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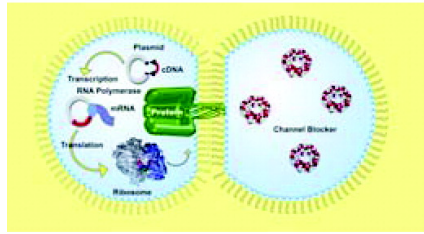
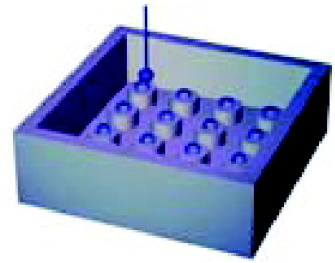
J. Am. Chem. Soc., **2008**, 130 (46), 15543-15548 • DOI: 10.1021/ja804968g • Publication Date (Web): 25 October 2008





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Screening Blockers Against a Potassium Channel with a Droplet Interface Bilayer Array

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Abstract: Droplet interface bilayers (DIBs) form between two lipid monolayer-encased aqueous droplets submerged in oil. Both major structural classes of membrane proteins, α-helix bundles and β barrels, represented by channels and pores, respectively, spontaneously insert into DIBs when freshly expressed by cell-free transcription and translation. Electrodes embedded within the droplets allow the measurement of transmembrane ionic currents carried by individual channels and pores. On the basis of these findings, we have devised a chip-based approach for the rapid screening of blockers against ion channels. The technique is demonstrated here with the viral potassium channel, Kcv.

Introduction

Membrane proteins account for approximately 25% of the polypeptides encoded within the human genome.[1] Of these, ion channels are an important class of proteins that mediate ion flow across cellular membranes and which, among other things, are responsible for the excitability of neurons.[2] The exploration of ion-channel function is central to the discovery of their roles in normal physiology and channel-related diseases (channelopathies).[3] But, despite their biological significance, the experimental investigation of ion channels remains an arduous task. For mechanistic studies, they are most often overexpressed in cells, which then are patched with micropipettes to allow macroscopic or single-channel current recording.[4] Purified ion channels can also be studied after reconstitution into model membrane systems, such as lipid vesicles or planar bilayers.[5] By contrast with work on cells, reconstitution allows the researcher to precisely control the composition of the lipid bilayer, which can affect channel function.[6−8] Further, interference from endogenous ion channels in the cells used for the expression of the channel of interest can be eliminated.

Several methods have been developed to incorporate ion channels into planar bilayers. These include the spontaneous insertion of detergent-solubilized channels,[9,10] planar bilayer formation from proteoliposomes containing the channel of interest,[11] and the fusion of proteoliposomes to a preformed planar bilayer.[12] In addition, a "probe" technique has recently been introduced, in which membrane proteins are directly transferred from a bacterial expression colony to a planar bilayer with an agarose-coated tip or a glass needle.[13,14] Despite the utility of these reconstitution methods, they are not universally applicable. Many ion channels are delicate and may denature, misfold, deoligomerize, aggregate or otherwise malfunction when subjected to these processes. Because of the challenges posed by ion-channel reconstitution, a method for simultaneous or closely coupled membrane protein expression and reconstitution would be highly desirable.

We and others have developed the droplet interface bilayer (DIB), in which two droplets under an oil/lipid mixture first become encased within lipid monolayers and are then joined to form a bilayer.[15−17] DIBs readily incorporate membrane proteins, and electrodes placed within the droplets enable electrical recording of channel activity at the single-molecule level.[16] In the present work, we show that membrane proteins freshly synthesized by *in vitro* (cell-free) transcription and translation (IVTT) outside or inside[18] a droplet are incorporated into the bilayer in a functional form, avoiding some of the issues associated with previous reconstitution methods. This approach works for both α-helix bundle and β-barrel membrane proteins.

