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Solid support membranes for ion channel arrays and sensors: application to rapid screening of pharmacological compounds

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Abstract

The use of solid supported membranes (SSM) was investigated for reconstitution of ion channels and for potential application to screen pharmacological reagents affecting ion channel function. The voltage-gated Kv1.5 K^+ channel was reconstituted on an SSM and a current was measured. This current was dependent on the presence of K^+, but not Na^+, indicating that the Kv1.5 K^+ channel maintained cation specificity when reconstituted on SSM. Two pharmacological reagents applied to Kv1.5 K^+ channels reconstituted on SSM had similar inhibitory effects as those measured using Kv1.5 in biological membranes. SSM-mounted ion channels were stable enough to be washed with buffer solution and reused many times, allowing solution exchange essential for pharmacological drug screening.

Keywords: Solid supported membrane, SSM; Kv1.5; Ion channel; Reconstitution; Rapid drug screening

1. Introduction

The use of solid supported membranes (SSMs) has become a popular method of studying biological processes of cellular proteins [1]. An SSM is constructed on a silanized glass slide, coated with a thin layer of chromium (10 nm) and then gold (150 nm). The gold surface is treated with long chain alkyl thiol, which is subsequently coated with a lipid monolayer. The bilayer formed by the lipid and the alkyl chain on the gold surface is similar to the planar lipid bilayer widely used to study ion channel activity, but it is much more stable [2]. It has been reported that some proteins behave similarly to their cellular counterparts when reconstituted on an SSM [3]. In addition, recent studies on the charge translocation by the Na/K ATPase on an SSM [4], cytochrome b_5 on a cushioned SSM [5], and rhodopsin on an SSM to study transducin activation [6] demonstrated similarity in behavior of these proteins on solid supported surfaces compared to that measured in biological membranes. The main advantages of using SSMs include their mechanical stability and ability to rapidly change the solution environment [7]. To our knowledge, reconstitution of ion channels on SSMs has not been studied prior to the present study.

Ion channels are expected to act similarly to the previously reported behavior of other biological proteins on SSMs. The voltage-gated Kv1.5 K^+ channel was chosen for this study. It is typically found in human and mammalian cardiovascular cells [8,9]. Kv1.5 is a delayed rectifier that controls the membrane potential of neurons and its biological activity in cells has been studied extensively [10]. Inhibitors and other effectors of Kv1.5 channels are available, and their effects on Kv1.5-mediated K^+ currents in cells have been highly studied [11,12].

Kv1.5 K^+ channels were reconstituted on SSMs, and a current was measured in response to holding potential. Several experiments were carried out to test the view that the current measured was Kv1.5-mediated and that thus functional reconstitution of Kv1.5 on SSM had been

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achieved. Cation selectivity of the current and effects of specific Kv1.5 inhibitors on the current were examined. Kv1.5 currents were K^+-dependent, virtually absent in the presence of Na^+, and inhibited by specific Kv1.5 inhibitors. These effects were similar to those observed with Kv1.5 in biological membranes, supporting the view that Kv1.5 K^+ channels were successfully reconstituted on SSM and appeared to maintain normal channel function. The strength and stability of SSM containing reconstituted functional ion channels suggest that it can be used to construct a screening device for pharmacological agents affecting ion channels.

2. Materials and methods

2.1. Membrane vesicle preparation

An Ltk^- cell line (mouse fibroblast cells) stably overexpressing Kv1.5 K^+ channels under the control of a dexamethasone promoter was used to prepare plasma membrane vesicles [13]. Expression of Kv1.5 was induced in Ltk^- cells by addition of dexamethasone to the medium. The dexamethasone-specific induction of channel expression is totally specific for Kv1.5 channels [13]. Cells were grown in 2 μM dexamethasone for 24 h prior to use. The cells were centrifuged for 5 min at 1000 rpm and resuspended in 1.0 ml of 20 mM HEPES (pH 7.5), 20 mM NaCl, 100 mM KCl, 1.0 mM EDTA, 0.02% NaN_3, 1 mM PMSF, 10 μg/μl leupeptin, and 50 μg/μl aprotinin. After freeze-thawing twice, the membrane fragments/vesicles were collected following centrifugation at 12,000×*g* for 20 min at 4 °C. Membrane vesicles were also prepared from uninduced Ltk^- cells (no dexamethasone incubation) transfected with Kv1.5 K^+ channel cDNA and from non-transfected Lkt^- cells.

