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A Storable Encapsulated Bilayer Chip Containing a Single Protein Nanopore

Xiao-feng Kang,*,† Stephen Cheley,†,§ Allison C. Rice-Ficht,† and Hagan Bayley*,‡

Contribution from the Department of Molecular and Cellular Medicine, The Texas A&M University System Health Science Center, College Station, Texas 77843-1114, and Department of Chemistry, University of Oxford, Oxford OX1 3TA, United Kingdom

Received December 3, 2006; E-mail: hagan.bayley@chem.ox.ac.uk; xfkang@medicine.tamu.edu

Abstract: A robust, portable chip containing a single protein nanopore would be a significant development in the practical application of stochastic sensing technology. Here, we describe a chip in which a single α-hemolysin (αHL) pore in a planar phospholipid bilayer is sandwiched between two layers of agarose gel. These encapsulated nanopore chips remain functional after storage for weeks. The detection of the second messenger inositol 1,4,5-trisphosphate (IP_3) was demonstrated with a chip containing a genetically engineered αHL pore as the sensor element.

Introduction

Engineered protein nanopores have been devised as sensor elements for stochastic sensing[1] and DNA detection.[2] A single pore is placed in a planar lipid bilayer, and the ionic current that passes through it under an applied potential is monitored. Individual analytes, ions or molecules that pass through the pore, or bind or react within it, are registered through a modulation of the current. In this way, α-hemolysin (αHL) pores have been used to detect cations[3] and anions,[4] small organic molecules,[5−7] reactive molecules,[8,9] DNA,[10−12] and proteins.[13−15] However, practical applications of this technology are restricted by the fragility of planar lipid bilayers, which are sensitive to mechanical disturbance and therefore cannot be transported, and typically last only a few hours under optimal conditions. By contrast, we have demonstrated that the αHL nanopore itself is extraordinarily stable; for example, it can operate at temperatures close to 100 °C.[16]

One way to overcome the instability of the bilayer is to do away with it by using solid-state nanopores, such as those fabricated from Si_3N_4[17−19] or Au-coated nanotubes in a plastic film.[20] While solid-state Si_3N_4 nanopores have been used to detect large DNA molecules, it will prove more difficult to functionalize these pores to facilitate the detection of specific analytes or classes of analytes, which has been a strength of the work with protein nanopores, to which genetic engineering and site-specific chemical modification techniques can be readily applied. Conical Au-coated nanotubes have been functionalized by chemical modification or protein deposition over the entire gold surface and then used to detect proteins that bind near the narrow mouth.[20] Binding is manifested as a complete and irreversible block of the current. Precise spatial control over the modification is not possible. Further, at present, single-molecule detection with Au-coated nanopores has not been demonstrated.

Another possibility is to improve the stability of the lipid bilayer. Examples include tethered lipid bilayers on solid supports,[21−23] polymerized planar bilayers,[24] and protected planar bilayers.[25,26] Although solid-supported bilayers possess high mechanical stability, they pose several difficulties for sensor applications with protein nanopores. For example, only one side of a membrane is accessible, and ions accumulate or become

† The Texas A&M University.
‡ University of Oxford.
§ Present address: Department of Chemistry, Michigan State University, East Lansing, MI 48824.

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depleted in the thin aqueous zone between the solid support and the lipid bilayer. At this point, single-channel currents have not been recorded in this configuration.[27] By contrast, single-channel currents have been observed in polymerized planar bilayers,[24] but here it is a difficult task to prove that the bilayers are actually more stable than unpolymerized bilayers, and, indeed, in this configuration, the stability may not be limited by the properties of the bilayer itself but by the solvent torus that is required to bridge the bilayer and edge of the aperture in the plastic support film.[24]

