets. $^{\neq\neq}P<0.02$ with respect to leached membrane. (C) Assay of bilayer formation using leached PLLA membranes shown as the summarized resistance measurements of leached membranes after adding phospholipids (PL) without or with gramicidin D (gramD) in the presence of KCl (permeant cation) or NMDGCl (impermeant cation) medium. Medium is 100 mM KCl or NMDGCl with 10 mM HEPES (pH 7.4). Membrane area was 0.3 cm^2. PL used were 3:1 POPS: POPE (40 mg/ml). Data are plotted as mean ± S.E. Number of experiments is indicated in brackets. $^{\neq}P<0.002$; $^{\#}P<0.005$ with respect to PL+KCl+gramD.

membranes. A variety of methods for biological protein reconstitution on synthetic supports has been successful [7–11] and even has allowed experiments to be performed which would not be otherwise possible [17]. However, the methods used are complex, lengthy and not easy to use or adapt for multi-sample/multi-array use, necessary for example for screening drugs.

Ion channel incorporation into planar lipid bilayers formed in a hole of the wall of a Delrin cup has been successfully used for many years by many investigators (including the present authors) [20–25] to investigate channel function. A simple "painting" method in which membrane vesicles are deposited in close proximity to or on the bilayer has been highly successful and reproducible [24–26] and was therefore adapted and used in the present studies. No special additional conditions were needed for functional reconstitution of ion channels. In the case of the ATPases [7–9] and bacteriorhodopsin [18], it is thought that the molecules are adsorbed onto the surface of the phospholipid bilayer. In contrast membrane vesicles containing ion channels seem to fuse with the phospholipid bilayer and thus become incorporated into the bilayer [19–26] as shown schematically in Fig. 1.

Phospholipid bilayers made with all the micro porous membranes (PC, PETE, PLLA) resulted in resistances across the membranes, indicating formation of good seals in the pores of the membranes by the phospholipids and suggesting successful formation of micro lipid bilayers in the pores. This is schematically shown in Fig. 1. The drop in resistance on addition of gramicidin D in the presence of the permeant cation K^+ showed that gramicidin D formed ion channels that span the bilayer and K^+ ion flow across the bilayer reduced