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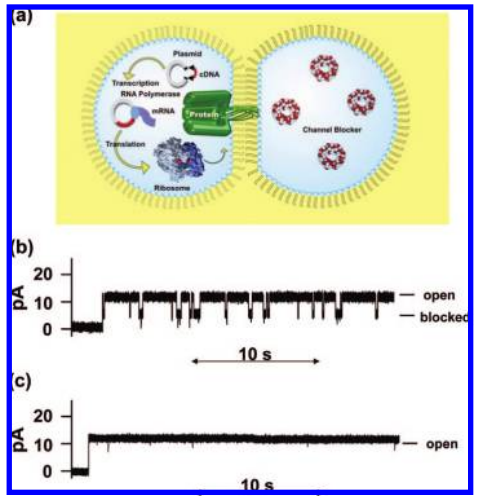

Figure 1. Expression and analysis of the pore-forming toxin staphylococcal α-hemolysin (αHL) in a DIB. (a) Schematic representation of cell-free transcription and translation (IVTT) to form αHL polypeptides within the aqueous droplet used to form a DIB. By using a plasmid containing the desired gene in an *E. coli* S30 extract, membrane proteins can be expressed and then examined by bilayer recording. The left-hand droplet contained the IVTT mixture as well as 5 mM HEPES, 250 mM KCl (to aid current recordings), at pH 7.0. The right-hand droplet contained 10 μM γ-cyclodextrin, which is a blocker of the αHL pore, in 5 mM HEPES, 250 mM KCl, pH 7.0. Both droplets contained 1 mM DPhPC lipids (as vesicles), to form a surface monolayer on each droplet. αHL is made as a monomeric polypeptide, which assembles into a transmembrane heptamer when the DIB is formed. (b) Single insertion event and current trace from a WT αHL pore incorporated into a DIB as described in (a), recorded at +50 mV. The γ-cyclodextrin in the opposing droplet caused transient current blockades. (c) Insertion event and current trace from a single WT αHL pore in the absence of γ-cyclodextrin. The droplets were suspended from Ag/AgCl electrodes to enable electrical recording.

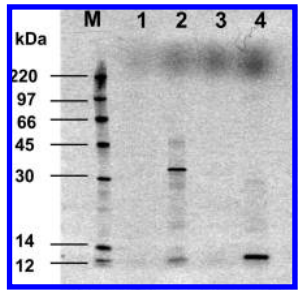

Figure 2. Cell-free expression of αHL and Kcv in the PURE system. Autoradiogram of a 12% Bis-Tris SDS-polyacrylamide gel showing the expression of αHL and Kcv after 1 h in the PURE IVTT system at room temperature and at 37 °C. Lane 1: αHL, room temperature; lane 2: αHL, 37 °C; lane 3: Kcv, room temperature; lane 4: Kcv, 37 °C. Protein synthesis did not occur at room temperature. The samples were not heated, so the gel also demonstrates that monomeric polypeptides were formed; heptamers of αHL and tetramers of Kcv are stable in SDS under the conditions of electrophoresis.[25,26,47]

Because of the long lifetime and high stability of DIBs, and because they can be dissociated and reformed,[16,17] we reckoned that the DIB system might be used for rapid screening. Accordingly, we demonstrate here rapid single-channel screening with a 16-element "DIB-chip" of blockers against the viral potassium channel Kcv, produced by IVTT. Potentially, our approach is applicable to a wide variety of ion channels, pores and other membrane proteins. Further, libraries of membrane proteins might be screened after cell-free expression of individual library members in droplets.

Results

Protein Synthesis and Incorporation into DIBs. As a demonstration of the compatibility of the IVTT approach with DIBs, we expressed and analyzed the bacterial pore-forming toxin staphylococcal α-hemolysin (αHL). In these experiments, protein synthesis and analysis were both performed at room temperature. The αHL pore is formed when seven monomers spontaneously assemble on a membrane surface.[19] The electrical properties of the αHL pore, such as unitary conductance,[20] orientation within the bilayer and blockade by small molecules, are well established.[21] A commercial IVTT kit (Promega) contains all the components necessary for coupled transcription and translation (see Supporting Information). The synthesis of wild-type αHL protein was initiated by adding plasmid DNA to the IVTT mixture, which also contained 250 mM KCl to enable subsequent current recordings (Figure 1a). Recently, a new method of DIB formation has been developed,[8] in which lipids (in the form of vesicles) are added to the aqueous phase, rather than to the oil phase. The vesicles fuse at the oil/water interface, resulting in monolayer formation around the droplets. Therefore, the IVTT mixture was supplemented with 1 mM 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) as vesicles, formed by extrusion through a 100-nm filter. Immediately following the addition of the αHL plasmid DNA to the IVTT/vesicle mixture, a 250 nL droplet of the mix was placed on a moveable Ag/AgCl electrode, controlled by micromanipulator, which was immersed in an oil bath composed of hexadecane and decane in a 10:1 ratio. A droplet was also placed on the opposing electrode, which contained 5 mM HEPES, 250 mM KCl at pH 7.0, as well as 10 μM γ-cyclodextrin (γCD). γCD is a reversible molecular blocker of the αHL pore[22] and was used as verification that the observed ionic currents were due to pore insertion rather than bilayer defects. Following a two to five minute period for monolayer formation, the droplets were joined to form a bilayer.[16]

