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hERG ion channel pharmacology: cell membrane liposomes in porous-supported lipid bilayers compared with whole-cell patch-clamping

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Abstract The purpose of this study was to obtain functional hERG ion channel protein for use in a non-cell-based ion channel assay. hERG was expressed in *Sf*9 insect cells. Attempts to solubilise the hERG protein from *Sf*9 insect cell membranes using non-ionic detergents (NP40 and DDM) were not successful. We therefore generated liposomes from the unpurified membrane fraction and incorporated these into porous Teflon-supported bilayer lipid membranes. Macroscopic potassium currents (1 nA) were recorded that approximated those in whole-cell patch-clamping, but the channels were bidirectional in the bilayer lipid membrane (BLM). Currents were partially inhibited by the hERG blockers E4031, verapamil, and clofilium, indicating that the protein of interest is present at high levels in the BLM relative to endogenous channels. Cell liposomes produced from *Sf*9 insect cell membranes expressing voltage-gated sodium channels also gave current responses that were activated by veratridine and inhibited by saxitoxin. These results demonstrate that purification of the ion channel of interest is not always necessary for liposomes used in macro-current BLM systems.

Keywords Ion channel assay · hERG · Liposome · Bilayer lipid membrane · Supported bilayer lipid membrane · Pharmaceutical screening

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Introduction

Pharmaceutical screening for compounds that modulate ion channel activity typically involve cell-based detection systems. New artificial bilayer lipid membrane (BLM) systems that use ion channels reconstituted into proteoliposomes offer greater flexibility for future technology. Although the protein is also sourced from cells overexpressing the protein of interest, once in liposomes it can often be frozen for later use. Previously, we developed a disposable porous-supported bilayer lipid membrane system using hydrated PTFE (Teflon) in which we measured the functional activity of voltage-gated sodium ion channels (Phung et al. 2011a). Because this device may have potential in emerging plate-based ion channel screening we wanted to test it in conjunction with other ion channels that are important for pharmaceutical screening. An ion channel against which potential drug candidates are tested for possible unwanted side effects is the human ether-á-go-go-related gene that encodes the hERG potassium ion channel and is of critical importance in heart function (Sanguinetti and Tristani-Firouzi 2006; Trudeau et al. 1995). When hERG ion channel function is impaired either by inherited mutations in the hERG gene or through drugs that block the pore, ventricular repolarization can be altered, predisposing individuals to life-threatening arrhythmias called "Long QT Syndrome" (Sanguinetti and Tristani-Firouzi 2006). It is, therefore, routine practice in the pharmaceutical industry to test drug candidates for modulatory effects on hERG early in drug development (Dubin et al. 2005; Redfern et al. 2003; Witchel 2011).

The standard method for testing drug candidates against hERG is whole-cell patch-clamping, which is highly informative but low throughput; even automated patch-

clamping is limited in throughput and expensive (Anson et al. 2004; Guo and Guthrie 2005; Scheel et al. 2011; Terstappen 2005). A variety of higher-throughput cell-based methods exist for hERG functional screening, for example, binding assays, ion-flux assays, (Schmalhofer et al. 2010; Zou et al. 2010) and fluorescence dye-based assays, for example, the membrane potential assay (Zheng and Kiss 2003). However, binding assays do not measure ion channel function, and ion flux, and membrane potential assays require follow up with electrophysiological assays to directly assess effects ion channel function (Witchel 2011). There is only one report of use of a voltage-clamp-based technique to record macroscopic hERG currents from BLMs (Leptihn et al. 2011), but it is low-throughput. Thus, there is still a need for functional ion channel assays that are predictive of pharmacological effect and are high-throughput and cost-effective. We therefore sought to determine how the hERG ion channel might be used in conjunction with the porous Teflon-supported bilayer lipid membrane system, because this functional assay is scalable.

The objective of this study was to use the porous-supported Teflon bilayer membrane system to record macroscopic potassium currents through hERG ion channels and to examine the effect of hERG modulatory drugs. Histidine-tagged hERG constructs were prepared and tested for function in HEK cells by patch-clamping. They were then over-expressed in insect cells for isolation, enrichment, and reconstitution into proteoliposomes. Because the protein could not be solubilised in non-ionic detergents a new method was developed to isolate the eukaryotic cell membranes and incorporate these directly into liposomes without prior solubilisation or extensive subcellular fractionation steps.

Materials and methods

Materials

Phosphoethanolamine (PE), phospho-L-serine (PS), and cholesterol (CH), were from Avanti Polar Lipids (Alabaster, AL, USA). Phosphatidylcholine (PC) was extracted from egg yolk (Singleton et al. 1965). Verapamil, clofilium, and all commonly used chemicals were purchased from Sigma (St. Louis, MO, USA). E4031 and anti-K_v 11.1 (HERG), an antibody that binds to the extracellular region, were purchased from Alomone Labs (Jerusalem, Israel). Nonidet P40 (NP40) was from BDH (Poole, England). Protease inhibitor (complete, Mini, EDTA-free) was from Roche Diagnostics (Penzberg, Germany). The Bradford assay was from Bio-Rad (Hercules, CA, USA). Commonly used materials for molecular biology and cell culture were from Invitrogen (Carlsbad, CA, USA).