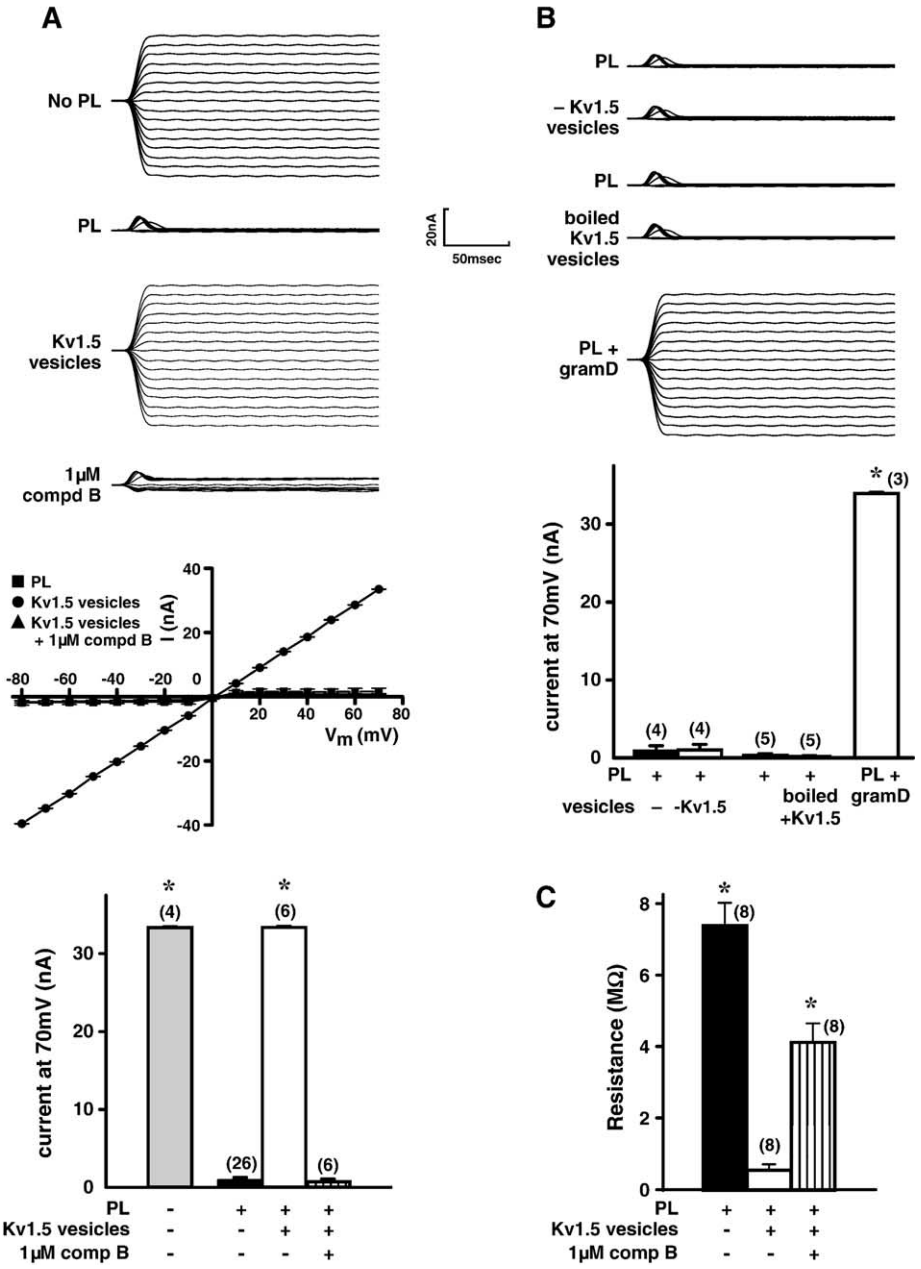

Fig. 5. Reconstitution of functional Kv1.5 K^+ channels in phospholipid-coated 50 μm pores in a plastic plate (A, B) and in phospholipid-coated leached PLLA membranes (C). For Panels A and B medium on both sides of the pore was 100 mM KCl with 20 mM HEPES (pH 7.4) and phospholipids (PL) used were 3:1 POPS: POPE (40 mg/ml). Well area was 0.3 cm^2. (A) Typical current recordings, $I-V$ curves and a summary of the data of experiments reconstituting membrane vesicles isolated from dexamethasone-induced Kv1.5 expressing Lkt^- cells (Kv1.5 vesicles) into phospholipids coating 50 μm pores. Sequential current recordings at different holding potentials from −80 to +70 mV are shown: before and after addition of phospholipids (PL); after addition of Kv1.5 vesicles to the PL and finally after addition of 1 μM compound B, an inhibitor of Kv1.5 K^+ channels. Data in the $I-V$ curves and in the summary are plotted as mean ± S.E., with number of experiments in brackets. *$P<0.001$ vs. PL alone or PL+Kv1.5 vesicles+compd B. (B) Control experiments using membrane vesicles isolated from Lkt^- cells transfected with Kv1.5 cDNA, but not induced with dexamethasone and therefore without Kv1.5 K^+ channels (−Kv1.5 vesicles); boiled membrane vesicles containing Kv1.5 K^+ channels (from dexamethasone-induced Lkt^- cells) and gramicidin D (gramD) reconstituted into the phospholipid bilayer. Current recordings are shown with and without vesicles or gramicidin D. Data in the summary are plotted as mean ± S.E., with number of experiments in brackets. *$P<0.001$ vs. PL alone, PL+vesicles (−Kv1.5 or boiled +Kv1.5). (C) Resistance was measured of leached PLLA membranes (area 0.3 cm^2) after adding phospholipids (PL) and after adding Kv1.5 vesicles to the PL and finally after addition of 1 μM compound B, an inhibitor of Kv1.5 K^+ channels. Medium was 100 mM KCl with 10 mM HEPES (pH 7.4). PL used were 3:1 POPS: POPE (40 mg/ml). Data are plotted as mean ± S.E. Number of experiments is indicated in brackets. *$P<0.001$ vs. PL alone, PL+Kv1.5 vesicles or PL+Kv1.5 vesicles+compound B.

the resistance. Since gramicidin D channels were formed, the phospholipids must be a single bilayer thick [15] in at least some regions. When the impermeant cation $NMDG^+$ was present instead of K^+ together with gramicidin D the resistance did not change demonstrating the ion selectivity of gramicidin D ion channels, which does not allow the passage of $NMDG^+$ or Cl^-. These experiments showed that lipid bilayer formation was successful with Millipore poly-