Immediately after bilayer formation, stepwise increases in the ionic current at +50 mV confirmed the insertion of αHL pores into the bilayer. Transient blockades of the current by γCD were also observed (Figure 1b). The unitary conductance of the αHL pore in a DIB was 240 ± 11 pS (n = 6) in 5 mM HEPES, 250 mM KCl, pH 7.0, at an applied potential of +50 mV. When adjusted for salt concentration and the magnitude and polarity of the voltage, the conductance is similar to the previously determined values of 798 pS (10 mM MOPS, 1 M KCl, pH 7.0, at −50 mV) in a DIB[16] and 450 pS (5 mM HEPES, 0.5 M KCl, pH 7.4, at −40 mV) in a conventional planar bilayer.[23]

IVTT Expression Systems. We also compared two commercial IVTT systems: the Promega S30 kit (see above) and the PURE system.[24] The Promega kit is based on an *Escherichia coli* lysate collected after centrifugation at 30 000 g. In place of the lysate, the PURE system contains *E. coli* 70S ribosomes, tRNAs and over thirty purified enzymes and accessory proteins

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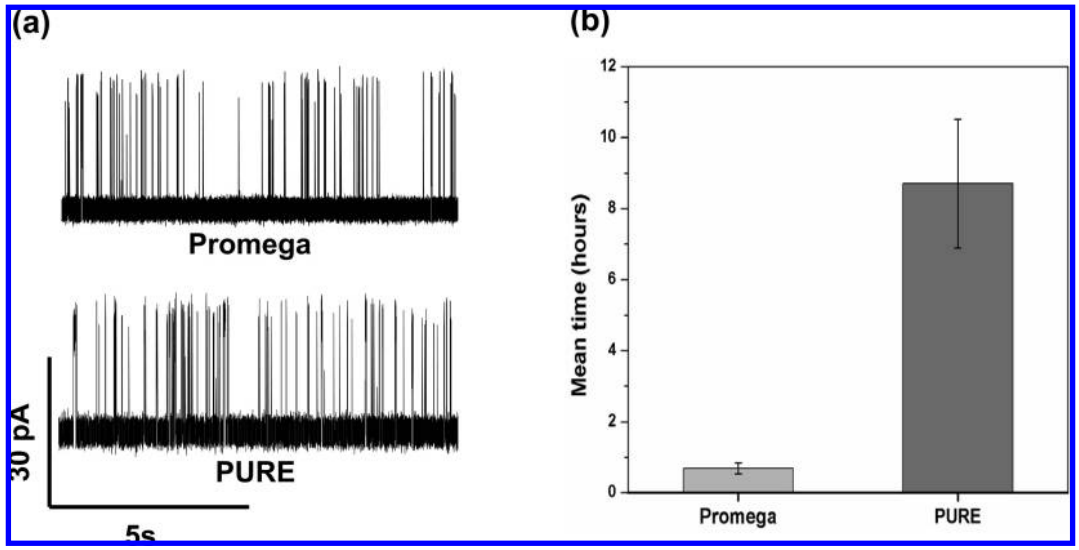

Figure 3. Comparison of Promega S30 and PURE IVTT expression systems for DIB current recording. (a) Single-channel recordings of Kcv in the DIB system (5 mM HEPES, 250 mM KCl, pH 7.0, 1 mM DPhPC as vesicles) at +100 mV are similar after expression with either kit. Channel openings are the upward spikes. (b) Comparison of DIB stability in the presence of either the Promega or PURE IVTT mixes. The lifetimes of bilayers with channel activity throughout the entire recording were determined under the same conditions as in (a) ($n = 10$).

