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Planar lipid bilayers in recombinant ion channel research

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ABSTRACT

There are a number of methods of investigating the function of recombinant proteins such as ion channels. However, after channel purification there are few methods to guarantee that the protein still functions. For ion channels, reconstituting back into planar lipid bilayers and demonstrating preserved function is a convenient and trusted method. It is cell free and even inaccessible, intracellular ion channels can be studied. We have used this method to study the function of recombinant channels of known subunit composition and have found it convenient for investigating the mode of action of ion channel modulators.

1. Introduction

Our knowledge and understanding of the function of ion channels has been enhanced by the use of recombinant expression systems. A wide range of systems are available, the choice of which, largely depends on the nature of the protein and subsequent experiments. For researchers investigating channel function, the expression system has to be compatible with standard electrophysiological techniques. For example, expression of ion channels in Xenopus oocytes can be investigated using two-electrode voltage clamp or patch clamp methods, whilst expression of ion channels in HEK 293 cells is amenable to patch clamp or planar lipid bilayer investigations. Recombinant ion channels are often expressed with a terminal tag to enable subsequent purification prior to biochemical and structural investigations. With these experiments, efficient expression, ease of purification and the yield of functional channels is important. However, how does one know if your precious, tagged, expressed and purified protein is still functionally intact? For ion channels, these questions can be answered using the planar lipid bilayers technique [1–8]. We have used this system to investigate purified Clostridium perfringens type A enterotoxin, pfCRT, BK, CFTR and VDAC proteins [9–11].

The planar lipid bilayers (PLB), also known as the Black Lipid Membranes (BLM), can be used by anyone familiar with patch clamp methods. It measures channel gating at the single channel level and makes measurements similar to excised patch or cell attached modes [12]. It is used to investigate (a) membrane vesicles [11], (b) synthetic ion channels [13], (c) purified ion channels reconstituted into proteoliposomes [2,14], and (d) ion channels purified using the newer SMALP technology [15]. It can measure transmembrane currents of 1 pA up to 20 nA and provides direct chemical and electrical access to both sides of the membrane. Some of the advantages of this method also include the ability to record inaccessible ion channels [16] and the ability to study stored membrane/protein preparation, obviating the need to record from living cells.

2. Material and methods

2.1. Equipment

The bilayer technique relies on creating an artificial membrane across a small aperture; the membrane forms from lipids that span and seal this hole. After bilayer formation, the channel is inserted into the membrane and single channel measurements are made (Fig. 1(A)). The schematic in Fig. 1(B) shows the bilayer recording system, which can be constructed by anyone familiar with patch clamp recordings. In order to carry out bilayer recordings, the experimenter needs (a) a lipid bilayer cup/chamber for bilayer formation (e.g. Warner Instruments chamber BCH-13A and cup

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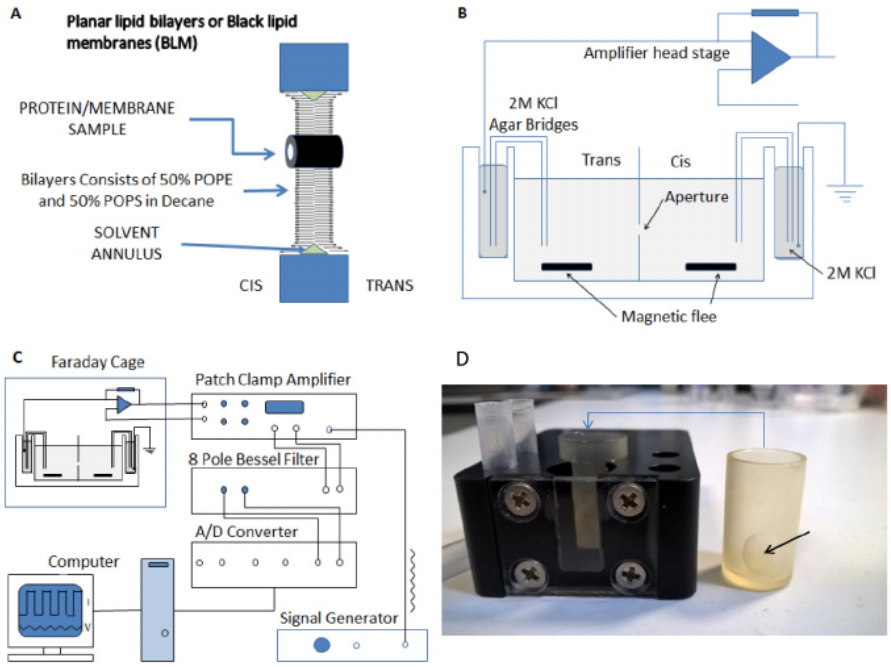

