Bilayer Reconstitution of Voltage-Dependent Ion Channels using a Microfabricated Silicon Chip

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ABSTRACT Painted bilayers containing reconstituted ion channels serve as a well defined model system for electrophysiological investigations of channel structure and function. Horizontally oriented bilayers with easy solution access to both sides were obtained by painting a phospholipid:decane mixture across a cylindrical pore etched into a 200-μm thick silicon wafer. Silanization of the SiO_2 layer produced a hydrophobic surface that promoted the adhesion of the lipid mixture. Standard lithographic techniques and anisotropic deep-reactive ion etching were used to create pores with diameters from 50 to 200 μm. The cylindrical structure of the pore in the partition and the surface treatment resulted in stable bilayers. These were used to reconstitute Maxi K channels in the 100- and 200-μm diameter pores. The electrophysiological characteristics of bilayers suspended in microchips were comparable with that of other bilayer preparations. The horizontal orientation and good voltage clamping properties make the microchip bilayer method an excellent system to study the electrical properties of reconstituted membrane proteins simultaneously with optical probes.

INTRODUCTION

Model membrane systems have proven to be useful tools for probing the biophysics of lipid bilayers and transmembrane proteins. Supported bilayers on mica, glass, and Si/SiO_2 substrates have been used for studies of both active and nonactive biomolecules using various optical and scanning probe microscopies (Sackmann, 1996). In the Langmuir-Blodgett (LB) technique, a monolayer is transferred to the substrate followed by the deposition of a vesicle with incorporated proteins (Kalb et al., 1992; Tatulian et al., 1995). As the vesicles spread, the lipids from the vesicle form the top bilayer leaflet. A simple variation of that technique deposits protein-containing vesicles directly onto a hydrophilic surface. In this instance, the vesicles spread and form bilayers on the substrate (Brian and McConnell, 1984). These two techniques have provided much insight to our understanding of lipid diffusion along translational (Kalb et al., 1992; Groves et al., 1997; Harms et al., 1999) and rotational (Harms et al., 1999) coordinates. The orientational characteristics (Tatulian et al., 1995; Salafsky et al., 1996) and functional properties (Salafsky et al., 1996) of reconstituted transmembrane proteins have also been described. Wagner and Tamm (2000) have recently reported on a method which increases the distance between the bilayer and the substrate by first depositing an LB film of a cushion polymer, followed by LB monolayer deposition, and then direct deposition of vesicles with incorporated protein. This development minimizes the interaction of transmembrane proteins to the substrate and, in effect, allows the proteins to carry out their activity as they do in a natural cell environment.

For the purpose of electrophysiological measurements of ion channels, the use of supported bilayers presents difficulties. Isolating a region of a bilayer requires the formation of a gigaohm seal between the bilayer and the supporting structure. Further, because there is only a $\sim$10–50 Å water film separating the bilayer from the substrate, solution exchange within this region is not feasible. To the best of our knowledge, no reports of voltage clamping of these types of supported bilayers have appeared in the literature.

Electrophysiologists interested in monitoring single-ion channel currents have developed a model membrane "painted-bilayer" technique in which isolated transmembrane proteins maybe reconstituted (Mueller et al., 1962). Bilayers are painted onto micropores that are punched through Teflon (DuPont, Wilmington, DE) or plastic sheets, and the presence of a bilayer is determined by measurement of the characteristic bilayer capacitance (Wonderlin et al., 1990; Labarca and Latorre, 1992). Artificial bilayer systems can be used to study permeation and gating properties of individual ion channels in a chemically isolated environment. This contrasts with the standard micropipette patch-clamp method, which measures channels in their natural cellular environment where other macromolecules may interfere with channel function. An additional advantage of the artificial bilayer preparation is the ease with which solution exchange can be achieved in both sides of the membrane.