2.2. SSM preparation

Glass slides were first plated with chromium (5 nm), and then gold (150 nm). The slide was then immersed in ethanol containing 1% octadecanethiol (w/w) for 48 h to attach alkyl thiol groups. After cleaning the gold plated slide with anhydrous isopropanol, epoxy resin was applied to the surface of the thiol-treated gold. Defects in the epoxy resin coating were used as the experimental chamber after coating the device with a lipid monolayer. A small area at the end of slide was left free of epoxy resin so that a silver wire could be soldered onto the surface of the gold plated slide. A 3:1 mixture of palmitoyl-oleoyl-phosphatidylserine (POPS) and pamitoyl-oleoyl-phosphatidylethanolamine (POPE) lipids, 10 mg/ml and 3.33 mg/ml in hexane, respectively, was used to form a Langmuir monolayer, which was then deposited on the thiol-treated gold slide using the Langmuir-Blodgett technique [14]. The experimental wells were constructed by mounting a plastic ring on the surface with silicon grease and sealed with a coating of clear nail polish around the inner edge. A silver wire was soldered to the surface of gold plated slide to provide electrical connection.

2.3. Ion channel reconstitution and current measurement

Membrane vesicles containing Kv1.5 K^+ channels were added to the lipid coated wells containing 125 mM KCl/10 mM K-HEPES pH 7.4. Currents were measured with an HS-2A headstage and Gene Clamp 500 amplifier (Axon Instruments, Foster City, CA). Channel currents were filtered at 60 Hz. Voltages ranging from −80 to +70 mV were applied in 10-mV increments for 200 ms, and electrical currents were recorded. pCLAMP version 5.5 was used to acquire data and Clampfit 8.0 (Axon Instruments) was used to compare current recordings. Similar measurements were made using membrane vesicles isolated from non-transfected Lkt^- cells and from transfected Ltk^- cells that had not been induced to express Kv1.5 with dexamethasone. Cation selectivity was measured by removing K^+ from the medium and replacing it with Na^+. Currents were first measured with K^+ present. SSMs were then washed with K^+-free, Na^+-containing medium and currents were measured with Na^+ present. Currents with K^+ present were remeasured. Statistical analysis was carried out using Student's *t*-test.

2.4. Whole-cell patch clamp electrophysiology

Whole-cell Kv1.5 current recordings were made at room temperature via the gigaseal patch clamp technique using an Axopatch-1D amplifier (Axon Instruments). Ltk^- cells overexpressing Kv1.5 channels were cultured for 24–72 h, induced to express Kv1.5 channels by 24-h incubation with 2 μM dexamethasone prior to use for patch clamp studies. Small, spherical cells approximately 10 μm in diameter were used for all patch recordings. Electrodes were made from TW-150F glass capillary tubes (World Precision Instruments, New Haven CT) and had resistances of 1.5–3.0 MΩ when filled with internal solution containing 110 mM KCl, 5 mM K_2ATP, 5 mM K_4BAPTA, 1 mM $MgCl_2$ and 10 mM HEPES, adjusted to pH 7.2 with KOH. The external solution contained 130 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$ 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, adjusted to pH 7.4 with NaOH. Series resistance was compensated following rupture of the seal. Currents were sampled at 1 kHz and filtered at 500 Hz. Cells were pulsed to +60 mV every 5 s from a holding potential of −70 mV in 20-mV increments. After stable control currents were obtained, inhibitors were perfused onto the cells at increasing concentrations until maximal inhibition was obtained for a given concentration. Whole-cell patch data were analyzed using Clampfit 8.0 in pCLAMP software (Axon Instruments). IC_{50} values for compounds were determined