Recently, a lipid membrane protected with a layer of photopolymerized poly(ethylene glycol) dimethacrylate (PEG-DMA) was reported to be of improved stability.[26] Protein pores were added after the protective gel had been formed, and one drawback of the approach is that this is a slow process, presumably because of the slow rate of diffusion of the protein through the gel. In this case, again, the improved stability of the system was not demonstrated convincingly. Beddow and colleagues used pre-cast gel slabs to protect both sides of a lipid bilayer, and incorporated the nicotinic acetylcholine receptor,[25] or the antibiotics valinomycin and gramicidin,[28] and examined their electrical properties. While the stability of the bilayer was significantly improved, the noise level was too high to observe single-channel currents.[25]

In the present work, we describe a chip in which a bilayer containing a single αHL pore is formed and then encapsulated by forming an agarose gel around it. These chips are mechanically stable and remain functionally intact when stored for weeks. Small analyte molecules can readily access the pore through the thin porous agarose layer.

Results and Discussion

Preparation and Characterization of an Encapsulated Bilayer Chip Containing a Single Protein Nanopore. Planar lipid bilayers have been used for more than three decades for the characterization of channels and pores. In one approach, which we extend here, a bilayer is formed by "folding" two lipid monolayers across a small hexadecane-treated aperture in a 10−25 μm-thick Teflon septum that separates the cis and trans compartments of a recording chamber.[29] To obtain a stable protein nanopore chip, we designed a bilayer scaffold with a three-layered structure, in which a 25 μm-thick Teflon septum with a 100-μm diameter aperture was glued between two 200 μm-thick polyester films. A 0.2 cm-diameter orifice was cut into each polyester film and used, ultimately, to contain the encapsulating gel (Figure 1).

The preparation of the encapsulated bilayer was carried out in a chamber that can be heated and cooled with a Peltier device.[16] After the scaffold had been clamped between the cis and trans compartments of the chamber, the aperture of the Teflon septum was primed with a 1:10 hexadecane/pentane

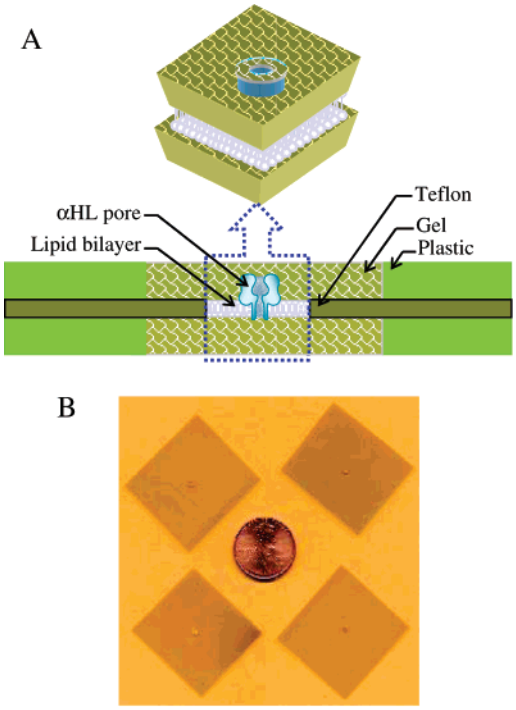

Figure 1. Chip containing a single encapsulated nanopore. (A) Schematic of the chip. The various components are not to scale. The scaffold is a three-layered structure; a 25 μm-thick Teflon septum with a 100-μm diameter aperture is glued between two 200 μm-thick polyester films each with a 0.2 cm-diameter orifice. After a lipid bilayer containing a single αHL pore has been formed in the Teflon aperture, agarose gel is allowed to set over the entire chip. The gel is then cut away so that thin layers remain in the polyester orifices on both sides of the bilayer. (B) The photograph shows four encapsulated bilayer chips, each containing a single WT αHL pore, after storage for 3 weeks at 4 °C.