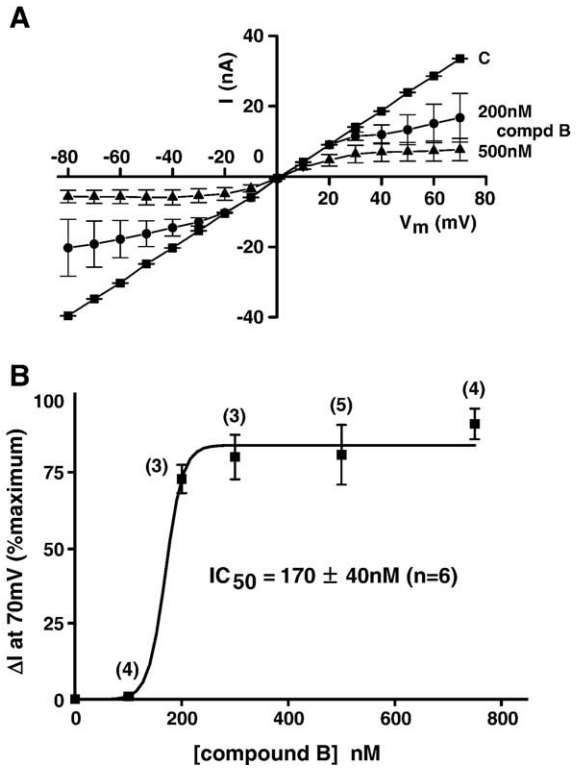

Fig. 6. Effect of varying compound B on the Kv1.5-mediated K^+ current. (A) Effect of increasing concentrations of compound B on Kv1.5 K^+ currents plotted as $I-V$ curves. Data are plotted as mean±S.E. with number of experiments in brackets. c, control. (B) Effect of compound B plotted as a dose–response curve with Kv1.5-mediated K^+ currents expressed as ΔI at 70 mV as % maximum. Data are plotted as mean±S.E. with number of experiments in brackets. From the sigmoidal plot, $IC_{50}=170\pm40$ nM ($n=6$); $\chi^2=35$, $P<0.001$.

carbonate membranes with pore sizes between 3 and 8 μm and an average thickness of 10–12 μm, PETE track-etched membranes with pore size of 3 μm and nominal thickness of 9μm and with porous PLLA membranes with pore sizes between 3 and 8 μm and a thickness of 10–15 μm. Gramicidin D and amphotericin B have been previously used to test for bilayer formation and stability by measuring currents or resistance changes [2,6,15], although cation selectivity of these compounds was not tested.

Lipid bilayers were also easily formed in 50 μm pores drilled in the wells of a 96-well plastic plate as measured by loss of currents upon bilayer formation. Kv1.5 K^+ channels were then successfully functionally reconstituted in this bilayer as measured by K^+ currents, which were inhibitable by the Kv1.5 K^+ channel inhibitor, compound B. Since there were no measurable currents using vesicles without Kv1.5 K^+ channels or vesicles with Kv1.5 K^+ channels that had been boiled, the K^+ currents were Kv1.5 K^+ channel-mediated. Gramicidin D-mediated K^+ currents were also measured across the bilayers in the 50-μm pores of the 96-well plate. Compound B-inhibitable Kv1.5 K^+ channels were also reconstituted in bilayers formed in porous PLLA membranes as measured by resistance changes although these changes were smaller than those measured using PC or PETE membranes. This could be due to the fact that PLLA pores were less uniform and varied in shape and therefore perhaps the lipid bilayers were less tightly sealed in the pore.

An essential component of these methods is the availability of an inhibitor of the transport protein being reconstituted. This is essential to establish that one is measuring function of the reconstituted protein per se (not a bilayer process and not an artifact) and that the reconstituted protein is functional.

These studies have shown that phospholipid bilayers can be formed on a wide variety of porous membranes. These membranes coated with phospholipids form supports which can successfully incorporate biological membranes containing proteins. These bilayer-incorporated membrane proteins maintain the functional properties of the native membrane proteins. These simple methods can provide composite membranes with the unique properties of biological materials. These stable permeable membrane-supported lipid bilayers can be used to incorporate a wide variety of membrane proteins in functional form, thus creating BSC membranes that mimic biological function. Use of 96 well plates with 50 μm holes (1/well) to form bilayers and reconstitute membrane transport proteins is particularly conducive to adaptation to large microarrays for high-throughput screening assays of drugs or compounds of biological interest and automation. These studies demonstrate a simple technology to create devices: functional BSC membranes that mimic biological function.

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