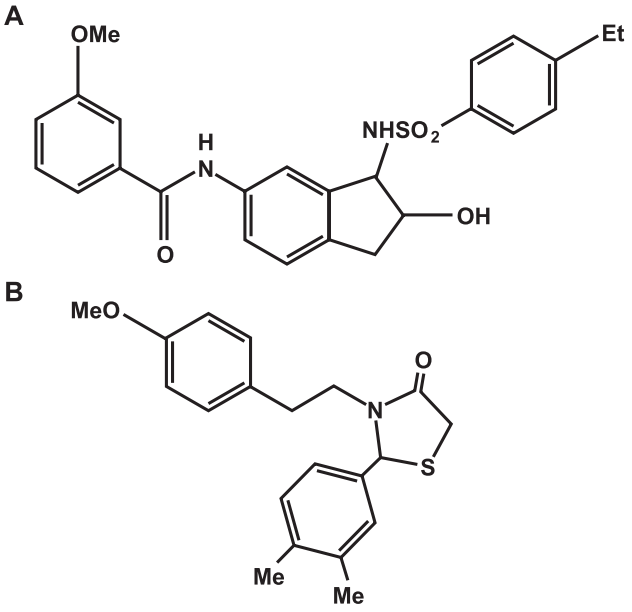

Fig. 1. Structure of inhibitor compounds. (A) Compound A is *N*-[(2*S*,3*S*)-3-[[(4-ethylphenyl)sulfonyl]amino]-2,3-dihydro-2-hydroxy-1*H*-inden-5yl]-3-methoxybenzamide and (B) compound B is 2-(3,4-dimethyphenyl)-3-[2-(4-methoxyphenyl)ethyl]-thiazolidin-4-one.

by nonlinear regression analysis using GraphPad Prism software (San Diego, CA).

2.5. Physical characterization

A current was recorded using K^+ medium in the absence of any added membrane vesicles at −80 mV for each well on the SSM surface. The surface area not covered by epoxy was determined mathematically by using a microscope and scale. The relationship between current and SSM area was determined.

2.6. Materials

Precleaned glass slides were obtained from Becton Dickinson Labware. Gold coating was performed by H.L. Clausing (Skokie, IL). Silver wire, 1-octadecanethiol and DMSO were from Aldrich (Milwaukee, WI). HEPES, KCl, and NaCl were from Sigma (St. Louis, MO). POPS and POPE were from Avanti Polar Lipids and dissolved in reagent grade *n*-hexane. Ag/AgCl reference electrode was obtained from Warner Instrument (New York, NY). Epoxy resin (5-min epoxy, no. 14250) was from Devcon. The inhibitors used were from Procter and Gamble Pharmaceuticals (Cincinnati, OH). Compound A, prepared according to the procedure provided in US patent #6,083,986 [15], is (*N*-[(2S,3S)-3-[[(4-ethylphenyl) sulfonyl]amino]-2,3-dihydro-2-hydroxy-1*H*-inden-5yl]-3-methoxybenzamide). Compound B, prepared according to the procedure provided in US patent #6,174,908 [16], is (2-(3,4-dimethyphenyl)-3-[2-(4-methoxyphenyl) ethyl]-thiazolidin-4-one. Structures of the two inhibitors are shown in Fig. 1.

3. Results

3.1. Reconstitution of Kv 1.5 into an SSM and cation selectivity

Before addition of membrane vesicles, the current across the SSM was measured at different holding potentials and plotted as an *I*/*V* curve. This is shown in Fig. 2A (□). Membrane vesicles containing dexamethasone-induced Kv1.5 K^+ channels were then added to the SSM, and after approximately 20 min, an increase in the current was evident at the same potentials (■). Typical current recordings of SSM without membrane vesicles (top) and with membrane vesicles containing induced Kv1.5 K^+ channels (bottom) are shown in the inset. At −80 mV, the increase was 7.76±3.10 (*n*=6) μA and at +70 mV, the increase was 8.06±3.18 (*n*=6) μA. These currents were significantly