Molecular biology

hERG (KCNH2) full length transcript variant 1 in the vector pCMV6-XL4 obtained from Origene (Rockville, USA) was transferred to mammalian and baculovirus expression vectors using Gateway™ (Invitrogen). The hERG sequence was amplified by PCR, using *Pfu ultra* DNA polymerase (Stratagene) and Gateway™-compatible primers to add *att* B sites, transferred into the donor vector, pDONR221 (Invitrogen), and sequenced. hERG constructs were then transferred to either mammalian vectors (pDEST26 and pDEST40) or used to generate baculovirus (BaculoDirect™; Invitrogen) in accordance with the manufacturer's instructions.

Patch-clamping

Expression of hERG in HEK cells

HEK cells were grown as described elsewhere (Dalziel et al. 2005). They were plated on to 24-well plates, grown to 95 % confluency, and transfected with 8 μg wild type hERG, hERG-NT-HT, or hERG-CT-HT, 0.8 μg pHygEGFP, and 29 μl OPTI-MEM® I reduced serum medium, then replated on to polylysine-coated cover slips 24 h later. Transfected cells were identified by their fluorescence under UV light.

Electrophysiological measurement for patch-clamping HEK 293 cells

Macroscopic currents were recorded from whole-cell membrane patches 2–3 days post-transfection of HEK cells. The pipette solution contained (mM): 140 KCl, 1 $MgCl_2$, 5 EGTA, 5 MgATP, 10 HEPES, pH 7.2. The bath solution contained (mM): 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.4. Pipettes were pulled from borosilicate glass using a five-stage microelectrode puller (Flaming/Brown; Sutter Instrument Company, Novato, CA, USA) to give resistances of 3–5 MΩ when filled with pipette solution. Whole-cell currents were recorded from voltage-clamped cells at a holding potential of −80 mV with an EPC-10 amplifier and Pulse version 8.53 data collection software (HEKA, Germany). Data were filtered at 5 kHz and sampled at 100 μs intervals. Fast capacitance compensation was used to cancel the fast transient. Data were analysed using Pulse Tools 8.67 and Sigma Plot 9.0 software.

Protein expression in insect cells and isolation and reconstitution into liposomes

hERG-HT expression in Sf9 cells

*Sf*9 cells were grown as described elsewhere (Dalziel et al. 2007; Zhang et al. 2007) in Grace's Insect medium (Sigma) supplemented with 10 % fetal bovine serum, 2 % yeastolate, 3.3 mg/ml lactalbumin (pH 6.2). Cells were routinely seeded at a density of 5×10^5 cells/ml and grown to 2×10^6 cells/ml in 50 ml medium, by using spinner flasks at 25 °C, then scaled up to 500-ml cultures at 2×10^6 cells/ml density before infection with the virus. Virus titre determined by plaque assay was 8×10^7 PFU/ml. For protein production, cells were grown to a density of 2×10^6 cells/ml then infected with baculovirus at an MOI of 1.5 and harvested at 66–69 h post-infection. Protein expression in *Sf*9 cells infected with wild type hERG or hERG-NT-HT or hERG-CT-HT recombinant baculovirus was verified, from a sample of 3.5×10^4 cells per lane, by SDS-PAGE. 2×10^6 cells were washed and resuspended in 1 ml PBS then diluted 1:60 with protein loading buffer (Invitrogen), boiled for 5 min, centrifuged, and 25 µl supernatant loaded per lane. Western blot analysis was performed after transfer of proteins by iBlot (Invitrogen) to a PVDF membrane, using anti-K_v 11.1 (HERG) antibody (1/500), a secondary peroxidase-conjugated goat anti-rabbit antibody (1/2,000), and visualised using chemiluminesence (ECL™, Amersham, UK).

Solubilisation of hERG-CT-HT protein

A sample of 5×10^4 *Sf*9 cells infected with hERG-CT-HT recombinant baculovirus was washed three times with Dulbecco's phosphate-buffered saline (DPBS, pH 7.1; Gibco, Invitrogen) and stored at −80 °C. To solubilise the protein, cell membranes in DPBS buffer with protease inhibitor were sonicated (Vibro-Cell; Sonics and Materials, Danbury, CT, USA) for two 30-s bursts at 60 % power. Cell fragments were centrifuged at 10,000×*g* for 40 min at 4 °C. The pellet was incubated in lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 % glycerol, one tablet of protease inhibitor per 10 ml) with 2 % nonidet P-40 or 2 % DDM for 1, 4, or 16 h, then centrifuged at 10,000×*g* for 40 min.

Reconstitution of cell membranes into liposomes

Thawed cells (1×10^9) were pelleted at 13,000 rpm for 2 min at room temperature, using a bench-top centrifuge, and resuspended in 8 ml lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 % glycerol, one tablet of protease inhibitor per 10 ml). Samples were sonicated for $6 \times$ 10-s bursts in an ice bath at 100 % power with a 1 min interval between each burst. After sonication, cell fragments were centrifuged at 10,000×*g* for 40 min at 4 °C. The pellet (cell fragments) was resuspended in 8 ml of lysis buffer containing 0.05 % NP40 and 0.5 % lipid mixture containing: phosphoethanolamine, phospho-L-serine, phosphatidylcholine, and cholesterol in the ratio of 5:3:1:1 (Avanti Polar Lipids, Alabaster, AL, USA) (final solution pH 7.9), sonicated as before, then centrifuged at 100,000×*g* for 60 min at 4 °C. The pellet (liposomes) was resuspended in 4.5 ml reconstitution buffer (15 mM HEPES, 0.5 mM EGTA, 300 mM NaCl, 200 mM sucrose; pH adjusted to 7.4) by vortex mixing, sonicated in an ice bath at 100 % power for two 10-s bursts, then stored in 100 µl aliquots at −80 °C until use. Proteoliposomes were analysed for the presence of hERG protein by SDS-PAGE; 25 µl proteoliposomes and loading buffer containing 2 % SDS were boiled for 10 min and 30 µl loaded per lane for Coomassie stained gels and 10 µl per lane for western blots.