mixture. Once the priming solution had dried, the chamber was placed in the chamber holder and 1.5% agarose at 45 °C in 0.1 M Tris·HCl, pH 7.4, containing 1 M NaCl (0.75 mL, prepared by heating and cooling) was added to both compartments. At this point, the solution level was below the orifice in the polyester films, and the temperature in the chamber was maintained at 45 °C to keep the agarose in solution. Next, 1% DPhPC (1,2-diphytanoyl-*sn*-glycero-3-phosphatidylcholine) in pentane (20 μL) was transferred to each compartment and allowed to spread on the surface of the solution. After ~2 min, during which the pentane evaporated, additional warm agarose solution of the same composition was added to both compartments to cause the solution level to rise above the aperture in the Teflon. The formation of a lipid bilayer across the aperture was monitored by observing the increase in capacitance to a value of 8−10 fF μm^{-2}. Following this, a WT αHL protein pore was introduced into the planar lipid bilayer. αHL is a 293-amino acid polypeptide secreted by *Staphylococcus aureus* as a water-soluble monomer that assembles into a heptameric pore.[30] Preformed heptameric αHL (0.05 ng) was added to the cis compartment, which was held at ground. The insertion of a single pore into the bilayer was detected within 1−30 min, by electrical recording, as a current jump of 44 ± 1.3 pA (n = 10) at an applied potential of −40 mV. We did not observe a delay in protein incorporation when using the agarose solution, as compared to incorporation in the absence of agarose under the

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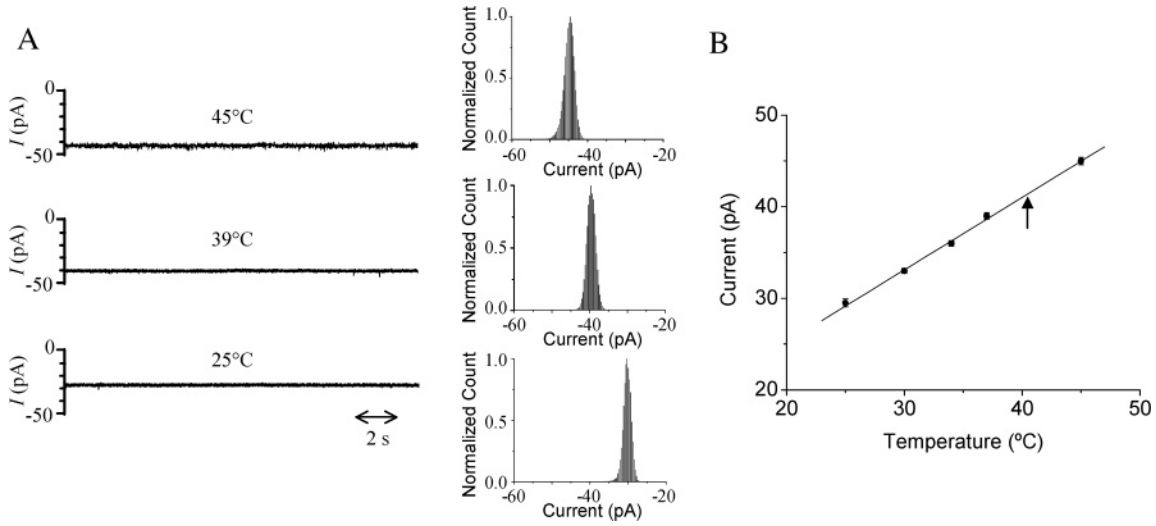

Figure 2. Single-channel current recordings during the formation of encapsulated bilayer chips. (A) Representative recordings and normalized histograms during formation of the agarose gel. A WT αHL pore was allowed to insert into a bilayer from the cis compartment, which was at ground, at an applied potential of −40 mV. A positive potential indicates a higher potential on the trans side of the chamber, and a positive current is one in which cations flow from the trans to the cis side. With respect to the αHL pore, this convention means that the cap domain is exposed to the cis compartment, while the entrance to the transmembrane β barrel at the tip of the stem domain is exposed to the trans compartment. The solution of 1.5% agarose in 0.1 M Tris·HCl, pH 7.4, containing 1 M NaCl, was initially at 45 °C. The solution was allowed to cool at ∼3 °C min^{-1} and gelled at 41 °C. Current traces were recorded at different temperatures. The corresponding amplitude histograms are shown (right). (B) Dependence of the single-channel current on temperature during the process of 1.5% agarose gelation in 0.1 M Tris·HCl, pH 7.4, containing 1 M NaCl. Current values at −40 mV were obtained from the peaks of normalized histograms. Ten separate experiments were performed, and the mean value is plotted. Bars showing the standard deviations fall close to or within the symbols. The gelation point is marked with an arrow.