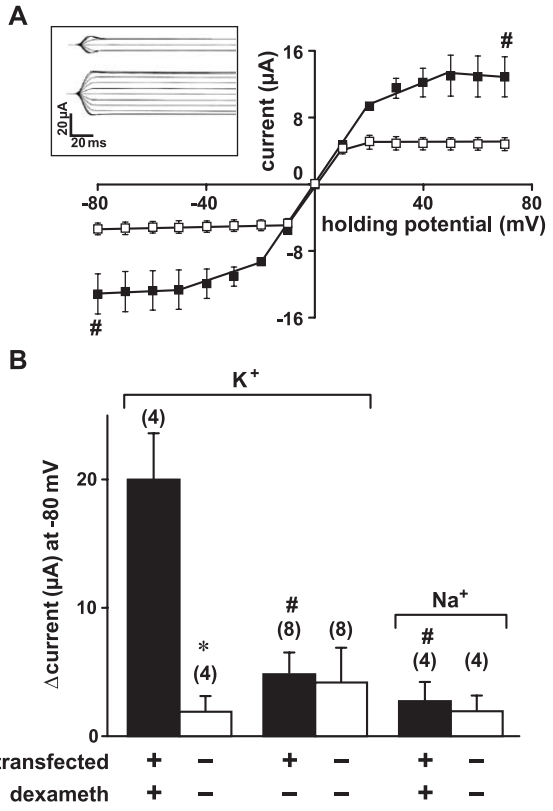

Fig. 2. (A) *I*/*V* relationship of K^+ channel currents recorded after reconstitution of membrane vesicles isolated from dexamthasone-induced Kv1.5 expressing Lkt^- cells into SSM (■) compared to currents measured with SSM alone (□). Data are plotted as mean±S.E. (*n*=6). $^{\#}P<0.01$ compared to SSM alone. SSM was made of a 3:1 mixture of POPS/POPE. Inset shows typical current recordings; top: SSM alone; bottom: SSM containing Kv1.5 K^+ channels. (B) Comparison of K^+ currents recorded at −80 mV using membrane vesicles isolated from Lkt^- cells transfected with Kv1.5 cDNA and treated with or without dexamethasone as well as membrane vesicles isolated from non-transfected Lkt^- cells. Data are expressed as Δcurrent since current measured in the SSM before membrane vesicle addition has been subtracted and is plotted as mean±S.E. (*n*). Also shown is cation selectivity in which Na^+ replaced K^+ in the medium ($+Na^+$). $^*P<0.005$ and $^{\#}P<0.01$ with respect to Δcurrent measured using membrane vesicles from dexamethasone-induced, Kv1.5-transfected cells.

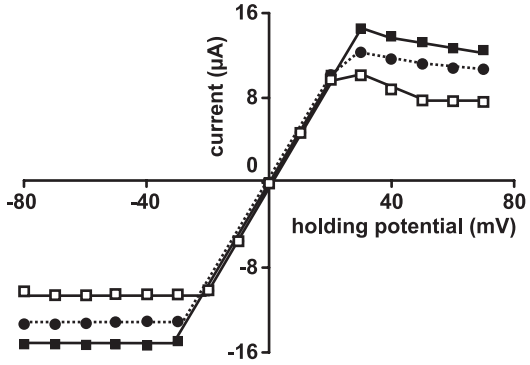

Fig. 3. A representative *I*/*V* relationship showing the effect of 100 nM compound A on Kv1.5 K^+ channel currents on SSM. (□) Current of SSM alone; (■) Kv1.5 K^+ current without compound A; (●) Kv1.5 K^+ current with 100 nM compound A.

(P<0.01) higher than the currents measured in the absence of membrane vesicles, but were not significantly different in magnitude from each other. The *I*/*V* relationship was not linear and showed similar rectification at both positive and negative holding potentials. A comparison of currents generated using membrane vesicles from Kv1.5-transfected Ltk^- cells±dexamethasone as well as from non-transfected Ltk^- cells are shown in Fig. 2B. Data are expressed as $\triangle I$. The current measured across SSM without any membrane vesicle addition (leak current) was subtracted from the current measured in the presence of membrane vesicles. Using membrane vesicles expressing dexamethasone-induced Kv1.5 K^+ channels, a large current (20.11±3.55 μA, n=4) was measured which was virtually absent when membrane vesicles from non-transfected Lkt^- cells were used (1.90±1.27 μA, n=4). This difference was highly significant (P<0.005). When membrane vesicles prepared from uninduced Kv1.5-transfected Lkt^- cells (not incubated with dexamethasone) were added to the SSM, a very small current increase of 4.88±1.66 μA (n=8) was observed. This current was also significantly lower (P<0.01) than that measured with membrane vesicles containing dexamethasone-induced Kv1.5 K^+ channels and was not significantly different from that measured using membrane vesicles from non-transfected Lkt^- cells.