Fig. 1. (A) Representation of BLM with an incorporated ion channel. (B) Diagrammatic representation of the connection of the bilayer chamber to the head stage. (C) A diagram of the complete bilayer set up. The integral, or as shown here, external signal generator, is used to assess the bilayer thickness on membrane thinning (i.e. bilayer formation). The square capacitive currents increase in size indicating planar lipid bilayer formation. (D) Warner Instrument's lipid bilayer cup/chamber (chamber BCH-13A and cup CP13A-250). The aperture in the cup (black arrow) for painting bilayers can be viewed through the glass panel after assembly with the chamber.

CP13A-250), (b) a vibration-isolated platform (e.g. an air table), (c) a light source, and (d) a magnetic stirrer, inside the Faraday cage. The *cis* and *trans* sides of the chamber are connected *via* KCl agar bridges and silver/silver chloride (Ag/AgCl) electrodes to the amplifier head stage. The amplifier used should be capable of bilayer recordings (e.g. Warner Instruments PC-501A or Bio-logic BLM-120). Two optional additions to the set up include (a) an eight pole Bessel Filter (Warner Instruments LPF-8) and, (b) a signal generator that can generate a triangular voltage wave form to measure membrane capacitance (some bilayer amplifiers, such as the BLM-120, have these as a built-in feature) (Fig. 1(C)). Single channel currents are digitised using an analogue to digital converter (e.g. CED micro1401 MkII), controlled by single channel analysis software (e.g. WinEDR, University of Strathclyde, UK), and stored on a PC running windows 7 or above. For mass storage of data, a data storage device is useful.

2.2. Preparing lipids

POPE (1-Palmitoyl-2-Oleoyl-*sn*-glycero-3-phosphoethanolamine (Avanti Polar Lipids)) and POPS 1-Palmitoyl-2-Oleoyl-*sn*-glycero-3-[phosphor-L-serine] (Avanti Polar Lipids) are dissolved in chloroform at 50 mg/ml. These solutions should be stored in the dark at −20 °C prior to use in bilayer experiments. To form bilayers add 7.5 µl of POPS and 7.5 µl of POPE to a sealable 2 ml glass volumetric flask. Evaporate the chloroform under a stream of nitrogen gas. Seal the volumetric flask with the stopper to minimise contact with air, re-suspend the dried lipid in 25 µl of *n*-decane. The lipid is ready to use in the formation of bilayers. These preparations are useable for 3–4 h, but avoid contact with air. (We have used other lipids, including cholesterol, to construct planar lipid bilayers). To condition the recording cup one µl of the reconstituted lipid is carefully spread around and over the aperture of the recording cup to form a bilayer. The cup is placed into the chamber and 1 ml of recording buffer added to the *cis* and *trans* compartments. We use a high free calcium (50 µM) buffer (150 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1.05 mM $CaCl_2$, 10 mM HEPES pH 7.2 with KOH); or a low free calcium (0.5 µM) buffer (150 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 0.75 mM $CaCl_2$, 10 mM HEPES pH 7.2 with KOH), in our recording chambers.

2.3. Forming a bilayer

There are a number of ways to encourage bilayer formation: the first is known as "lifting a bilayer". Gently aspirate the recording buffer in the *cis* chamber using a 1 ml pipette and then, gently dispense to the recording chamber. Dragging the surface of the recording solution across the aperture encourages a redistribution of lipid and bilayer formation. The second method is known as "painting a bilayer". This is done using a ball of glass formed on the end of capillary tubing by heating the glass with a Bunsen burner. This clean glass probe can then be dipped in the stock lipid and applied directly to the hole, this is often enough to encourage bilayer formation.