Pores fashioned from microtomed indentations of high density polyethylene plastic have the advantage of reducing the access resistance because of thin rim apertures, as compared with pores drilled into Teflon partitions. A stable horizontal-bilayer orientation has also been demonstrated for the plastic partitions (Wonderlin et al., 1990), and such an orientation is desirable for optical experiments. A disadvantage of using plastic partitions is that their dimensions

Received for publication 13 February 2001 and in final form 12 July 2001.
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0006-3495/01/10/2389/06 $2.00

are not as easily controlled from partition to partition. Finally, it is difficult to control the bilayer to solid interfacial properties in these types of partitions because the surface chemistry of Teflon or plastic is typically not amenable to chemical modification.

Advances in silicon processing techniques, coupled with progress in the organic chemistry of SiO_2 surfaces, have enabled the coupling of silicon micromachined devices with biological materials (Jaklevic et al., 1999; Voldman et al., 1991). The straightforward silicon-processing control over micron-scale dimensions makes silicon an ideal substrate for constructing microlaboratories, used for studies ranging from macromolecule recognition to cell manipulation. In this paper, we present an experimental approach for preparing a suspended bilayer on micromachined silicon wafers, and we demonstrate that single ion channels can be incorporated into such a bilayer. These channels can be voltage-clamped, and optical and chemical access to the bilayers is readily achieved. Much of the variability in conditions associated with the plastic and Teflon partitions is removed by using lithographic/etching techniques to control the pore size and by silanizing the silicon oxide surface to optimize the membrane/pore chemical interface. Schmidt et al. (2000) have recently reported on a microchip based technique in which electrophoretic focusing is used to overlay a vesicle onto a narrow pore micromachined through a Si_3N_4 diaphragm. Introduction of water-soluble bacterial pore channels into the aqueous solution led to demonstrable channel activity under voltage clamp. That work, although bearing some similarities to what is reported here, is substantially different with respect to device architecture, membrane preparation, and the issues of chemical and electrical access to either side of the bilayer.

MATERIALS AND METHODS

Pore preparation

Pores 50, 80, 100, and 200 μm in diameter were micromachined in silicon wafers using well established, semiconductor processing techniques. Phosphorous doped, 101.60 mm diameter $\times$ 280–320 μm thick, 111-crystal orientation, N-type wafers from WaferNet (San Jose, CA) were thinned to $\sim$200-μm thickness with a deep reactive ion etcher using the Bosch 59 process (Ayon et al., 2000). This step reduced the amount of time required to etch the pores through the wafer. Next, the wafers were cleaned by ultrasonication in an acetone bath for 5 min and then in 2-propanol for 5 min, and immediately rinsed thoroughly with deionized (DI) water (18 MΩ) and air-dried under flowing N_2. A dehydration bake was performed by placing the wafer on a 150°C hot plate for at least 5 min, and the wafer was cooled to room temperature. Negative photo-resist (NPR) SU8–5 and SU-8 developer were both purchased from MicroChem Corporation (Newton, MA)(Loechel., 2000). SU8–5 NPR, which produces a thick (15–20 μm) and robust etch-resistant film, was spun onto the wafer (ramping at 180 rpm/s from 0 rpm to 900 rpm where it was held for 30 s and then ramped back down to 0 rpm at 180 rpm/s) using a programmable spin coater (Headway Research, Inc., Garland, TX). This was followed by a preexposure bake at 95°C for 15 min. A 2-min lithographic exposure (350 W mercury arc lamp; 365 nm) was carried out using a mask aligner (Karl Suss, Munich, Germany). Next, a postexposure bake was done at 95°C for 30 min, and the wafer was cooled to room temperature. The wafer was then placed in SU-8 developer for $\sim$3–4 min, and rinsed with DI water and dried under flowing N_2. The wafer was then glued onto a carrier wafer (bottom side) using a few drops of AZ5214 photo-resist from Clariant Corporation (Sunnyvale, CA). This step was done to maintain a constant base pressure during the etching process. The bonded-pair of wafers was then placed in a PlasmaTherm SLR770 ICP deep reactive ion-etcher (Unaxis Corporation, St. Petersburg, FL) in which the patterned wafer with photo-resist was exposed to a cycle of SF_6 and C_4F_8 plasmas, resulting in a deep anisotropic etch, at an etch rate of $\sim$2.5 μm/min. The wafers were then separated from one another by dissolving the AZ5214 with acetone. The SU8–5 NPR photo-resist was removed by first immersing the wafer in an oxidizing solution (2:1 concentrated H_2SO_4:H_2O_2(30%)) for $\sim$15 min, and then immersing it in an identical but fresh solution of the same mixture for 30 min. Next, the wafer was rinsed with DI water dried under flowing N_2. The wafer was diced into chips (4 mm $\times$ 4 mm), resulting in one pore per chip. Finally, an oxide coating (SiO_2) was applied to both sides of the chip using a PlasmaTherm 790 Series plasma-enhanced chemical vapor deposition device (Unaxis Corp.). The deposition time is 20 min, which produces an oxide thickness of $\sim$1 μm.