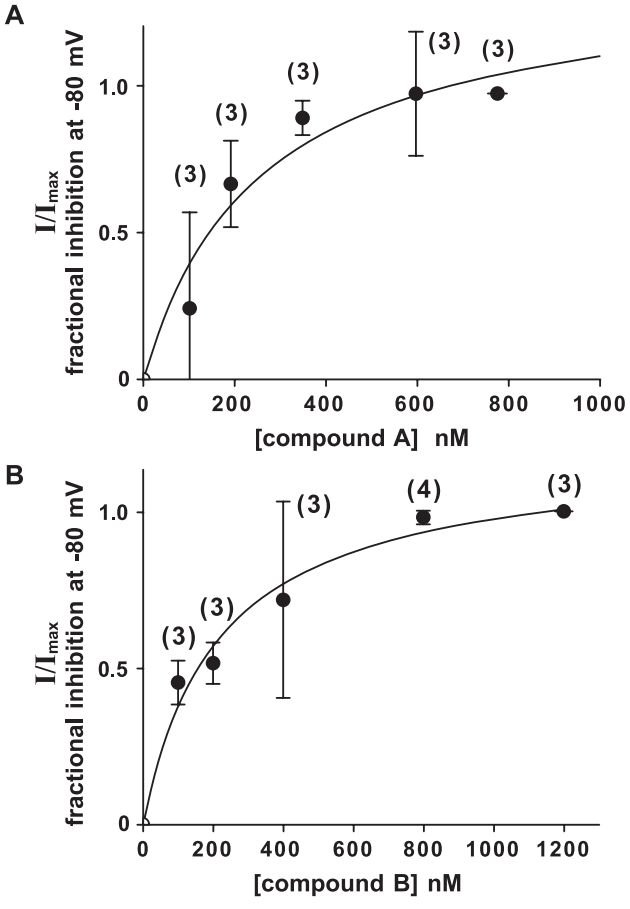

Fig. 4. Effect of varying (A) compound A and (B) compound B concentrations on Kv1.5 K^+ channel currents on SSM. The fractional inhibition of the current at −80 mV holding potential, I/I_{max}, is plotted as mean±S.E. (n). IC_{50} for compound A is 218 nM. IC_{50} for compound B is 265 nM.