2.4. Checking for bilayer formation

The *cis* and *trans* side of the bath is connected to the amplifier head stage *via* the agar bridges as shown in Fig. 1. The *cis* side should normally be at ground potential. Assess bilayer formation by applying 1–10 mV triangular voltage pulses across the *cis* and *trans* compartments. The passive bilayer properties are easy to evaluate under voltage clamp conditions. The bilayer leak conductance is equal to the base line shift divided by the holding voltage. For example, 1 pA of leak current at 100 mV *trans* membrane potential equates to a leak conductance of 10 pS (leak conductance = 1 pA/100 mV = 1×10^{-12} A/0.1 V = 1×10^{-11} S or 10 pS).

The capacitance of a membrane is described by the equation:-

$$C = \frac{\varepsilon A}{d}$$

where C is the capacitance in Farads, ε is the dielectric constant of the lipid, A is the area of the bilayer, and d is the thickness. Current flow onto a capacitor, such as a planar lipid bilayer, is given by the equation

$$I = C\frac{dV}{dt}$$

where I is the capacitive current and dV/dt is the rate of change of the voltage across the membrane. We can combine both equations to give

$$I = \frac{\varepsilon A}{d}\frac{dV}{dt} = kA, \text{ where } k = \frac{\varepsilon}{d}\frac{dV}{dt} \text{ and } d \text{ and } \varepsilon \text{ are constants}$$

A triangular voltage waveform has a constant rate of change and, if we change the voltage across our bilayer in a triangular manner, then dV/dt will be constant. More importantly, the dielectric constant of the lipid (ε) is fixed, as is d, being determined by the chain length of the phospholipid. Consequently, the equation can be simplified to $i = kA$. Using a triangular pulse, the current output of the amplifier is a square wave directly proportional to the area (A) of the bilayer. The current output can be calibrated by placing a 100 pF capacitor between the input and ground of the patch clamp amplifier head stage.

We have formed bilayers across 250 μm apertures. The cups we use are Warner Instruments CP13A-250 cups, which fit snugly into the associated chamber (BCH-13A). The bilayer can have its voltage clamped between −150 and 150 mV and for a 250 μm aperture should have a leak conductance of less than 10 pS and a capacitance of greater than 150 pF.

2.5. Recording ion channel activity

Once a stable bilayer has been formed, then the channel under study can be incorporated into the bilayer. Approximately 2 μl of membrane preparation is added to the grounded *cis* side of the chamber and, because membrane preparations can sink to the bottom of the recording chamber, it is important to stir the preparation slowly. This keeps membrane vesicle preparations suspended and aids channel insertion. The membrane potential is held at a voltage of +/− 50 mV whilst observing single channel currents on the computer screen. If no channels insert, then, after 15 min, a further 2 μl of membrane preparation can be added.

Sometimes membrane vesicle fusion with the bilayer has to be encouraged and there are a number of ways to help this process. The first method is to ensure an osmotic gradient across the PLB by adding non-electrolyte to the *cis* side of the bilayer, a 3:1 osmotic gradient is a good starting point. Secondly, stirring the recording solution encourages membrane vesicle fusion by reducing the thickness of the unstirred layer close to the PLB. Thirdly, blowing the membrane vesicle preparation onto the PLB from a pipette placed close to the bilayer may help. Lastly, the presence of acidic phospholipids in the PLB itself may encourage channel incorporation. Once stable channel activity is observed, the electrophysiological characteristics of the channel can be investigated.

2.6. Improving the signal to noise ratio

Electromagnetic interference is best dealt with by grounding of equipment to a common ground point within the Faraday cage. Care should be taken to switch off mobile phones, as these can interfere with recordings, and a good air table helps to reduce mechanical noise. A lot of the background noise that arises from bilayer experiments is capacitive in nature, a consequence of a large membrane surface area, so using a smaller aperture may help to reduce this source of noise. Alternatively, if the channel gating kinetics are slow, then the signal to noise ratio can be improved by judicious use of an in line, low pass, 8 pole Bessel filter (Warner Instruments LPF-8). 8 pole Bessel filters have a sharper cut off than that of the more common 4 pole filters, which are often built into patch clamp amplifiers, so for measuring small channels, an in line filter can help.

2.7. Preparing membranes or purified protein for bilayer experiments

Membranes can be prepared from native tissue, or more usually, from cells expressing the protein of interest. We have used baculovirus to express proteins, such as BK channels and pfCRT protein, in Hi5 insect cells, as this system can yield large amounts of membrane protein for bilayer recordings and protein purification. We have also used HEK 293 cells for stable expression of BK channels for both patch clamp and bilayer experiments [10,17]. We use this expression system routinely because it produces dependable membranes for electrophysiology, protein biochemistry and protein purification.