Supported bilayer lipid membranes

Preloading of proteoliposomes into porous Teflon supports and BLM formation

Porous Teflon-supported bilayer lipid membranes (s-BLM) were formed as described elsewhere (Phung et al. 2011a, b). The inside and outside of the s-BLM contained 0.3 ml and 2 ml sterile filtered solution, respectively, 140 mM KCl and 10 mM HEPES, pH 7.2. Proteoliposomes from cells expressing hERG-CT-HT were diluted 1:100 with bath solution and preloaded into porous Teflon membranes, by vacuum filtration, before formation of a bilayer lipid membrane. The BLM-forming solution contained 5 % PC and 2 % cholesterol.

Electrophysiological measurement for supported bilayer lipid membranes

Electrical impedance spectroscopy Electrical impedance spectra (EIS) were used to verify formation of a BLM. They were obtained using a PCI14/300 Potentiostat and a FAS2 Femtostat manufactured by Gamry Instruments operating under Gamry Framework, Echem Analyst™ and EIS300 analysis software (Gamry Instruments, Warminster, PA, USA). Spectra were recorded for frequencies between 1 mHz and 100 kHz at 0 V potential with an AC modulation amplitude of 10 mV. Nine sample points were taken per tenfold increase in frequency. For impedance recordings the measuring (*trans*) and reference (*cis*) electrodes were silver/silver chloride wires, and the counter wire (*cis*) was platinum.

Voltage-clamp Macroscopic currents from hERG channels reconstituted into the supported bilayer membrane were recorded using an EPC-10 amplifier with an integrated interface controlled by HEKA Pulse software and data were analysed using HEKA PulseFit and PulseTools v8.8 (HEKA, Lambrecht/Pfalz Germany). Silver/silver chloride electrodes connected the cell to the amplifier to record ionic currents. In the experiments EIS and voltage-clamp measurements were made on the same preparation of s-BLM. The cell was enclosed in a Faraday cage, supported on a vibration isolation table, in which the pre-amplifiers for both EIS and voltage-clamp were also placed. Data were filtered at 1 kHz and sampled at 500-μs intervals, and analysed using TAC X4.2.0 software. Conductance voltage data were junction potential adjusted (P. Barry, JPCalc software).

Voltage protocols Four different voltage protocols were used (Fig. 1): a tail-current protocol (I) used in whole-cell patch-clamping; an activation protocol (II), two current–voltage protocol (III) to account for channels being in both orientations, and a leak subtract protocol (IV) to more closely approximate that used in pharmacological assays.

Solutions BLM bath solution: 140 mM KCl, 10 mM HEPES, pH 7.2. All drugs were water-soluble and diluted from stocks dissolved in bath solution (verapamil 25.0 mM; clofilium 0.2 mM, and E4031 0.2 mM).

Statistical analysis Results are expressed as mean ± SEM. Statistical comparisons were made using either a Student's *t* test or one-way anova nested design in Genstat v12 (VSN International, Hemel Hempstead, UK).

Results and discussion

Function of histidine tagged hERG in HEK cells

Because a histidine tag had been added to hERG for purification, its effect on ion channel function was assessed and compared with that of unmodified (wild type) hERG. HEK cells expressing hERG-NT-HT or hERG-CT-HT gave robust voltage-activated currents, similar to that observed for wild type, when *voltage protocol I* was used (Fig. 2a–c). E4031, an anti-arrhythmic drug, is an open-channel blocker that is selective for hERG (Spector et al. 1996). It can therefore be used to subtract endogenous currents or as a positive control (Anson et al. 2004; Guo and Guthrie 2005). E4031-subtracted currents (Fig. 2c) were used to characterise peak amplitudes for the tail currents at −20 mV as a function of the preceding activation potentials. The data were fitted with the Boltzmann equation and that potential that resulted in half-maximum activation ($V_{1/2}$) was determined for each construct. $V_{1/2}$ was 15.1 ± 1.7 mV for hERG-NT-HT ($n = 4$), and 14.3 ± 3.0 mV for hERG-CT-HT ($n = 6$), which were not significantly different from that for wild type 24.2 ± 4.8 mV, ($n = 5$), and the slope was also unchanged. Thus either construct could be used. hERG-CT-HT was chosen for subsequent experiments because hERG-NT-HT appeared to deactivate more rapidly.

hERG expression in *Sf*9 insect cells

hERG-C-HT expression in *Sf*9 insect cells was confirmed by western blot analysis (Fig. 3a). Protein expression was maximized by establishing the optimum multiplicity of infection (MOI) and post-infection time for harvest of infected *Sf*9 insect cells. An MOI of 1 after 66 h of infection gave the highest level of protein expression and was routinely used.