Microchip surface treatment

A hydrophobic surface on the partition was necessary to promote surface wetting by the *n*-decane solvent used to dissolve the phospholipid mixture. Therefore, a silanization step was done just before painting the pore with the phospholipid bilayer. If >1 day elapsed between the silanization and application of the bilayer steps, the chips were cleaned by ultrasonication in an acetone solution for 5 min and then in 2-propanol for another 5 min, and immediately rinsed thoroughly with DI water (18 MΩ) and air-dried under flowing N_2 before silanization. The silanization was performed by placing the chips vertically on an aluminum foil base in a 300 ml Kimax crystallizing dish from Fisher Scientific (Tustin, CA) and depositing 100 μl of tri-*n*-butylchlorosilane from Pfaltz and Bauer (Waterbury, CT) on the jar bottom. The jar was immediately capped with Pyrex (Corning, Garden Grove, CA) watch glass also purchased from Fisher Scientific, and sealed with Teflon tape. The vessel was then placed in an oven at 160°C for 24 h.

Protein-vesicle isolation

Maxi K channel C-Less (C14S, C141S, and C277S) (h*Slo*) mutant R207Q-N200C was expressed in *Xenopus laevis* oocytes. This mutant was selected because of the relative ease in resolving single-channel currents and for labeling experiments using the engineered cysteine. Oocytes were injected with 50 nl of 0.2 mg/ml mRNA in water (Tseng-Crank et al., 1994; Adelman et al., 1992). They were maintained at 18°C in standard oocyte solution (in mM: 100 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.3) supplemented with 50 μg/ml gentamycin for $\sim$5 days until homogenization. The preparation of the vesicles was identical to that of Perez et al. (1994) and a brief description of the procedure will now be provided. Batches of 20 to 30 oocytes were first rinsed with a 10% w/v sucrose solution dissolved in K-Buffer (600 mM KCl, 5 mM K-PIPES, pH 6.8). The rinsed oocytes were placed in a 1-ml Pyrex tissue grinder from Kontes Duall Glass (Hayward, CA) and 10 ml/oocyte of 10%w/v sucrose dissolved in K-Buffer supplemented with protease inhibitors (100 μM phenylmethylsulfonylfluoride, 1 μM pepstatin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μM *p*-aminobenzamidine) from Sigma (St. Louis, MO). Next, the oocytes were mechanically homogenized with a matching rod also from Kontes Duall Glass. The homogenate was placed on top of 20% w/v sucrose: 50% w/v sucrose (both dissolved in K-Buffer with protease inhibitors) gradient in a Sorvall centrifuge tube purchased from Fisher Scientific (Tustin, CA). The tube was placed in a swinging bucket holder, which was then mounted on the Sorvall RP55S rotor. The first centrifu-