2.7.1. Cell culture

HEK 293 cells, stably expressing hSloα1, are cultured in DMEM, supplemented with 10% foetal bovine serum, non-essential amino acids, 5 μg/ml blasticidin and penicillin–streptomycin. HEK 293 cells, expressing both hSloα1 and hSloβ1 are cultured in MEM with 10% foetal bovine serum, non-essential amino acids, 5 μg/ml blasticidin and 1 mg/ml geneticin. The cells are grown to confluence and the size and number of culture flasks increased, until cultures consist of 20 × 175 cm^2 confluent flasks.

2.7.2. Membrane preparation

The tissue culture media is aspirated from the 20 × 175 cm^2 confluent flasks. Cells are detached from the bottom of the flasks with cell dissociation solution (non-enzymatic, Sigma) [18] and centrifuged at 500×*g* for 15 min to create a large pellet. The pellet is re-suspended in 5 × the pellet volume of Buffer 1 containing protease inhibitors (Table 1).

A pre-cooled, nitrogen cavitation apparatus is loaded with the cell suspension and sealed. The cavity is filled with nitrogen gas to a pressure of 1000–1500 psi and left on ice for 15 min. After 15 min, bleed the apparatus, collect the burst cells in a 50 ml tube, and repeat the bursting step once more. After nitrogen cavitation, dilute the cell lysate to 25 ml with ice-cold Buffer 2. Centrifuge the diluted suspension at 500–1000×*g* for 10 min to remove cell debris and unbroken cells. The supernatant is gently layered on top of a 10 ml sucrose cushion (Buffer 3) and centrifuged at 30,000×*g* for 30 min. The cloudy suspension resting on top of the sucrose cushion should be collected. Transfer the collected interface to a fresh ultracentrifuge tube. Dilute the collected interface with Buffer 4 to fill the ultracentrifuge tube and centrifuge at 100,000×*g* for 45 min at 4 °C. The supernatant is discarded and the glassy pellet is re-suspended in 1.5 ml of Buffer 5. This is best done with a 5 ml syringe and a 22 G and then 25 G syringe needle. Store ready for use in 50–100 μl aliquots. These membranes can be used directly in bilayer experiments or the protein can be further purified using metal affinity chromatography (IMAC).

3. Results

The analysis of single channel data obtained from BLMs is similar to analysing single channel data obtained during patch clamp experiments. Experiments can be done at a range of transmembrane voltages and in the presence of physiological and pharmacological ligands. One can obtain a direct measure of single channel currents, and thus, single channel conductance, as well as a

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Table 1
Buffers for membrane preparation.

Buffer 1:	Buffer 2:	Buffer 3:	Buffer 4:	Buffer 5:
10 mM Tris–HCl	10 mM Tris–HCl	10 mM Tris–HCl	10 mM Tris–HCl	Tris–HCl
250 mM sucrose	25 mM sucrose	35%(w/v)sucrose	250 mM sucrose	150 mM NaCl,
				1.5 mM $MgCl_2$,
200 mM $CaCl_2$	1 mM EDTA	1 mM EDTA	–	20% glycerol
Pierce protease inhibitors mini tablets	–	–	–	Pierce protease inhibitors mini tablets
pH 7.4,	pH 7.4	pH 7.4,	pH 7.4,	pH 7.0,

measure of open times, single channel closed times and open probability.

3.1. Bilayer recordings of recombinant BK channels

We have used this BLM system to analyse the stoichiometry and pharmacology of BK channels. In particular, we have investigated the effects of the hSloβ1 subunit on the properties of the BK channel. An example of a recording from a BK channel is shown in Fig. 2A. This channel exists in the closed or open (conducting) state. Before starting our recording, we always check for multiple channel insertions by observing channel activity at a range of voltages. At negative voltages, the channels should be closed and at positive voltages, the BK channel should open, whilst at intermediate voltages, multiple openings and closings will be observed. From this, the number of inserted channels can be determined. Ideally, only one ion channel should insert into the bilayer, as this makes determining the open probability easy.