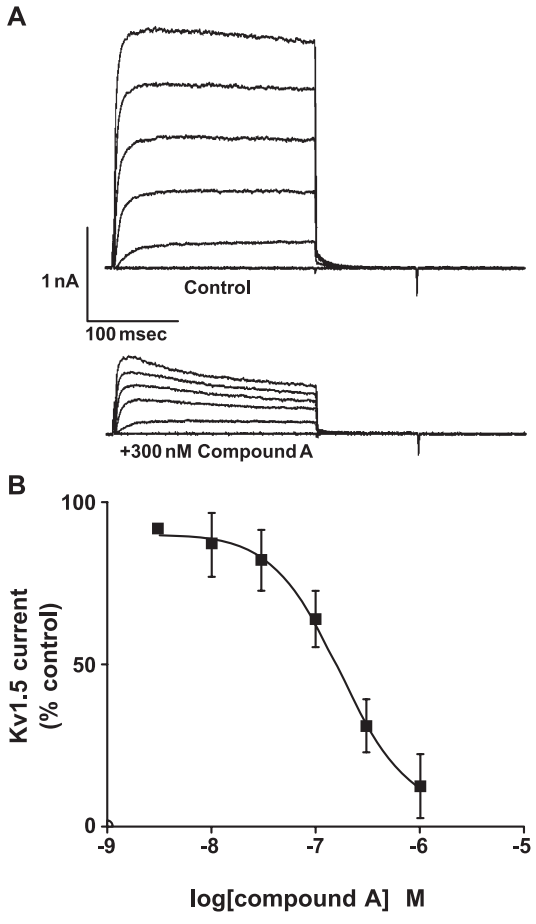

Fig. 5. Effect of compound A on Kv1.5 K^+ currents measured by whole-cell patch clamp of dexamethasone-induced Lkt^- cells expressing Kv1.5 K^+ channels. (A) Typical current recordings without (control) and with 300 nM compound A. (B) The dose–response curve plotted as % control. Data are plotted as mean±S.D. (n=10) and IC_{50} is 170 nM.

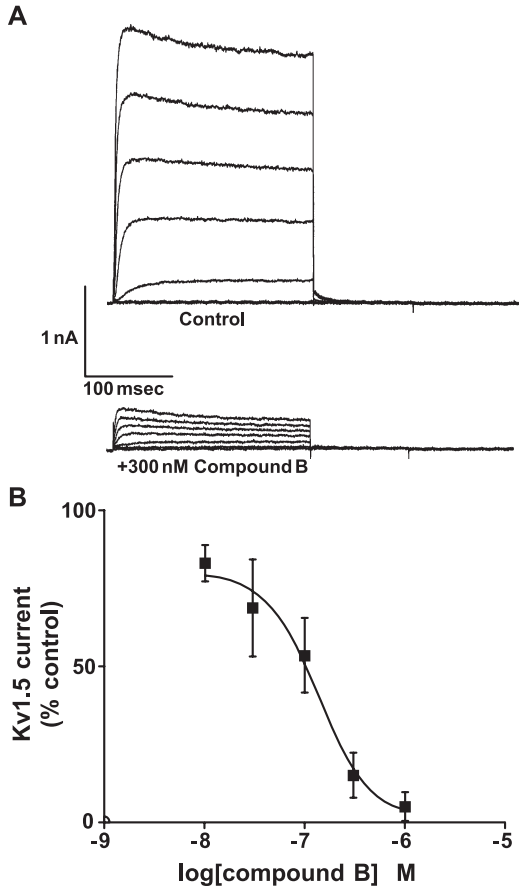

Fig. 6. Effect of compound B on Kv1.5 K^+ currents measured by whole-cell patch clamp of dexamethasone-induced Lkt^- cells expressing Kv1.5 K^+ channels. (A) Typical current recordings without (control) and with 300 nM compound B. (B) The dose–response curve plotted as % control. Data are plotted as mean±S.D. (n=3) and IC_{50} is 137 nM.

To investigate whether the increased current was occurring through Kv1.5 K^+ channels successfully and functionally reconstituted on the SSM, cation selectivity was examined. The effect of K^+ removal and replacement with Na^+ on the current measured at −80 mV was investigated. Current was measured first with K^+ present and then with Na^+ present. The results obtained are shown in Fig. 2B. When K^+ was removed from the medium, the current decreased significantly (P<0.01) from 20.11±3.55 (n=4) to 2.73±1.56 (n=4) μA. Using membrane vesicles from non-transfected Lkt^- cells in which Kv1.5 channels were absent, currents were low and similar whether in KCl or NaCl medium. These findings indicate that Kv1.5 K^+ channels reconstituted on the SSM were functional and highly selective for K^+ over Na^+.