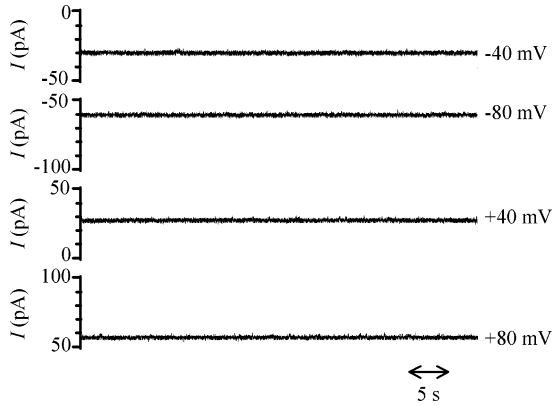

Figure 3. Currents recorded through a stored bilayer chip containing a single WT heptamer. The chip had been stored for 3 weeks at 4 °C. Current traces were recorded in 0.1 M Tris·HCl buffer, pH 7.4, containing 1 M NaCl at 25 °C.

same conditions. Following the insertion of a pore, the chambers were cooled at a rate of ∼3 °C min^{-1} to allow the agarose to form a gel. Current recording was continued during this process (Figure 2). The unitary conductance of an αHL pore in gelled agarose at 25 °C was 29.5 ± 0.9 pA at −40 mV (n = 10), which is similar to the value for an αHL pore recorded under the same conditions, in the absence of agarose (30.2 ± 1.0 pA, n = 8). No additional pores inserted after gelation. The single-channel current decreased linearly with falling temperature with a slope of 0.96 pA deg^{-1} (n = 10) during the process of gelation (Figure 2B). The temperature dependence of the unitary conductance in the absence of agarose was very similar: 0.94 pA deg^{-1}.[16] This suggests that agarose molecules do not enter the αHL pore and that the variation in conductance with temperature largely reflects the variation in conductivity of the salt solution.[16] Accordingly, the cis and trans openings of the αHL pore are 29 and 20 Å in diameter, respectively, while the mesh size of 1.5% agarose gel at 25 °C is approximately 50−100 nm in diameter.[31,32] After the agarose had gelled completely, the single nanopore chip could readily be removed from the chamber by carefully cutting the gel away from the polyester films with a thin stainless steel blade, leaving a protective layer of agarose within the larger diameter (0.2 cm) openings (Figure 1). For storage and transport, the two sides of the sandwich chip were sealed with a plastic adhesive strip over a central Teflon pad (0.22 cm in diameter), which prevented adhesion of the strip to the gel.

Table 1. Number of Intact Nanopore Chips Remaining after Mechanical Vibration for 5 min on a Platform Shaker[a]

vibration rate (rpm)	gel-protected αHL chips (4 chips were tested at each rate)	unprotected αHL pore (4 separate experiments for each rate)
10	4	0
35	4	0
70	4	0
90	4	0
105	2	0
120	1	0
150	0	0

[a] Freshly made chips containing single WT αHL pores were clamped between the cis and trans compartments of the chamber. After 1.5 mL of 0.1 M Tris·HCl, pH 7.4, containing 1 M NaCl was added to each compartment, the chamber was placed on a platform shaker for 5 min at different vibration levels. Electrical recording was then used to determine whether the WT pore was still present and intact. In the case of chips that failed, the recorded current was greater than that expected for a single WT pore, but lower than that expected for current flow through the aperture itself, suggesting that a partly blocked aperture had formed. For comparison, unprotected single αHL pores were formed in a conventional bilayer chamber, and then carefully moved to the platform of the shaker. In this case, the bilayer was completely broken by mechanical vibration at any rate, producing an open aperture.