Solubilisation of hERG protein

Attempts to solubilise hERG-CT-HT protein from insect cell membranes using different detergents (Nonidet P-40, DDM) were not successful. No hERG band was observed in protein samples extracted from the supernatant when cell membranes had been exposed to 2 % of either NP40 or DDM for 16 h (Fig. 3b). Because we wanted to maintain ion channel function we did not want to use harsher conditions that might denature the protein. We therefore generated proteoliposomes directly from cell membranes without further purification, which did not require the protein to be soluble. We also added lipids to the cell membranes to incorporate these into the proteoliposomes as they formed.

Generation of liposomes from hERG cell membranes

Protein isolated from insect cells was incorporated into the cell–proteoliposomes and assessed by SDS-PAGE (Fig. 3c). The total protein yield was 0.706 ± 0.06 μg/ml cell culture ($n = 3$). Because the preparation was not purified protein, quantification of hERG protein was not possible. Instead the total protein from the cell fraction was determined using the Bradford assay, in accordance with the manufacturer's instructions (Bio-Rad). A procedure was designed to determine the optimum (total) protein-to-lipid ratio for efficient liposome formation from cell membranes using the current response measured after incorporation into Teflon-supported BLMs. The 1:1 ratio proteoliposome mix contained 50 μg/ml total protein and 5 mg/ml lipids. The support matrix was first preloaded with the liposomes (Phung et al. 2011a) and then the BLM was

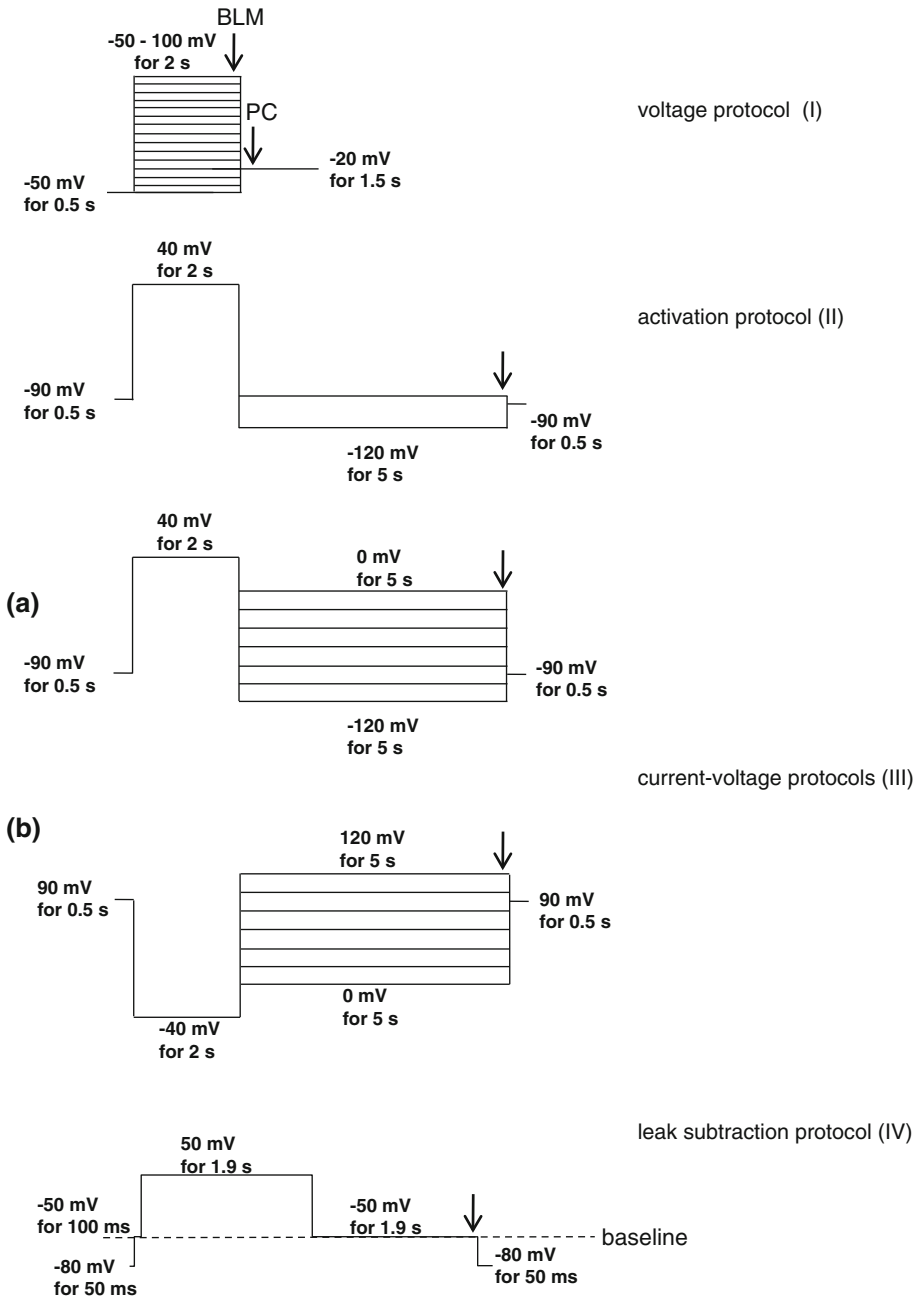

Fig. 1 Voltage protocols. Measurements were made for patch-clamp (PC) or bilayer lipid membrane (BLM) experiments, where indicated by the *arrow*, and were from a baseline of 0 pA, except for voltage *protocol IV* as indicated

formed over the support matrix. Protein-to-lipid ratios of 1:1, 1:10, 1:100, and 1:1,000 were tested for hERG-CT-HT-infected insect cells and compared with that for uninfected cells at 1:1 and 1:100 protein-to-lipid ratios. Because of practical limitations in adding large amounts of lipid to the mixture, the amount of lipid added was held constant at 0.5 % lipid mixture containing: phosphoethanolamine, phospho-L-serine, phosphatidylcholine, and cholesterol in the ratio of 5:3:1:1 (with 0.05 % NP40), and the amount of protein varied. Currents were activated by voltages that were measured in solutions containing 140 mM K^+ on both sides of the BLM (Table 1).