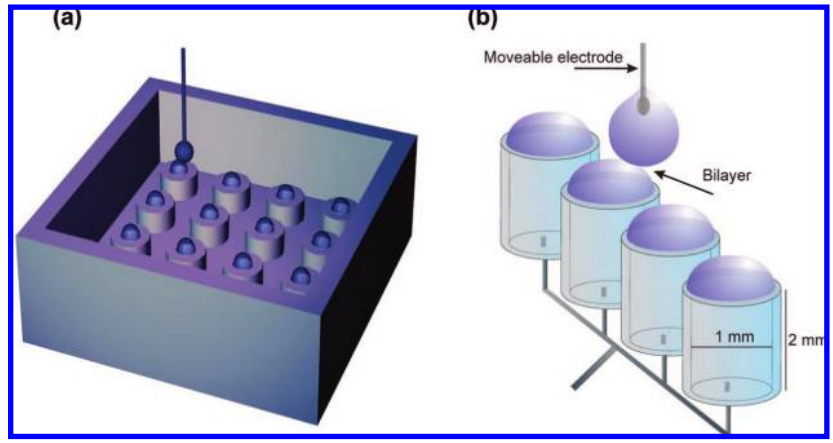

Figure 4. Ion-channel screening chip. (a) Simplified schematic of the chip. Each well holds 1.5 μL of solution and presents a convex surface under the oil. A droplet containing the desired IVTT mixture is suspended from the moveable Ag/AgCl electrode, which is grounded. Ion channel blockers and a control (buffer only) are distributed in the wells, each of which contains a Ag/AgCl electrode connected to the working end of the patch clamp amplifier. A DIB is formed between the IVTT droplet and the control well to verify normal channel function. Subsequently, the DIB is separated and the droplet is moved to the next well, and so on. (b) Detail showing the wiring of 4 of the 16 wells.

(see Supporting Information). In both cases, the addition of salts above 50 mM is not recommended. Nevertheless, we added 250 mM KCl and found that for both systems, protein was synthesized at levels sufficient for our experiments. While the Promega system produces protein at both room temperature and at 37 °C, this was not the case with the PURE system, which required incubation at 37 °C (Figure 2).

The properties of proteins expressed using the Promega and PURE kits were closely similar. For example, Kcv, a potassium channel from chlorella virus PBCV-1, exhibited a unitary conductance in 250 mM KCl at +100 mV of 227 ± 13 pS ($n = 10$) when expressed by the PURE system and 213 ± 22 pS ($n = 10$) in case of the Promega system (Figure 3a), which are comparable to reported values when scaled according to the applied potential and the salt concentration.[25,26]

The stability of DIBs formed in the presence of the PURE system was far greater than the stability of DIBs obtained with the Promega kit. PURE system DIBs lasted an average of 8.7 h ($n = 10$) and Promega DIBs an average of 0.7 h ($n = 10$) under a continuous applied potential of +100 mV (Figure 3b). The bilayer tends to rupture with the Promega system, perhaps due to the presence of polyethylene glycol (PEG)[27] and lipid components[28] in the S30 extract. Therefore, the PURE kit was chosen for subsequent screening experiments.

Rapid Screening. We have previously demonstrated the potential of the DIB system for rapid screening.[16] A droplet containing the αHL pore was sequentially connected to a droplet for recording, disconnected by pulling on the droplet's electrode, and then used to record from a second droplet. Here, we expand this concept by implementing rapid single-molecule screening with a 16-element chip of an ion channel produced by IVTT. We made a chip with a 4 × 4 array of wells, each containing a Ag/AgCl electrode. Each well was loaded with 1.5 μL of a potential ion channel blocker in 5 mM HEPES, 250 mM KCl,