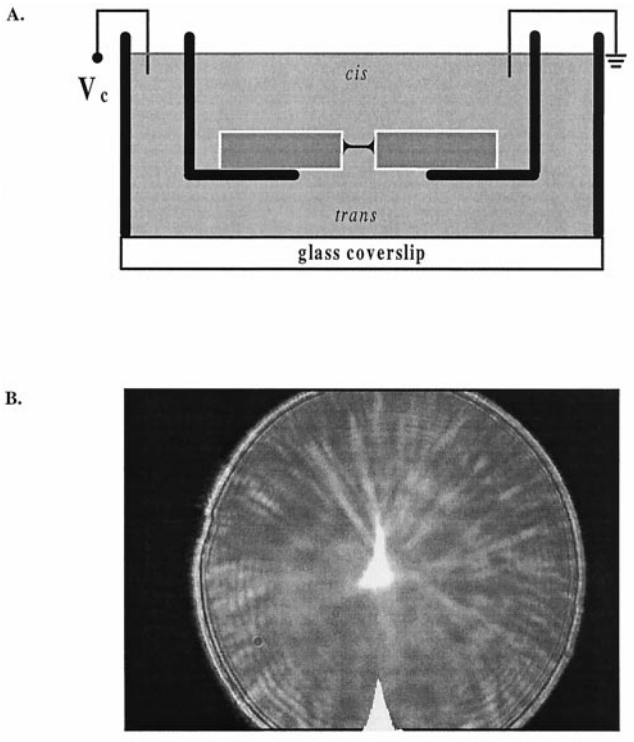

FIGURE 1 (*A*) A cross-sectional view of the microchip/chamber set-up (not to scale). The silicon wafer is shown as the darker gray disk resting on top of the black inside chamber. The white rim represents the functionalized SiO_2 surface. The hourglass-shaped structure in the wafer pore represents the bilayer and surrounding annulus. The bottom of the inside chamber is sealed to the microchip with Vaseline. (*B*) A bottom view of a bilayer suspended on a 100-μm diameter pore silicon microchip. Illumination is from the top. The light being collected is transmitted and, therefore, the bilayer does not look black.

gation was done at 30,000 rpm (61,000 $\times$ *g* average) for 30 min at 4°C with a swinging bucket rotor. The band at the 20:50 interface after the first centrifugation was extracted with a syringe with a 20.5-gauge needle. Removing excess material from the band can lead to unstable bilayers that rupture during incorporation. The extract was diluted three times with solution A (300 mM sucrose, 100 mM KCl, 5 mM K-MOPS, pH 6.8) and the first centrifugation sequence was repeated. The vesicle preparation needs to be carried out in either ice or at 4°C to minimize protease activity. The pellet precipitate recovered after this step was aliquoted to 4-μl portions in Eppendorf tubes that were then submerged in liquid N_2 and stored in a freezer at -80°C. To produce a greater proportion of unilamellar vesicles before use, the vesicle preparation was ultrasonicated for 5–15 s using the special ultrasonic cleaner from Laboratory Supplies Company (Hicksville, NY).

Reconstitution into lipid bilayers

The sample chamber used in these experiments was a Teflon cylinder threaded within a larger cylinder, with the silicon chip placed horizontally and sealed on the base of the inside cylinder (Fig. 1*A*) with Vaseline purchased from Fisher Scientific. The inside cylinder, or top compartment (*cis* side) is held at virtual ground and the bottom compartment (*trans* side) is the voltage-controlled side. A lipid mixture was prepared by dissolving a phosphatidylethanolamine:phosphatidylcholine:phosphatidylserine (PE:PC:PS) from Avanti Polar Lipids (Alabaster, AL), ratio of 5:3:2 in 25 mg/ml (Perez et al., 1994). The bilayer and then the vesicles are painted onto the *cis* side of the wafer using separate glass rods. Voltage-clamping was performed with a home-built bilayer clamp as described by Alvarez (1986). Briefly, the current-to-voltage converter consisted of a low-input voltage noise OPA 111 operational amplifier (Burr-Brown, Tuscon, AZ) with a 1 gigaohm feedback resistor (Kobra, Springfield, MA). The signal was amplified with a gain of 10 pA/mV and then fed at full bandwidth into a 16-bit analog-to-digital converter at a rate of 5 μs per point. The signal was processed with a PC44 board (Innovative Technologies, Moorpark, CA), that was interfaced with a Pentium-based computer (Intel Corp., Santa Clara, CA). Before final sampling, the data were digitally filtered using a steep finite impulse response algorithm (cutoff frequency set at twice the sampling rate). Measuring capacitance with the signal from a triangle wave generator (150 mV peak to peak; 140-ms period) monitored bilayer formation. To promote vesicle adhesion, we used the following solution compositions: 250 mM KCl, 5 mM K-MOPS, 0.1 mM $CaCl_2$, pH 7.4 solution on the *cis* side and 50 mM KCl, 5 mM K-MOPS, 0.1 mM $CaCl_2$, pH 7.4 solution on the *trans* side. Incorporation of ion channel containing vesicles was marked by the onset of single channel currents in response to a pulse train (100 mV for 100 ms followed by -100 mV for 100 ms). To prevent further incorporation, ionic concentrations were symmetrized by adding a 3.64 M KCl, 5 mM K-MOPS, 0.1 mM $CaCl_2$, pH 7.4 solution to the *trans* side and no agitation was done to speed up mixing.