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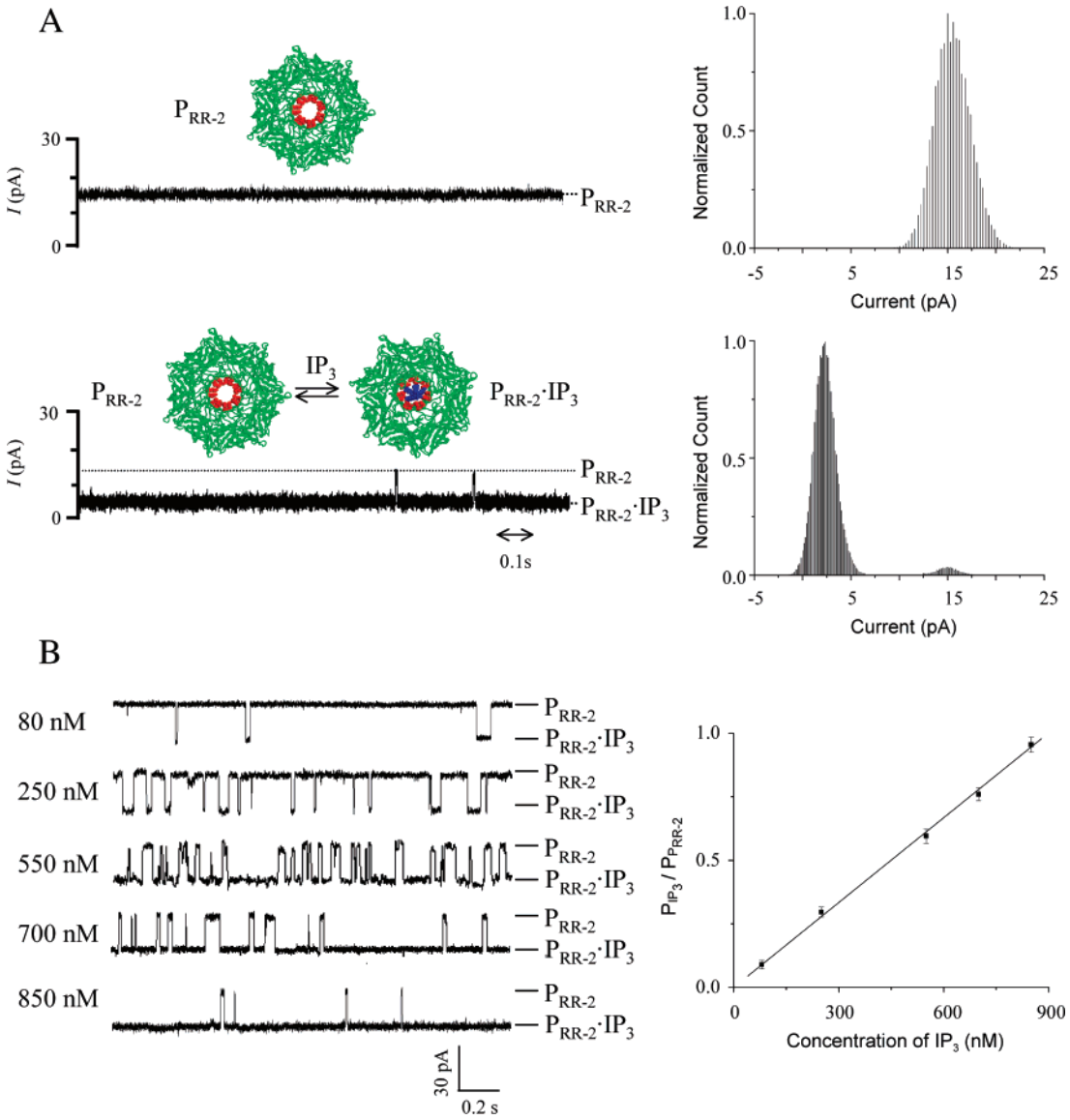