If more than one channel inserts into the bilayer then the open probability for BK channels can be determined from the current amplitude histograms by fitting the appropriate number of Gaussians to the all points histogram and determining the area under each Gaussian curve (Fig. 2(B)). The open probability is given by the equation

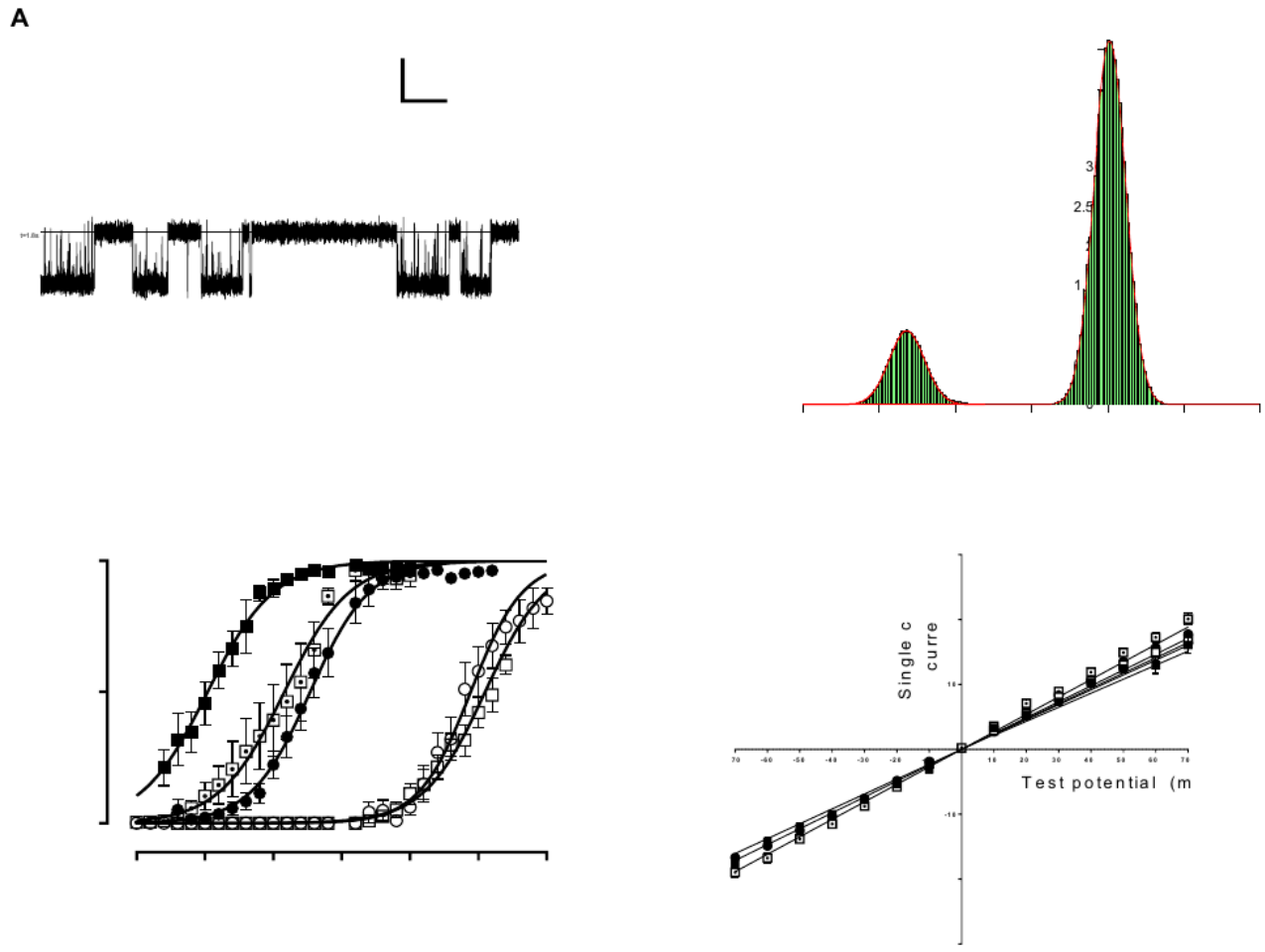

Fig. 2. (A) Single channel currents recorded from a bilayer treated with membranes purified from HEK 293 cells expressing both the pore forming hSloα1 and hSloβ1 subunits, the holding voltage is −50 mV and the free Ca^{++} concentration was 0.5 μM in this recording. The single channel current is approximately 13 pA, giving a single channel conductance of approximately 260 pS. (B) The all points current amplitude histogram reveals the open probability to be 0.18. (C) The activation of BK channels in bilayers. The graph plots the effect of transmembrane potential on the open probability of a single BK channel (hSloα1 alone) in the presence of 0.5 μM, ( ) or 50 μM, ( ) free calcium. Recordings from membranes purified from HEK 293 cells expressing both the pore forming hSloα1 and β1 subunits at a free Ca^{++} of 0.5 μM ( ), ( ) 5 μM and ( ) 50 μM are also shown. Increasing voltage and increasing calcium ion concentration moves open probability voltage relationship to a more hyperpolarised potential. (D) The single channel current amplitude in bilayers. The graph plots the amplitude of the single channel current against transmembrane potential.

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$$NP_o = \frac{\sum_{i=0}^{N} iA_i}{\sum_{i=0}^{N} A_i}$$

where N = the number of channels in the bilayer, A_o is the area under the Gaussian curve corresponding to the closed current; A_1 is the area under the Gaussian curve corresponding to a single open channel and A_i is the area under the Gaussian curve to the current corresponding to i open channels. From estimates of the number of channels (N) in the bilayer, P_o can be calculated.

$$P_o = NP_o/N$$

In practice, this method only works if (a) the number of ion channels is low, (b) the channels do not display multiple subconductance states, (c) it is possible to estimate N and (d) if the signal to noise ratio is high. If too many channels insert into the bilayer, then it may be necessary to dilute the sample or, very briefly, ultra-sonicate to break up membrane aggregates.