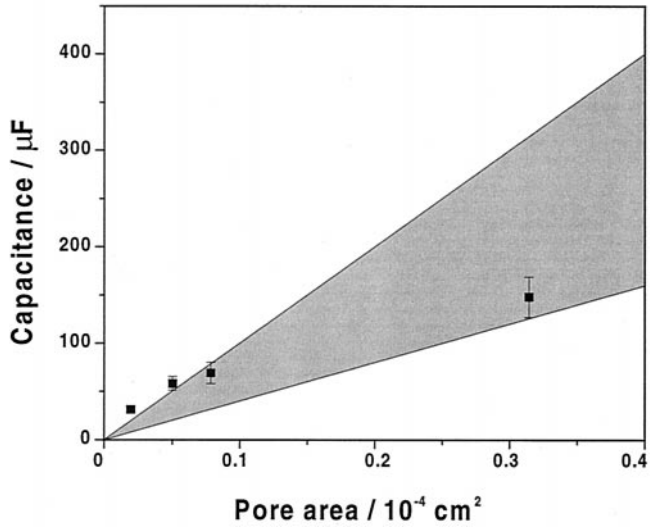

FIGURE 2 Capacitance measurements for bilayers suspended on a silicon microchip. The shaded area represents the literature bilayer capacitance range of 0.4 to 1 μF/cm^2. The observed trend indicates that the annulus fraction of the pore decreases as the area of the pore decreases which is typically accompanied by a decrease in bilayer thickness.

RESULTS AND DISCUSSION

Bilayer suspended on a microfabricated silicon chip

Silicon processing capabilities allow the preparation of such precise structures on the micron scale. A unique feature of this microchip is the SiO_2 film that can be functionalized with silane. The surface hydrophobicity produced by the alkyl chains of the silane enhances the attraction between the *n*-decane and the substrate surface to ensure tight seal formation between the annulus solvent and aperture. The