hERG channels are either closed, open, or inactivated, depending on transmembrane potential (Vandenberg et al. 2004). Channels are closed at negative potentials. Membrane depolarization (+) slowly activates (opens) the channels, which then inactivate rapidly. Repolarization (−) of the membrane reverses the transitions between these channels states. The channel currents were observed at potentials more positive than the holding potential in the patch-clamping system. However, hERG channels reconstituted into Teflon-supported BLM could be oriented in either direction. Therefore ionic currents could be observed at both positive and negative potentials. A symmetrical

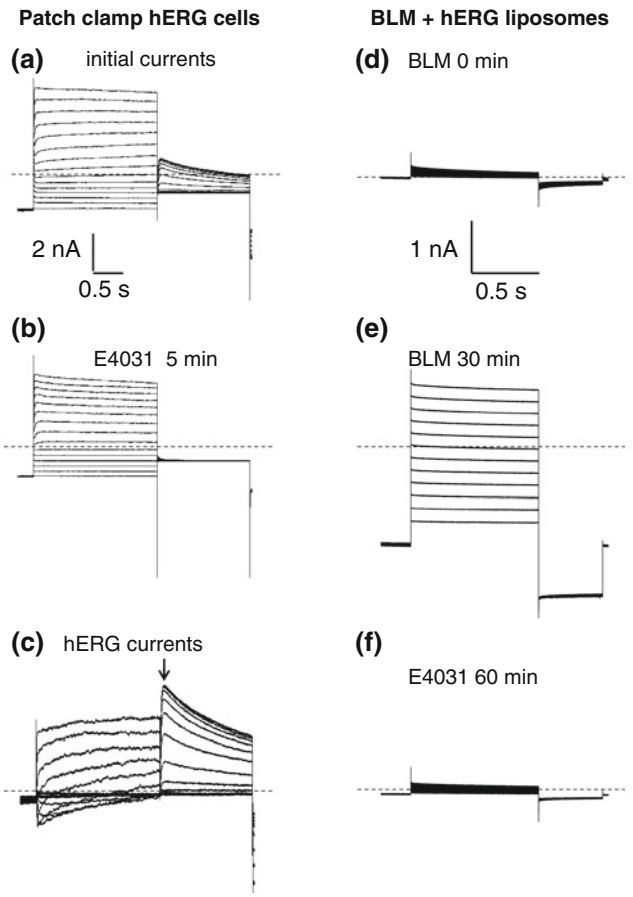

Fig. 2 hERG current responses from patch-clamping compared with Teflon-supported BLMs. Macroscopic current recordings from hERG activated using *voltage protocol I* measured by whole-cell patch-clamping of HEK cells expressing hERG (**a**–**c**) and by using BLMs on Teflon supports containing hERG *Sf*9 cell liposomes (**d**–**f**). *Scale bars* are shown for (**a**–**c**) and (**d**–**f**). **a** Initial currents; **b** currents after 5 min exposure to 5 μM E4031; **c** putative hERG current obtained by subtracting E4031 inhibited currents from the control. **d** The current recorded immediately after formation of a BLM on a cuvette preloaded with hERG cell liposomes; **e** that after 30 min of liposome incorporation; **f** that after 60 min of exposure to E4031