3.2. Effect of Kv1.5 K^+ channel inhibitors

To further support the view that the measured current was due to the presence and function of Kv1.5 K^+ channels on the SSM and not due to a nonspecific leak in the SSM, the effect of two specific inhibitors of Kv1.5 K^+ channels, compounds A and B, was measured. Fig. 3 shows an example of the effect of 100 nM compound A on the current measured at varying holding potentials with Kv1.5 K^+ channels reconstituted on the SSM. The current decreased over the range of holding potentials outside of the range of ±20 mV. Fig. 4A shows the effect of varying concentrations of compound A on Kv1.5 K^+ channel current expressed as fractional inhibition (I/I_{max}). Compound A dose-dependently inhibited the Kv1.5 K^+ channel current at −80 mV with half-maximal inhibition, IC_{50}=218 nM (n=3). Similar inhibition of Kv1.5 K^+ channel currents at −80 mV was observed with compound B (Fig. 4B) with IC_{50}=265 nM (n=3). Figs. 5A and 6A show Kv1.5 K^+ channel currents recorded by whole-cell patch clamp of dexamethasone-induced transfected Lkt^- cells without (control) and with 300 nM of inhibitor compounds A and B, respectively. A similar level of inhibition of the current was observed with both compounds. Dose–response curves are shown in Figs. 5B and 6B. Using whole-cell patch clamp, IC_{50} was 170 nM for Compound A and 137 nM for Compound B, values similar to those calculated from experiments using Kv1.5 K^+ channels incorporated into SSMs.

Stability and robustness of the SSM-mounted Kv1.5 K^+ channels and reversibility of the inhibitor effects were also examined. Fig. 7 shows a typical experiment in which

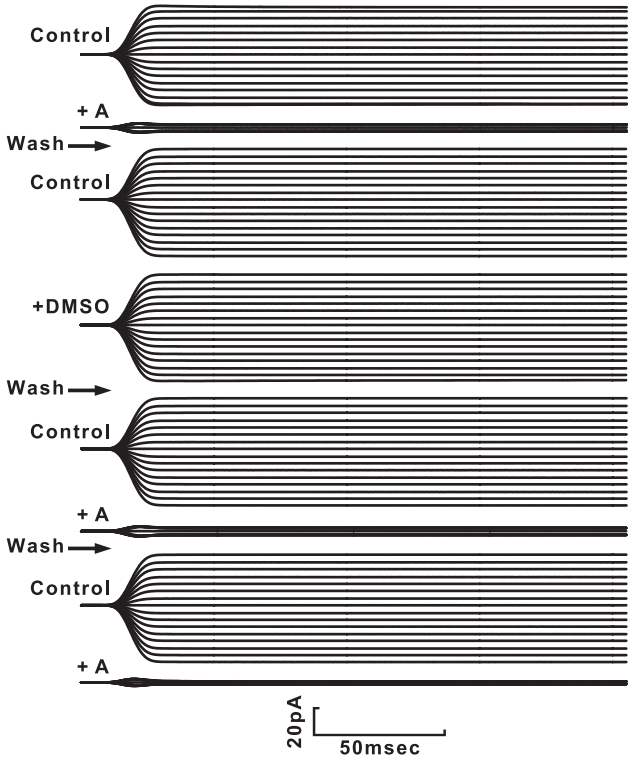

Fig. 7. Typical sequential current recordings from SSM-mounted Kv1.5 K^+ channels obtained over a period of 3.5 h. Currents without and with 774 nM compound A are shown, as well as a DMSO control recording. In between recordings, as indicated, the SSM-mounted channels were washed three times with fresh medium. In this experiment the medium was 130 mM K-methanesulfonate, pH 7.4.

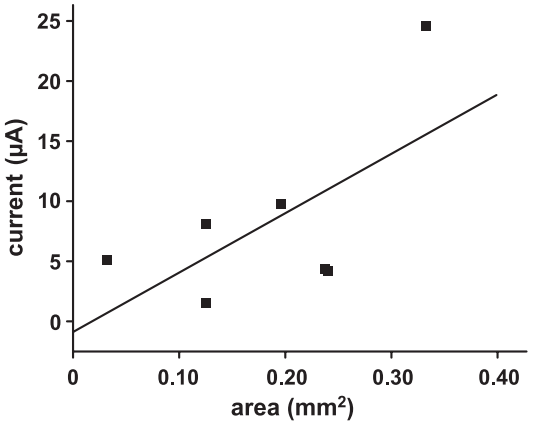

Fig. 8. Relationship between size of SSM surface and current measured of SSM alone, i.e., in the absence of any membrane vesicles, using the K^+ buffer at −80 mV holding potential. The line was calculated by linear regression: R=0.633 (n=7).

current recordings were carried out over a period of 3.5 h. The effect of 774 nM compound A was tested and retested three times after washing the SSM with medium. A DMSO control was also carried out. Compound A at 774 nM caused similar current inhibition each time it was tested and currents measured after washes were similar to the first current recording before inhibitor was added. No deterioration of currents was observed over 3.5 h. DMSO had no effect. Thus, SSM-mounted Kv1.5 K^+ channels were very stable. Inhibitor effects were reversible and the SSM with Kv1.5 K^+ channels could be reused.