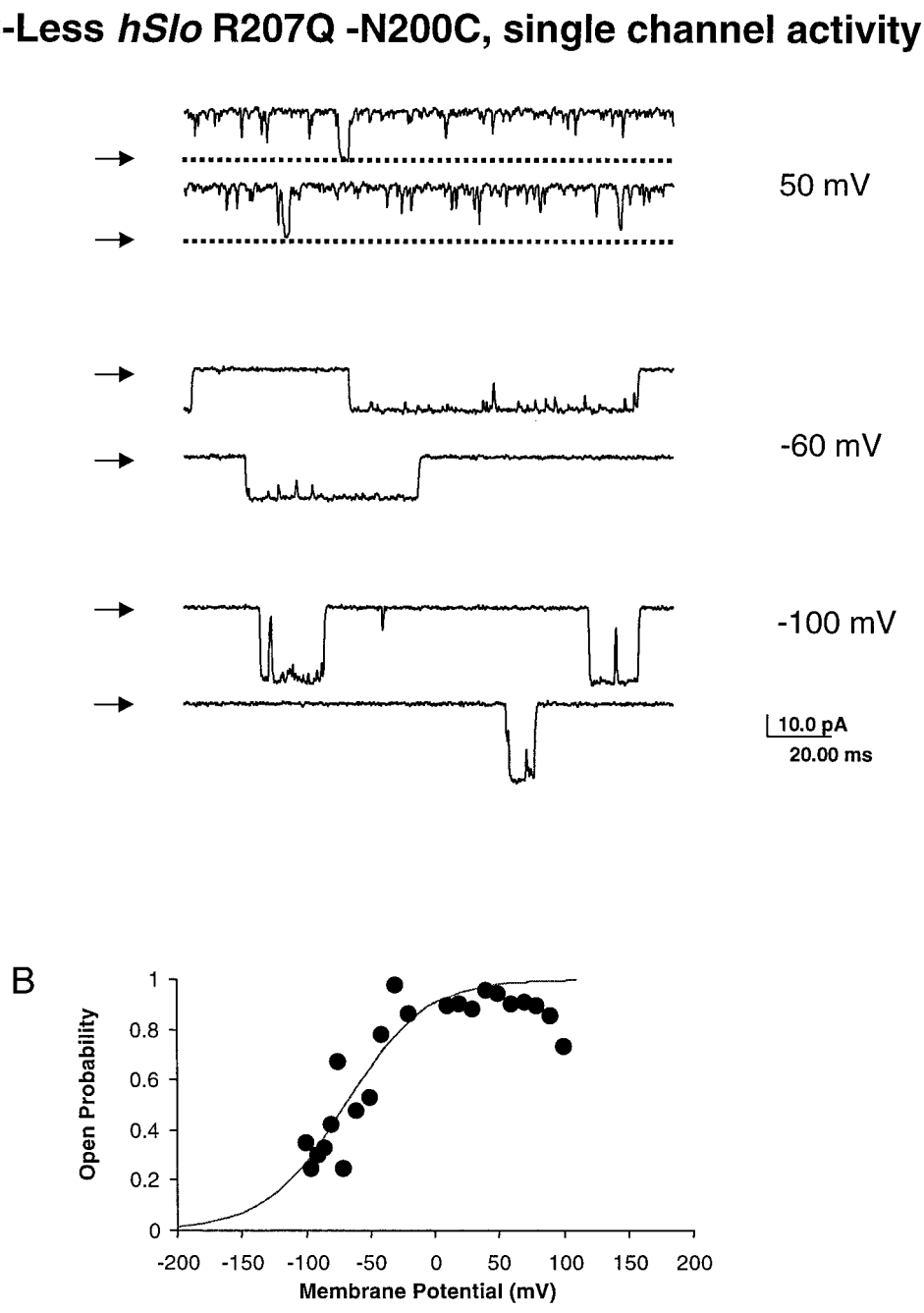

FIGURE 3 (*A*) Recordings of an active Maxi K ion channel reconstituted in a bilayer suspended within a silicon microchip. The arrows indicate the closed state. (*B*) The open probability (P_o) of a single ion channel versus the membrane potential (V) is plotted. 3 s are averaged per point. The fit is to a standard Boltzmann function: $P_o = (1+\exp(-q\cdot(V-V_{1/2})/kT))^{-1}$, where q is the gating charge, $V_{1/2}$ is the potential of half activation, and kT has its usual thermodynamic meaning. Values for the fit were $V_{1/2} = -69.4$ mV and $z = 0.81$.

resulting horizontal bilayers proved to be extremely robust, even in the presence of modest fluid level differences between the two chambers. Optical pictures demonstrated that the bilayer is centered both within the pore and docked near the midpoint between the top and bottom surfaces of the silicon wafer (Fig. 1 *B*). Electrophysiological measurements of bilayer properties were computed from the current response to a small (< 5 mV) step in voltage. Capacitance values were computed as the ratio of total integrated charge to the voltage change, and normalized with respect to the full area of the pore, after subtracting off a small component (19 pF) attributable to the chamber and recording apparatus.