Fig. 3(A) illustrates a classic open probability (P_o) vs voltage plot that has been fitted to a Boltzmann function. Recombinant hSloα1 subunits displayed typical characteristic features of native BK channels, single channel conductance was 260 ± 2 pS, (n = 10), and the P_o increased in response to depolarisation. Fitting a Boltzmann function to the P_o vs voltage curve for single channel recordings yielded $V_{0.5}$ of −26 mV and a slope of 19 mV.

Next, single channel currents were recorded from bilayers treated with membranes purified from HEK 293 cells expressing both the pore-forming hSloα1 and hSloβ1 subunits, in the presence of 50 μM Ca^{++}. Fitting a Boltzmann function to the P_o vs voltage curve for single channel recordings yielded $V_{0.5}$ of −100 mV and a slope of 21 mV. The presence of the hSloβ1 subunit shifted the half activation voltage 74 mV in the hyperpolarising direction, whilst the single channel current remained unaffected. Not all the channels from purified membranes from HEK cells expressing both the pore-forming hSloα1 subunits and hSloβ1 subunits displayed increased voltage sensitivity. A subset of channels analysed displayed similar characteristics to the hSloα1 subunit expressed alone. At high Ca^{++}, this channel population displayed a $V_{0.5}$ of −36 mV and slope 17 mV, similar to the hSloα1 subunit expressed alone, and clearly distinguishable from the more active species (Fig. 3(A), fx2, n = 3). These results indicate two populations of channel, one with hSloα1 alone characteristics and one with hSloα1 and hSloβ1 characteristics.

To date, we have conducted 65 recordings from membranes derived from HEK cells expressing both the α and hSloβ1 subunits, 18 of these had characteristics of channels composed solely of α subunits. If we assumed all channels can accommodate up to four possible hSloβ1 subunits, and hSloβ1 subunit association with the pore forming α subunits are similar and independent, then the number of β1 subunits associated with BK channels should follow a binomial distribution (4, Φ):-

Probability of a β1 subunit not associating with a BK channel

$$= \Phi_0 = (18/65)^{0.25} = 0.725 \quad (1)$$

The probability of β1 subunit association in our recombinant expression system would be

$$\Phi = 1 - \Phi_0 = 1 - 0.72542 = 0.274$$

This gives the probability of there being 0, 1, 2, 3 or 4 hSloβ1 subunits associated with hSloα1 channel complexes to be 0.2769, 0.4193, 0.2380, 0.060, 0.0057, respectively. The mean number of hSloβ1 subunits per channel being = np = 0.274 × 4 = 1.09 with a variance = np(1 − p) = 4 × 0.274 × 0.725 = 0.796.