3.3. Physical characterization

As shown in Fig. 2A, there was a current associated only with the lipid coating without any membrane vesicles present. These leak currents were measured and plotted against the corresponding SSM area. The diameter of the area varied from 200 to 700 μm as estimated by microscopic examination. The results are shown in Fig. 8. There was a moderate correlation of the SSM current (without membrane vesicles) with the area of the SSM as estimated microscopically. For each individual lipid bilayer used for an experiment, this leak would be a constant related only to the area of the bilayer. Under ideal conditions, the lipid monolayer obtained with the Langmuir–Blodgett technique is tightly packed resulting in high capacitance. Evidence of leak current is present. In the experimental setup used, the amount of insulation achieved with a lipid coating was proportional to the area (Fig. 8).

4. Discussion

Voltage-gated Kv1.5 K^+ channels were reconstituted on SSMs as indicated by an increased K^+ current upon the addition of membrane vesicles containing Kv1.5 K^+ channels. A reduction in the K^+ current was also demonstrated with the addition of inhibitors to Kv1.5 K^+ channels as well as with the replacement of K^+ with Na^+. The IC_{50} values for the inhibitors using Kv1.5 reconstituted on SSM were comparable to their patch clamp equivalent [15,16]. This suggested that the SSM reconstituted Kv1.5 channels had responded to the inhibitors as if they were in a biological membrane. The potential advantages of using SSM-mounted ion channels are their mechanical and physical stability compared to that of unsupported membranes, which are not easily washed and reused, and the system appears to be amenable to automation. The testing wells containing SSM-mounted ion channels could be simply washed with buffer and reused many times. This suggests that it may be possible to use this system for rapid screening of pharmacological compounds.

The inhibitory effects on Kv1.5 K^+ currents appeared to be hyperbolic, suggesting a simple bimolecular interaction between the drugs and the channel, without effects on the membrane itself. Effects on the membrane per se would be expected to be linear, rather than hyperbolic. This essentially rules out an effect of the compounds on the SSM lipids.

Kv1.5 K^+ channels in cells are rectified [10]. However, the I/V relationship indicates that ions move at both positive and negative holding potentials. This behavior could be explained if the orientation of Kv1.5 was a mixture of inside-out and outside-in Kv1.5 channels. The resultant channel current would be the sum of the activities of the channels in both orientations and thus would show rectification at positive and negative holding potentials. This is likely the case since Kv1.5 K^+ channels were introduced to the SSM surface as membrane fragments.

Currents observed with this method are in the microampere range whereas typical single channel currents are in the picoampere range. There are many active Kv1.5 ion channels on the surface of the SSMs. The ion currents measured are large (the sum of those occurring through the channel proteins in the SSM), as expected from the large surface area of the SSM.

There are reports of water being present between a bilayer film and a solid support [17], where water exists as a thin layer of 10–20 Å. A theoretical model introduced by Sparr and Wennerström [17] reveals that membranes are permeable to water under certain conditions [17], which may be similar to the SSM.

In summary, reconstitution of ion channels on SSMs was demonstrated. A low leak current increased when Kv1.5 K^+ channels were introduced on the SSM surface. This was indirect evidence that ion channels were reconstituted. Moreover, the level of the K^+ current could be reduced by removal of K^+ (and replacement with Na^+) or by addition of ion channel inhibitors. The IC_{50}s obtained using SSMs were comparable to those obtained from patch clamp studies. Kv1.5 K^+ channels reconstituted on SSMs maintained cation specificity as seen in cells. Ion channels mounted on SSMs can potentially substitute for the time-consuming

patch clamp method for rapid screening of pharmacological reagents.

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