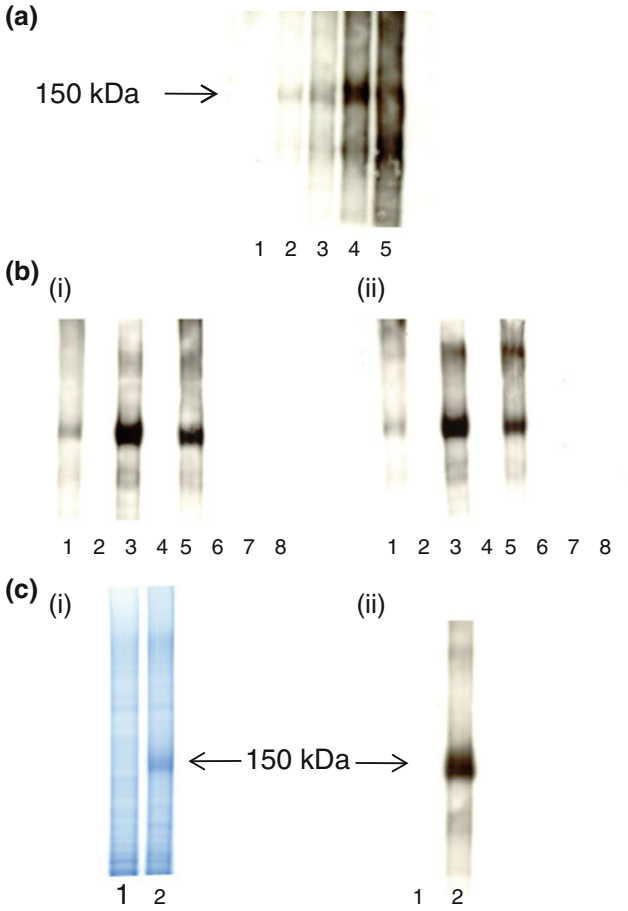

Fig. 3 Expression and isolation of functional hERG channels. **a** Western blot of hERG expressed in *Sf*9 cells at different post-infection times (*Lane 1*: 24 h; *2*: 40 h; *3*: 48 h; *4*: 66 h; *5*: 88 h), (3.5×10^4 cells per lane). **b** Western blot of protein extracted from the cell pellet and supernatant from 1.4×10^4 cells expressing hERG exposed to 2 % NP40 (*i*) or 2 % DDM (*ii*) for 1–16 h (*Lane 1*: 1 h pellet; *2*: 1 h supernatant; *3*: 4 h pellet; *4*: 4 h supernatant; *5*: 16 h pellet; *6*: 16 h supernatant; *7*: 16 h uninfected control *Sf*9 cell pellet; *8*: 16 h uninfected control *Sf*9 cell supernatant). **c** SDS-PAGE showing hERG in unpurified cell membrane liposomes in a (*i*) Coomassie blue-stained gel and (*ii*) the corresponding western blot from 1:1 ratio proteoliposome. (*Lane 1*: liposomes from uninfected *Sf*9 cells, *Lane 2*: cells infected with hERG-CT-HT)

voltage protocol II was used to measure the channel currents at positive and negative potentials in this study. Currents less than 50 pA were omitted from the analysis, because these also occurred in controls and were therefore likely to be a result of endogenous channels. To preclude possible BLM instability over the duration of the experiment, data were excluded if the BLM capacitance changed by more than 12 nF. Impedance spectroscopy methods were based on a model circuit described elsewhere (Phung et al. 2011a). The average BLM resistance was 8.0 ± 1.0 GΩ ($n = 71$). The results (Table 1) showed that the most current responses were recorded for protein-to-lipid ratios of 1:1 and 1:10, with few or no responses for protein-to-lipid ratios of 1:100 and 1:1,000. The mean current response for liposomes generated from uninfected cell membrane liposomes was 27.6 ± 10.6 pA after 30 min and was not inhibited by verapamil. Results from experiments that gave current responses 30 min after BLM formation were not significantly different in amplitude and were therefore combined, giving a mean current of 1.0 ± 0.4 nA ($n = 7$) at −120 mV (*voltage protocol II*). Inhibition by verapamil was not significantly affected by the protein-to-lipid ratio. The liposome preparation method was standardised by using a protein-to-lipid ratio of 1:1 in subsequent experiments.

hERG pharmacology in Teflon-supported BLMs

To establish what proportion of the current was due to hERG and to further assess the functional integrity of the

Table 1 Liposome composition in relation to hERG function in BLM

Protein-to-lipid ratio	BLM tested	Increased current in BLM[a]	Percentage inhibition[b] (*n*)
hERG-CT-HT			
1:1	12	5	43 ± 7 (4)
1:10	12	4	64 ± 9 (4)
1:100	12	1	60 (1)
1:1,000	12	0	0
Uninfected cells			
1:1	12	0	0
1:100	11	0	0

Data are mean ± SEM

[a] Number of BLMs for which current responses were recorded 30 min after BLM formation

[b] Current response inhibited by 50 μM verapamil

recombinant hERG channels, the effect of pharmacological modulators on the current response was examined. Because we did not know the orientation of the ion channels in the liposomes, membrane-permeable blockers (E4031, verapamil, clofilium) were chosen and applied to both chambers. E4031 was used to identify the HERG component of the current recordings (Anson et al. 2004; Guo and Guthrie 2005) using a voltage protocol similar to that used for the patch-clamp experiments (*voltage protocol I*) and similar solutions were used for visual comparison of currents (Fig. 2d–f), however measurements were taken at the end of the activating pulse (rather than at the start of the tail current). It was found that 5 μM E4031 reduced currents in 6/8 experiments after 60 min, inhibiting by 48.1 ± 13.0 %, ($n = 7$) (Fig. 4f). This is less inhibition than expected, and it was slower; in one experiment, however, E4031 inhibited the current by 90 %, indicating that in this case a large