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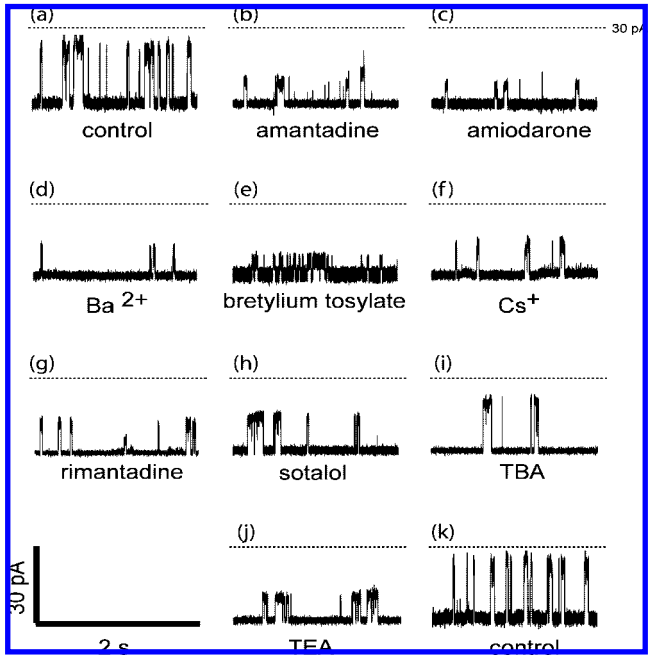

Figure 5. Rapid screening of 9 potential K^+ channel blockers with a droplet expressing Kcv. (a) Control, Kcv activity at +100 mV in 5 mM HEPES, 250 mM KCl, pH 7.0; (b−j) Short segments of three- to four-minute current recordings of Kcv in the presence of various blockers at the concentrations indicated in Table 1. (k) Final control after reconnecting to the buffer-only well; no blocking activity was observed. The entire screen takes ~1 h. Channel openings are the upward spikes. The dashed line is at 30 pA.

Table 1. Apparent Single-Channel Current Values for Kcv in the Presence of Various Blockers

blocker	concentration	apparent single-channel current (pA)[a]
Amantadine	0.5 mM	12 ± 2 (n = 4)
Amiodarone	10 μM	9 ± 1 (n = 4)
Ba^{2+}	20 μM	15 ± 2 (n = 4)
Bretylium tosylate	0.5 mM	5 ± 1 (n = 4)
Cs^+	10 mM	14 ± 2 (n = 4)
Rimantadine	60 μM	13 ± 2 (n = 4)
Sotalol	40 μM	13 ± 1 (n = 4)
Tetrabutyl ammonium (TBA)	100 nM	20 ± 1 (n = 4)
Tetraethyl ammonium (TEA)	15 mM	8 ± 1 (n = 4)

[a] The current passed by Kcv in the absence of a blocker was 23.5 ± 1.5 pA (n = 4). The recordings were performed at +100 mV, in 5 mM HEPES, 250 mM KCl, pH 7.0.

pH 7.0, 1 mM DPhPC (as vesicles), forming a convex meniscus (Figure 4). The established potassium channel blockers amantadine,[29] Cs^+,[30] Ba^{2+},[30] n-tetrabutylammonium (TBA),[31] and tetraethylammonium (TEA)[32] were used to validate the blocking of Kcv. In addition, the efficacy of three antiarrhythmic drugs: amiodarone,[33] bretylium tosylate[34] and sotalol,[35] was examined.

First, protein synthesis was performed with the PURE system at 37 °C for 1 h. Then, a droplet of the solution was placed on a moveable electrode, which was attached to a micromanipulator (10 μL of protein is sufficient to form 40 droplets). The droplet was first connected to a buffer well (5 mM HEPES, 250 mM KCl, pH 7.0, 1 mM DPhPC) as a control. The currents flowing through individual Kcv channels were recorded as the channels became incorporated into the bilayer. In the typical experiment shown (Figure 5), the channels exhibited a conductance value of 232.5 ± 6 pS in 250 mM KCl at +100 mV.[25,26] The Kcv-containing droplet was then disconnected from the buffer droplet and connected to the droplet in the next well, which contained a blocker, and from which a recording containing hundreds of events was made over 3 to 4 min. The apparent unitary conductance value decreased in presence of the blocker (Table 1 and Figure 5b). "Fast" blockers associate and dissociate rapidly from a channel compared with the bandwidth of the recording and hence an averaged signal is observed rather than individual blockades.[2] The Kcv-containing droplet was then disconnected from the well containing the blocker and reconnected to the buffer well. The behavior of Kcv was closely similar to that seen in the initial recording, which confirmed that the Kcv-containing droplet had not been contaminated by the blocker. In this way, nine molecular species were screened for their ability to block Kcv (Figure 5b−j). The entire screen was completed in less than 1 h. The screen was performed four times to demonstrate reproducibility. Control (buffer well) recordings carried out between the examination of each blocker (not displayed for every case) showed that the properties of Kcv and the salt concentration in the droplets did not change during the experiment (Figure 5k). Leakage of the blockers from the droplets was examined and found to be insignificant in all cases except for the highly hydrophobic amiodarone, where a small decrease in efficacy was noted after 45 min. It may be possible to reduce leakage by changing the lipid or lipid mixture used to make the DIBs, but clearly the investigator must remain on guard with respect to the possibility of losses from the droplets.