Square current responses to triangular waveforms yielded identical values of capacitance. The range of bilayer capacitance measured for the 100- and 200-μm diameter were similar to the range in literature value of 0.4 to 1 μF/cm^2 represented in the shaded area in Fig. 2 (Labarca and Latorre, 1992). Bilayers suspended in the narrower diameter pores (50 and 80 μm) exhibited capacitance values that were slightly larger than 1 μF/cm^2 (Fig. 2) which is contrary to the classical view of capacitance versus pore area relationships. The classical view of bilayers is that the annulus will occupy a higher percentage of the area in the smaller pores as compared with the larger area pores. In such a scenario, the capacitance values would produce the opposite trend of what we observe. We believe that the increase in specific capacitance for smaller pores in our system is caused by an increase in percentage of bilayer area for small pores which is typically accompanied by a decrease in bilayer thickness from solvent extrusion (White, 1985). This is because the very hydrophobic nature of the microstructure surface influences solvent extrusion to a greater degree in our system, as compared with other partitions.

In addition, we do not observe channel incorporation in the 50- and 80-μm pores. There are two possible explanations for this effect. First, channel reconstitution in nearly solvent-free bilayers is not favored. Second, the smaller pores have high aspect ratio and the bilayers are positioned too far from the outer surface, preventing the vesicle glass rod from coming into intimate contact with the bilayer. However, channel reconstitution in the 100- and 200-μm diameter pores was as reproducible as the plastic partitions with similar noise characteristics. That is, as expected from the increase in absolute capacitance with pore diameter, the root-mean-square current noise amplitude increases as a function of pore area, from 0.5 to 1.9 pA for 100- and 200-μm diameter pores, respectively. The characteristic access resistance of the pores in the absence of bilayers is $\sim$50 KΩ. Membrane resistance of the suspended bilayer typically exceeded 5 GΩ.

Maxi K recordings

To demonstrate the use of our technology, we used a large conductance mutant Maxi K channel C-less (C14S, C141S, and C227S) R207Q N200C. The R207Q mutation shifts the range of gating charge movement to voltages that are more easily clamped than in the wild-type channel (leftward shift of Q-V). Maxi K mutant channel recordings were taken using a 5:3:2 ratio of PE:PC:PS, respectively, at 25 mg/ml dissolved in *n*-decane (Fig. 3 *A*). Vesicles were applied after the bilayer was formed and electrically monitored for several min to check stability. Similar to what has been previously reported (Perez et al., 1994, Labarca and Latorre, 1992), ion channel fusion into the bilayer is promoted by a 5:1 [K^+] concentration gradient with respect to *cis:trans* compartments. To discourage multiple incorporation events, the asymmetrical [K^+] concentrations were equalized after incorporation to a final [K^+] concentration of 250 mM by adding 3.64 M KCl to the *trans* solution.

We incorporated single Maxi K channel mutants into seven bilayers (Fig. 3 *A*). Four bilayers were formed in a plastic partition as described by Wonderlin et al. (1990). Three remaining preparations used etched microchips. In all cases, the channel incorporated with the extracellular portion of the channel facing the *cis* compartment. The conductance of the ion channel for identical 250:50 mM KCl *cis:trans* conditions was 197.68 $\pm$ 2.89 pS ($n = 5$) independent of partition. The voltage dependence of the probability is shown in Fig. 3 *B* and is similar to the dependence determined by Diaz et al. (1998). The high symmetrical [Ca^{2+}] resulted in some blocking of the ion channel resulting in a decrease of the open probability from its maximum value at large depolarizations.

CONCLUSION

We have demonstrated that standard silicon microchips with phospholipid bilayers can serve as useful tools to study active ion-channel proteins. The dimensions of the silicon wafers may be tailored to meet specific experiment requirements. The drive in our group is to couple the Si microchip with our ultrasensitive microscopes with high numerical apertures. In addition, we also believe that because of the relative ease of suspending the bilayer on the microchip, these results may provide a first step toward a high-throughput drug screening device.

We thank Dr. Riccardo Olcese for invaluable discussions with regard to single-channel analysis and Dr. Ligia Toro for providing us with the Maxi K clone. We also thank Dr. Ramon Latorre for very useful bilayer discussions. We also gratefully acknowledge the assistance provided by the UCLA Nanoelectronics Research Facility staff.

This research was supported by the William M. Keck Foundation and National Institutes of Health Grant GM 30376.

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