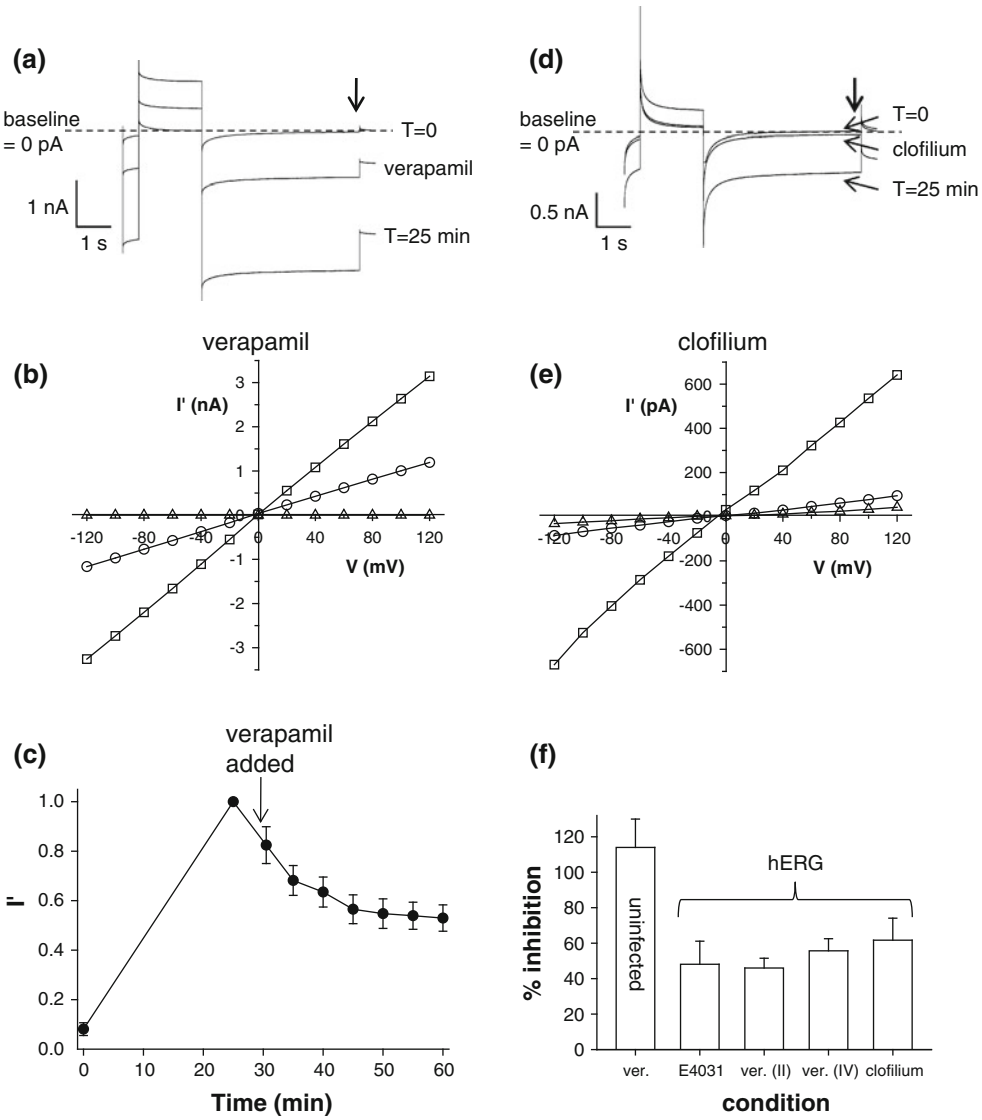

Fig. 4 Macroscopic hERG current responses in Teflon-supported BLMs. **a**, **d** Representative recordings of voltage-activated currents (*voltage protocol II*) immediately after BLM formation ($T = 0$), after 25 min ($T = 25$ min), used as a control recording, and 30 min after addition of **a** verapamil and **d** clofilium. **c** Time-dependence to verapamil inhibition relative to the controls at 20 min, $n = 15$ (1 × lipid), −80 mV (*voltage protocol II*). **b**, **e** Representative IVs (*voltage protocol III*) from single experiments are shown immediately after BLM formation (*triangles*), after 30 min to allow for liposome fusion (*squares*), and after 30 min of exposure to inhibitor (*circles*)—50 μM verapamil **b** or clofilium **e**. **f** Histogram showing effects of inhibitors on current responses for hERG cell membrane liposomes. The inhibitory effect of verapamil on currents from uninfected cell membrane liposomes is shown for comparison. Currents recorded at −120 mV (*voltage protocol II*), unless indicated. Data are mean ± SEM

component of the current response was due to hERG (Fig. 2f).

Although not specific to hERG channels, verapamil, a calcium-channel inhibitor and pore blocker, is commonly used in hERG pharmacological studies because it inhibits rapidly with high affinity (Duan et al. 2007a, b; Zhang et al. 1999). By monitoring the effect of verapamil (50 μM) over time we found that 30 min was sufficient to reach steady state inhibition (Fig. 4a) under our experimental conditions. Verapamil inhibited the current response to *voltage protocol II* by 46.0 ± 5.5 % ($n = 15$), after 30 min (Fig. 4b), which was less than expected. Clofilium, a quaternary amine compound that binds in the pore was also used because, despite being slow acting, it is a potent inhibitor of hERG channels (Gessner and Heinemann 2003; Perry et al. 2004; Suessbrich et al. 1997). It was found that 1 μM clofilium inhibited the current response by 61.7 ± 12.4 % ($n = 4$) after 30 min at −120 mV (Fig. 4d, e). This was less than the 90–100 % inhibition expected (Gessner and Heinemann 2003; Perry et al. 2006; Suessbrich et al. 1997).

Because electrophysiology studies with hERG use a variety of voltage protocols we wanted to determine how much the voltage protocol used would affect the degree of inhibition by verapamil. *Voltage protocol IV* (activation measured after 1.9 s at −50 mV) was used immediately before *voltage protocol II* (activation measured after 5 s at −120 mV) to record inhibition by verapamil. The mean control current response 30 min after BLM formation was 1.6 ± 1.0 nA ($n = 5$) determined at −50 mV. Verapamil reduced the current response by 55.7 ± 6.8 % after 30 min after use of *voltage protocol IV* (Fig. 4f), which was not significantly different from that for *voltage protocol II*. The degree of inhibition observed is similar to that reported previously and may reflect voltage dependence of the block (Duan et al. 2007a, b), especially with channels in both orientations. The low current recorded for uninfected cell membrane liposomes of ~30 pA after 30 min, and lack of inhibition by verapamil (Fig. 4f), further supports the recorded hERG membrane liposome currents as being largely due to hERG channels.