Figure 4. Response of chips containing the P_{RR-2} pore to IP_3 after storage. The chips had been stored for 3 weeks at 4 °C. (A) Current traces were recorded in 0.1 M Tris·HCl, pH 7.4, containing 1 M NaCl at 25 °C with 40 μM IP_3 in the cis compartment. The applied potential was +20 mV. Representative current traces (left) and the corresponding normalized histograms (right) are shown. (B) Single-channel current responses of a stored P_{RR-2} chip to various concentrations of IP_3 (cis compartment) at an applied potential of +40 mV. Representative traces (left) were recorded in 0.1 M Tris·HCl, pH 7.4, containing 1 M NaCl at 25 °C. Plot of $P_{IP_3}/P_{P_{RR-2}}$ versus IP_3 concentration (right). $P_{IP_3}/P_{P_{RR-2}} = (1 - P)/P$, where P is the probability that the pore is unoccupied. P_{IP_3} and $P_{P_{RR-2}}$ were obtained from the peaks of normalized single-channel current histograms. Three separate experiments were performed, and the mean value is plotted. The binding constant K_f was determined by fitting a straight line and using the relationship $P_{IP_3}/P_{P_{RR-2}} = K_f[IP_3]$. The correlation coefficient is 0.98.

The encapsulated bilayer chips were easily reassembled into the recording chamber after removing the seal. In addition, the following features of the chips were observed:

(1) Once fabricated and sealed, encapsulated bilayer chips containing a single αHL nanopore are stable and storable for at least 3 weeks at 4 °C (Figure 3) with a 30% failure rate in subsequent functional assays at room temperature. By contrast, conventional bilayer cannot be stored at all (100% failure).

(2) The agarose gel layer provides an aqueous environment and protection for the lipid bilayer containing an αHL pore, permitting the investigator to empty the solution in the chamber completely, and refill it during an experiment.

(3) The chips are robust. After an experiment, the used nanopore chip can be removed and reassembled into the chamber for a subsequent experiment. Assembly and disassembly could be repeated at least three times.

(4) The chips endure mechanical disturbance; they survived the motion of a platform shaker for at least 5 min at 90 rpm (Table 1).

(5) Encapsulated bilayer chips containing a single αHL nanopore can be used once at elevated temperature (e.g., 85 °C) after storage at 4 °C. In this case, the agarose layers melt away. The agarose might be removed in this way and the chip returned to room temperature, for the detection of analytes that cannot diffuse rapidly through the gel.

Application of the Single Nanopore Sensor Chip. To determine whether stored chips could be used for stochastic sensing, we examined their ability to detect inositol 1,4,5-trisphosphate (IP_3), a second messenger molecule implicated in a variety of signal transduction events. Previously, we showed that an engineered pore, P_{RR-2}, can be used to detect nanomolar concentrations of IP_3 in solution.[4] P_{RR-2} is a homoheptameric