4. Discussion

The PLB method produces single channel recordings comparable to other electrophysiological methods. For instance, the voltage and calcium sensitivity of the BK channel appear similar to that reported using whole cell patch clamp [17]. Here, we show that two distinct oligomeric BK channel populations with different voltage sensitivities were discernible in purified membranes from HEK cells that co-expressed both hSloα1 and hSloβ1 subunits. The first population was similar to purified membranes from HEK cells that expressed just hSloα1, whilst the second, more numerous population, behaved like an hSloα1 and hSloβ1 channel complex. No channels with intermediate properties were observed, suggesting that the hSloβ1 subunit effects are all or none in nature and in agreement with other studies [19,20].

The PLB method has a significant contribution to make to our understanding of ion channel gating. It is a useful adjunct to traditional electrophysiological methods, especially when investigating recombinant channel proteins. The method is uniquely useful when it comes to investigating the role of lipid constituents on channel gating [21] and investigating the function of purified channels. Indeed, it is potentially the only method available to study intracellular ion channels [22], such as ryanodine receptors, and has other advantages such as the ability to investigate stored protein and provide direct access to both sides of the channel. However, there are limitations to the method, the most obvious being that PLBs are inherently noisier than classical patch clamp methods.

4.1. Noise limitations

PLB noise arises from voltage clamping the potential across a membrane with a large capacitance. This is a direct consequence of the equation for capacitive currents.

$$I = C\frac{dV}{dt}$$

This equation predicts that high frequency voltage fluctuations will produce augmented current noise with increasing membrane capacitance and increasing bandwidth. To reduce the noise, decrease the membrane capacitance, use a low noise amplifier, use a low noise voltage command and decrease the bandwidth. Reducing the aperture in your bilayer chamber may help to reduce the capacitance, but this will decrease your chance of channels inserting. Often experiments are driven by voltage commands using a digital to analogue converter. This is a potential source of voltage noise, especially if the D/A converter has a 12 bit rather than the preferable 16 resolution. Judicious use of an eight pole Bessel filter will help to improve the signal to noise ratio. Firstly, it is important for the A/D converter to sample at a rate at least 5-fold faster than the cut of frequency of the low pass filter in order to avoid problems with aliasing. Secondly, if your channel gating kinetics are slow, then heavy filtering may help the signal to noise ratio. Thus, channels with a small conductance and faster gating kinetics will be difficult to study using PLB.

4.2. Choice of lipids for bilayer recordings

Another problem is choice of lipids for the PLB. Your channel of interest may require particular lipids for channel activity to be observed [23]. For example, the nicotinic receptor requires cholesterol in the bilayer for optimal channel activity [24] whilst prokaryotic ion channels may well require phosphatidylglycerol [25,26]. If the lipid requirements of your channel are not known, then it may be best to start with a mixed lipid extract from soybeans that includes a wide range of phospholipids. In addition,