The inhibitory effect of three diverse blockers of hERG channels demonstrates that the method of hERG proteoliposome generation from cell membranes produced ion channels that were functional and responded as expected to pharmacological modulators. The slow onset of block by inhibitors relative to that in patch-clamp experiments suggests that the location of the receptors in bilayers is difficult to access which may be because of poor access to the bilayer, because it is composed of many small bilayers present in the interstices of the porous Teflon. Expression and isolation of recombinant hERG protein expressed in *Sf*9 cells has not been reported previously. However, a detergent-free method has been reported that incorporates cell membrane fragments from human embryonic kidney cells expressing hERG into droplet bilayers, and results in macroscopic current responses inhibited by E4031 (Leptihn et al. 2011).

To compare the current level in BLMs produced by ion channels in cell membrane liposomes with those in detergent-solubilised cell membranes for liposome production, we used the voltage-gated sodium channel (VGSC). Recombinant VGSC protein expresses at only low levels in the *Sf*9 baculovirus system (Zhang et al. 2007). Because we had previously enriched for his-tagged voltage-gated sodium channels (VGSC) using immobilized metal affinity chromatography (IMAC) after extraction from *Sf*9 cell membranes with non-ionic detergent (NP40) (Zhang et al. 2007), we prepared cell membrane liposomes from insect cells expressing VGSCs for functional comparison in Teflon-supported-BLM (Phung et al. 2011a, b). Because the Na^+ current through VGSC channels is rapidly activated by voltage and inactivates rapidly it would be difficult to separate this from the capacitive transient given the large BLM area; recordings were therefore measured at steady state, as reported elsewhere (Phung et al. 2011a). Currents from continuous recordings were measured at −80 mV, before and after addition of the activator veratridine and 20 min after addition of the inhibitor saxitoxin. Only very small current responses were detected for liposomes from uninfected cell membranes. Veratridine (100 μM) increased the current twofold and saxitoxin (200 nM) reversed this effect (Fig. 5). The veratridine-activated currents ranged from 80 to 550 pA (BLM resistance 300–700 MΩ) for cell membrane liposomes compared with currents of 1–10 nA for VGSC-enriched liposomes (Phung et al. 2011a). We therefore inferred that liposomes produced from the former IMAC method to enrich for VGSC contained more functional protein than those from the current cell liposome method. Because VGSC are expressed at much lower levels than hERG in *Sf*9 cells, it seems that VGSC protein must be enriched for first rather than used directly from cell membranes. This suggests that the cell–liposome method may be best suited to proteins that express at high levels but cannot be easily solubilised.

Conclusions

In this paper we report a method of producing eukaryotic ion channel proteoliposomes without isolating the protein from the cell membranes in which they are expressed, circumventing the need to solubilise these very hydrophobic proteins. This avoids possible denaturation caused by strong detergents, preserving their function for pharmacological studies in BLMs. This technique is broadly

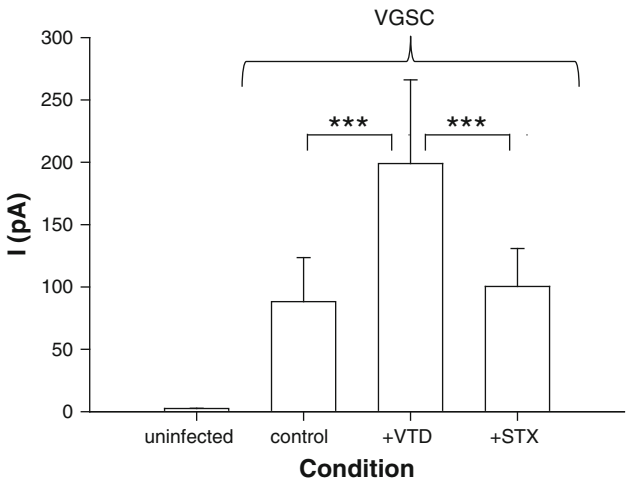

Fig. 5 Unpurified cell liposomes from voltage-gated sodium channels (VGSC). Histogram showing mean current in Teflon-supports preloaded with uninfected or VGSC-unpurified cell membrane liposomes on which bilayers were formed. Liposomes from uninfected cells are shown 30 min after formation of a stable bilayer determined by impedance spectroscopy, $n = 5$ (SEM bars not visible). The control current (before addition of veratridine) is compared with that recorded 20–30 min after addition of 100 μM veratridine and 20 min after addition of 200 nM saxitoxin, $n = 7$. Data are from 1 min of continuous recording at −80 mV; $^{***}p < 0.001$. Data show mean ± SEM

applicable to other eukaryotic ion channels. The activity of the ion channel of interest can be distinguished from that of endogenous channels by controlling the ionic composition of the solution, applying relevant voltage protocols, and use of specific inhibitors. These findings suggest that when an ion channel of interest is expressed at high levels compared with endogenous channels, production of liposomes from unpurified membrane preparations can be used in Teflon-supported BLM applications and therefore have potential for use in future pharmacological ion channel assays.

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