The chip was also used to examine the concentration dependence of inhibition by three drugs (bretylium tosylate, rimantadine, and sotalol). Inhibition constants (K_i) were determined by plotting the normalized single-channel current versus the concentration of blocker (Figure 6) and fitting the data to a saturation function. The K_i values in 250 mM KCl at an applied potential of +100 mV were found to be: 34 μM (bretylium tosylate), 88 μM (rimantadine) and 72 μM (sotalol). The concentration dependencies of the three drugs toward Kcv were determined within 1 h.

Conclusions

We have shown that membrane proteins in a submicroliter volume can be screened for inhibition by blockers by using a multielement chip. The DIB chip promises to be a valuable addition to the ion-channel screening toolbox, as demonstrated by our Kcv screen. It might also be used to screen inhibitors against pores, such as those formed by bacterial toxins.[36,37] We have also evaluated the use of protein prepared by cell-free synthesis (IVTT) for use in this system. When using the

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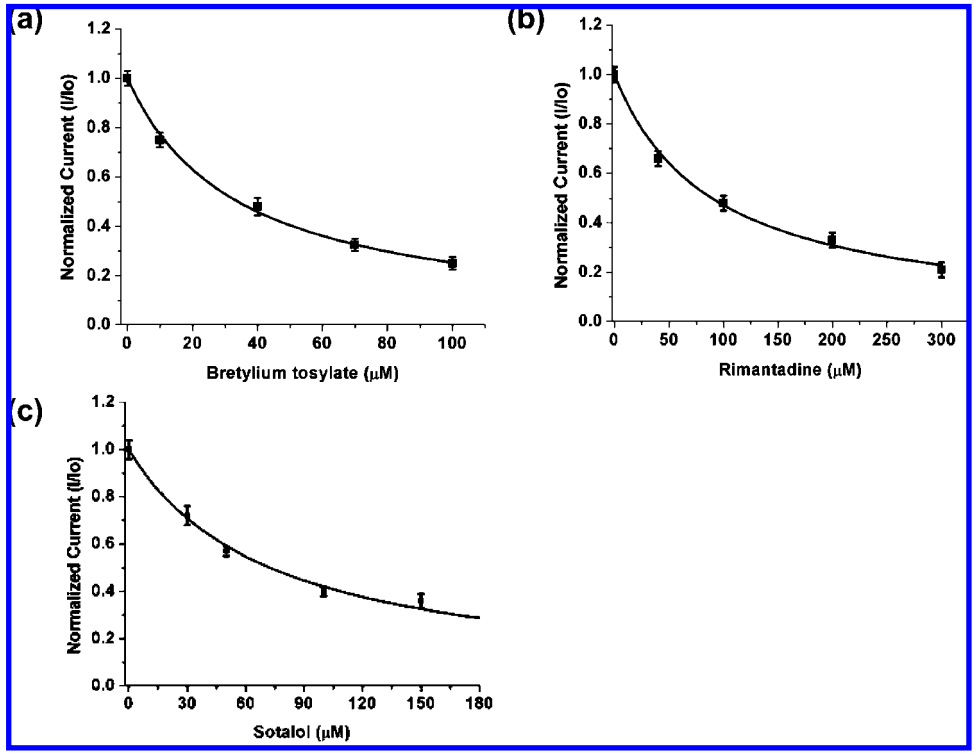