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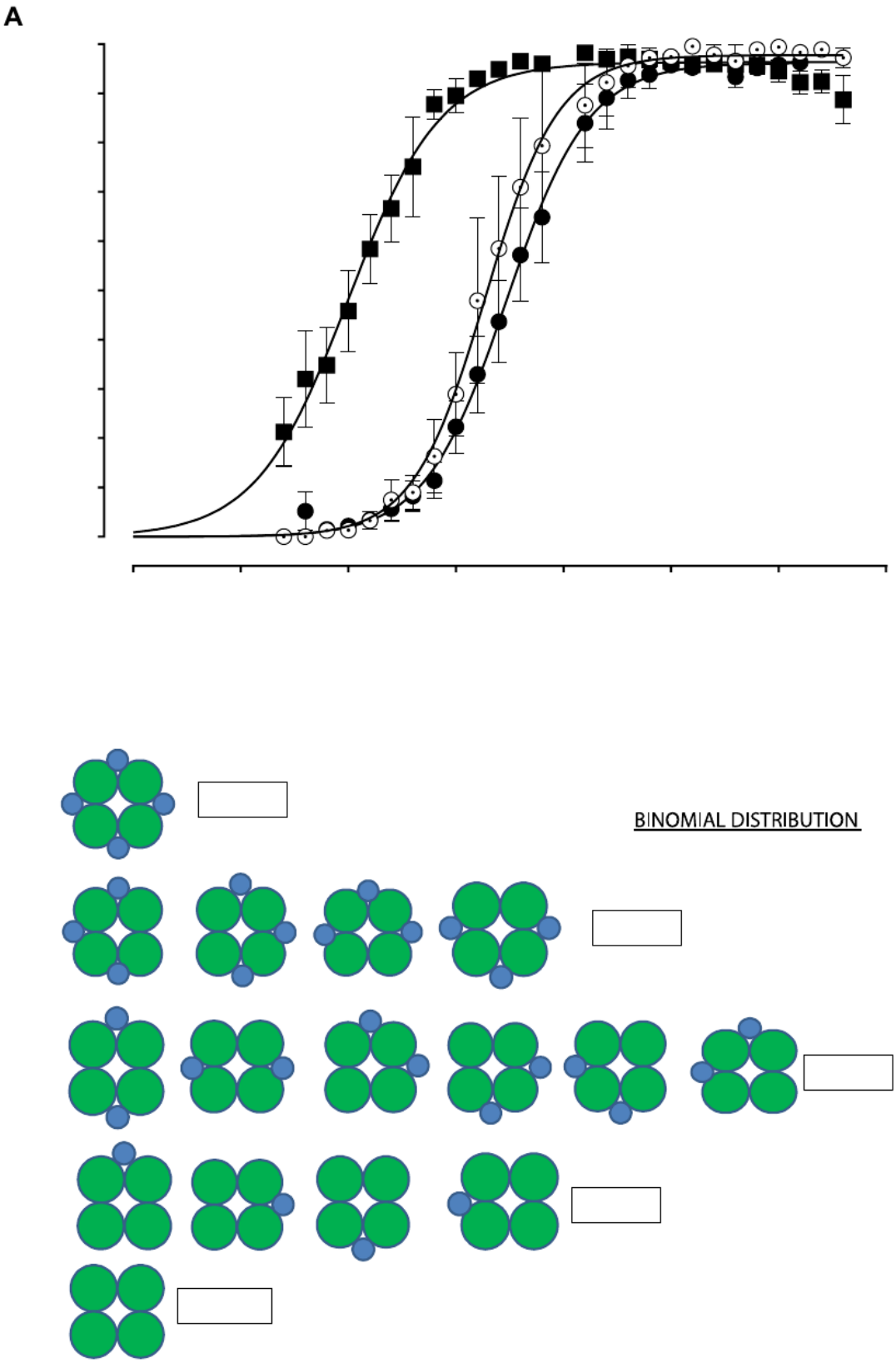

Fig. 3. (A) The dependence of BK channels open probability in bilayers on transmembrane voltage. ( ) The effect of transmembrane potential on the open probability of a single BK channel composed of recombinant hSloα1 subunits in the presence 50 μM free calcium (n = 10). ( ) The effect of transmembrane potential on the open probability of a single BK channel composed of recombinant hSloα1 and hSloβ1 subunits in the presence of 50 μM free Ca^{++} (n = 9); occasionally, a second channel species with hSloα1 alone characteristics can also be observed (n = 3, ⊙). (B) Shows the calculated hSloβ1 subunit stoichiometry associated with the BK channel. These ratios were estimated from 65 recordings from membranes purified from HEK 293 cells expressing both the pore-forming hSloα1 and hSloβ1 subunits.

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PLBs will contain traces of organic solvent, such as decane, which may affect the recordings.

4.3. Contamination

For PLB electrophysiologist, there is always a chance that recorded activity is due to a contaminant. Consequently, it is important that reconstituted channels are thoroughly characterised and compared to their native counterparts. This is difficult to carry out for intracellular proteins because these proteins are challenging to investigate using traditional patch clamp methods. One useful approach involves the use of specific pharmacological agents to validate the ion channel identity. For example, ryanodine can be used to validate ryanodine receptors identity. Other characteristics, which are useful markers of ion channel identity, include gating kinetics, unitary conductance, and ion channel selectivity.

In conclusion, PLBs are the ultimate test bed for studying recombinant ion channels. With careful attention to detail, this method can be successfully used to investigate their physiological, pharmacological and biophysical properties. The method can be used to investigate (a) purified membranes, including intracellular membranes, (b) purified protein, as well as those purified using newer SMALP technologies and (c) can be used on stored membrane/protein, obviating the need to record from living cells. These represent major advantages for the planar lipid bilayer technique in recombinant channel research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ymeth.2018.03.003.

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