pore made from the αHL mutant M113R/T145R. P_{RR-2} contains two rings of arginine side chains, which project into the lumen of the transmembrane β barrel. The high density of positive charge confers the ability to bind phosphate esters. Chips containing single P_{RR-2} pores were prepared and stored for 3 weeks at 4 °C, as described above. After reassembly into the chamber, the chips were used to detect IP_3 in 0.1 M Tris·HCl, pH 7.4, containing 1 M NaCl (Figure 4A). The IP_3 was added directly to the cis chamber. The signal reached a stationary state within a few seconds, which is consistent with the rate of diffusion of a small molecule through a 200 μm aqueous gel. In this case, attainment of the stationary state is likely to be aided by electrophoresis of the IP_3 toward the mouth of the pore in the positive applied potential. At 25 °C and an applied potential of +20 mV, IP_3 (40 μM, cis) reduced the current flowing through a single P_{RR-2} from 15.0 ± 0.4 pA ($n = 3$) to 2.3 ± 0.1 pA ($n = 3$), which are similar to the values in the absence of agarose gel (14.4 and 2.2 pA, at +20 mV).[4] However, at +20 mV, the apparent affinity of IP_3 toward the gel-encapsulated P_{RR-2} pore is somewhat greater ($K_f = (3.1 \pm 0.4) \times 10^8$ M^{-1}, $n = 3$) than it is toward the P_{RR-2} pore in the absence of agarose ($K_f = (1.2 \pm 0.1) \times 10^8$ M^{-1}, $n = 3$). The higher binding affinity is ascribed to the extended dwell time of IP_3 (τ_{off}) in the pore: $(8.0 \pm 0.4) \times 10^{-2}$ s ($n = 3$) in the absence of an agarose layer, as compared to $(2.1 \pm 0.1) \times 10^{-1}$ s ($n = 3$) with the agarose. The basis of the small difference in dwell time is unclear. In addition, as expected, the affinity of IP_3 to the gel-encapsulated P_{RR-2} pore is dependent on voltage. By comparison with the K_f value at +20 mV, K_f was decreased 258-fold to $(1.2 \pm 0.1) \times 10^6$ M^{-1} at +40 mV ($n = 3$).

The response of the stored gel-encapsulated P_{RR-2} pore to IP_3 followed the expected dependence on concentration for a binary interaction. The ratio of the time P_{RR-2} was in the occupied state to the time spent as the unoccupied pore was determined[4] and was found to increase linearly with IP_3 concentration (Figure 4B).

Conclusions

In this work, we have developed a protein nanopore sensor chip, in which a lipid bilayer containing a single protein pore is encapsulated between two layers of agarose gel. The chip is robust, storable for weeks, and portable. The approach will open up new possibilities for fundamental studies of membrane channels and pores, and the application of engineered protein pores in commercial biosensors, including their ability to act as detectors for various separation techniques.[33] The approach taken here might also be combined with additional recent developments in which probes have been used to place single copies of channels and pores in planar bilayers, either from preparations of purified protein[34] or directly from bacterial colonies.[35] A logical next step would be to use these technologies to make single pore arrays for rapid screening of membrane proteins, stochastic sensing, or DNA sequencing.

Materials and Methods

Reagents and Materials. Inositol 1,4,5-trisphosphate hexasodium salt (IP_3) and agarose (type VI-A, gel point 41 °C, gel strength ≥1200 g cm^{-2} for a 1.5% gel) were from Sigma (St. Louis, MO).

WT and Mutant αHL Pores. The assembly and purification of heptameric protein pores were carried out as reported previously.[4,36] The wild-type (WT) pores were derived from the αHL construct RL2[36] and P_{RR-2} from M113R/T145R.[4]

Planar Bilayer Recording and Data Analysis. Planar bilayer current recordings were performed with a patch clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA). The signal was low-pass filtered with a built-in 4-pole Bessel filter at 5 kHz and was sampled at 20 kHz with a computer equipped with a Digidata 1322A A/D converter (Axon Instruments). Data were analyzed and prepared for presentation by using pClamp 7.0 software (Axon Instruments) and Origin 5.0 (Microcal, Northampton, MA). Current amplitudes were obtained from the peaks of normalized histograms.

For the determination of kinetic constants, three separate experiments were performed. Mean inter-event intervals and residence times (τ values) for IP_3 were obtained from the residence time histograms by fitting the distributions to exponential functions.[4] Kinetic constants for the binding of IP_3 to P_{RR-2} pores were calculated by using $k_{off} = 1/\tau_{off}$, $k_{on} = 1/(\tau_{on}[IP_3])$, and $K_f = k_{on}/k_{off}$.

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