Figure 6. Concentration dependence of the inhibition of Kcv by various blockers. Inhibition curves in presence of (a) bretylium tosylate, (b) rimantadine and (c) sotalol. The normalized current (I/I_o) is the current recorded for a single Kcv channel at the specified concentration of blocker (I, the apparent unitary conductance during fast block) divided by the current in the absence of blocker (I_o). I/I_o was plotted versus the concentration of the blocker, [B]. The error bars represent the standard errors from four trials. The data were fit to a saturation function,[2] $I/I_o = 1/(1 + [B]/K_i)$, by using Origin software, to obtain the dissociation constant of the blocker (K_i). The K_i values of bretylium tosylate, rimantadine and sotalol were found to be 34 μM, 88 μM, and 72 μM, respectively. The currents were recorded in 5 mM HEPES, 250 mM KCl, pH 7.0, at an applied potential of +100 mV.

Promega IVTT system, we observed a lag time before full-length protein was detected[38] (see Supporting Information). Therefore, DIB formation can be accomplished before full-length proteins have been made within a droplet. Because the Promega system works at room temperature, proteins can be expressed and allowed to fold in the proximity of the bilayer, which may favor correct insertion. Unfortunately, the Promega IVTT mix destabilizes DIBs with respect to prolonged use. Because of this, we selected the PURE expression system for screening the K^+ channel Kcv. Since the PURE system works only at 37 °C, we made Kcv in a plastic tube before forming droplets, producing enough protein for 40 experiments in 10 μL of solution. These experiments were successful, but in other cases the potential advantage of synthesizing the protein in the presence of a bilayer will be nullified and it may be beneficial to incorporate a heating element into the present device.

In a recent study, an odorant receptor, OR5, was expressed by IVTT adjacent to a tethered bilayer. The receptor inserted in a preferred orientation, as determined by antibody binding. Infrared difference spectroscopy suggested that the inserted protein could bind ligand.[39] The direct observation of G protein-coupled receptor activity might be possible by merging this technique with ion-channel recording in tethered bilayers. However, this is yet to be realized. Further, the ionic reservoir under tethered bilayers is quickly exhausted during ion-channel recording experiments.[40]

Various proteins, such as Kcv,[26] can insert into bilayers in both possible orientations, which can usually be distinguished by voltage-dependent noise or rectification. Where this is the case, inhibitors that act from either end of the protein can be examined. In the present work, based on rectification, we believe we are examining the intracellular aspect of Kcv,[26] although the opposite assignment has been made.[41] In other cases, such as the αHL pore or anthrax protective antigen, in which the protein contains a large extramembraneous domain, insertion is almost exclusively in a single orientation, which prevents the examination of blockers that act at one of the two ends with the DIB chip. To examine the opposite orientation, it will be necessary to use alternative means of protein insertion, such as vesicle fusion, or to build on approaches where the channel or pore in the desired orientation can be dragged from one pool of inhibitor to another.[42]

We anticipate that the screening process can be streamlined by using a larger array chip and by automation. Further, the screening of membrane-protein libraries would be facilitated if the proteins could be produced within droplets from single plasmids, for example by isothermal amplification of the DNA[43,44] in parallel with IVTT. However, the IVTT kits used in our experiments perform best with prokaryotic proteins. For

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example, they do not contain the biological machinery for the efficient incorporation of polypeptides into membranes or for post-translational modification.[45] Progress in the analysis of medically relevant eukaryotic membrane proteins might be made by supplementing existing IVTT mixes. For example, the addition of cAMP-dependent protein kinase could be used to activate the CFTR chloride channel.[46] With these improvements, cell-free expression combined with DIB arrays can be envisaged as a general expression/ rapid analysis system for ion channels.

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Acknowledgment. The plasmids encoding αHL and Kcv were gifts from Dr. Stephen Cheley. We thank Dr. Andrew Heron, Dr. Amy Mason, and Dr. Lakmal Jayasinghe for helpful discussions and experimental assistance. R.S. was supported by a Clarendon Scholarship in partnership with a Lady Noon Award. W.L.H. is an American Rhodes Scholar. This work was funded by a Royal Society Wolfson Research Merit Award (H.B.), the Medical Research Council (H.B.), and the National Institutes of Health (H.B.).

Supporting Information Available: Full experimental details are provided. The expression of αHL in a cell-free system is shown in Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

JA804968G