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Expression of human BK ion channels in *Sf*9 cells, their purification using metal affinity chromatography, and functional reconstitution into planar lipid bilayers

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Abstract

This report describes a procedure for purification of large conductance calcium-activated potassium (BK, maxi-K) channels using immobilised metal affinity chromatography (IMAC) under non-denaturing conditions. An amino-terminal histidine fusion tag was added to *hSlo*, the human BK channel, and expressed in *Sf*9 insect cells. Following IMAC purification and production of proteoliposomes, protein function was assessed electrophysiologically in planar bilayer lipid membranes. Single channel openings had conductances of 250–300 pS and were inhibited by paxilline, demonstrating that the BK channels remained functional following IMAC purification. This method to obtain functional human ion channels will be useful in assays to screen potential pharmaceuticals.
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Keywords: IMAC; His-tag; Ion channel; *Sf*9 insect cell; Purification; Planar lipid bilayer

1. Introduction

Biological membranes and their associated integral membrane proteins are responsible for a broad range of important physiological functions including sensing the environment, accumulation of nutrients, capture and transduction of energy, neural signalling, extrusion of unwanted compounds, and the maintenance of homeostasis [1]. The biological sensing mechanisms that underlie these functions may be utilised in new applications in biotechnology including bio-nanotechnology. However, development of this technology is limited by the availability of membrane proteins that are functional for use in artificial bilayer lipid membranes. Expression of proteins in heterologous systems provides a source of human membrane proteins that may be purified for use in new assays being developed to screen for potential pharmaceuticals.

This research has focussed on the large conductance calcium-activated potassium (BK) channel because it is physiologically and medically important, having roles in bladder control, regulation of blood pressure, and motor function, and is therefore a potential target for new pharmaceuticals [2,3]. Moreover, its molecular function is well characterised electrophysiologically and its large conductance gives channel activity that is easily detectable [4]. The BK channel consists of seven membrane-spanning α domains that make up the BK α pore-forming subunit [5]. There are four variants of an accessory β-subunit that alter the pharmacology and gating kinetics of the *Slo* α-subunit: β1 is highly expressed in smooth muscle; β2 is highest in the ovary; β3 in the testis and β4 in all neural tissues [6]. BK channels are activated by the binding of Ca^{2+} ions to the intracellular portion of the protein and by membrane depolarisation. They have a conductance of ca. 200–350 pS and contribute to the repolarisation of the membrane potential following an action potential [7]. Traditionally, BK channels have been extracted and purified from muscle, and reconstituted into planar bilayer lipid membranes for electrophysiological analysis of their function [8–11]. However, the routine study of human BK channels in this way would require that they be expressed in cultured cells

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for subsequent purification. While *hSlo* has been expressed in *Xenopus laevis* (frog) oocytes and purified for use in a lipid bilayer formed on a silicon microchip [12], this technique is labour-intensive and not amenable to being upscaled. The ability to purify human BK channels that are functional is important because it would provide a source of protein for use in artificial bilayer systems that will be the "next generation" of electrophysiological assays to screen for potential pharmaceuticals against target ion channels.

The objective of this work was to purify human BK channel protein in a functional form for reconstitution into bilayer lipid membranes. The strategy used to achieve this involved modification of the *hSlo* cDNA sequence to add a tag of six histidine residues to the N-terminus of the protein to enable purification using immobilised metal affinity chromatography (IMAC) because this method can be fast, efficient, and upscaled to large volumes. In addition, the small size of the fusion tag reduces the probability that it will affect protein function. Since maintaining protein function was imperative, we used non-denaturing conditions during extraction and purification. The *hSlo*-HT gene was expressed in *Sf*9 insect cells using the baculovirus system, which was confirmed by immunodetection and function verified by patch-clamping. *hSlo*-HT was purified using IMAC and incorporated into liposomes which were fused to planar lipid bilayers. The function of *hSlo*-HT was assessed by electrophysiological measurements following reconstitution into planar lipid bilayers and the effect of a BK channel inhibitor investigated.

2. Experimental

2.1. Materials

The baculovirus vector pFastBac-HT, the Bac-to-Bac baculovirus expression system, *Sf*9 cells, Mlu1 and Not1 restriction endonucleases, molecular weight standard markers, Bis–Tris gels and buffers, foetal bovine serum, yeastolate, and streptomycin/penicillin were purchased from Invitrogen (Carlsbad, USA). Coomassie blue stain was from Bio-Rad (Hercules, USA). Anti-BK_{Ca} channel antibody was from Alomone Labs (Jerusalem, Israel). Peroxidase-conjugated goat anti-rabbit antibody (ECL™) was from Amersham (England). Protease inhibitor cocktail tablets, EDTA-free, were from Roche (Basel, Switzerland). Grace's insect medium, lactalbumin, Triton X-100, Tween 20, *n*-octane, KCl, HEPES (*N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid]), HEDTA (*N*-(2-hydroxyethyl)ethylene-diaminetriacetic acid) were purchased from Sigma–Aldrich (St. Louis, USA). Talon™ metal affinity resins were purchased from Clontech (Mountain View, USA). SpectraPro Float-A-Lyzer dialysis tube was from Spectrumlab (CA, USA). Phosphatidylethanolamine (PE), phosphatidylserine (PS), and cholesterol (CH) were from Avanti Polar Lipids. Phosphatidylcholine (PC) was extracted from egg yolk [13]. Methanesulfonic acid was from Alfa Aesar (Karlsruhe, Germany). Borosilicate glass (Harvard Apparatus, Kent, UK) for pipettes was purchased from SDR (Middle Cove, Australia). Paxilline was produced by *Penicillium paxilli* cultures and purified using methods previously described [14]. All other reagents were from BDH (London, UK) and were of analytical grade or higher.

2.2. Expression of hSlo-HT in Sf9 insect cells

An N-terminal poly-histidine (His) tag was added to *hSlo* (Genbank accession number U11058) by sub-cloning it from the mammalian vector pcDNA3 [5] into the baculovirus vector pFastBac-HT. *hSlo* was excised at Mlu1 and Not1 restriction endonuclease sites. The Mlu1 digest was treated with Klenow DNA polymerase 1 to fill in the recessed 3′ end prior to Not1 digestion, then the fragment was ligated into pFastBac-HTa at Stu1 and Not1 sites to achieve in-frame cloning. This was confirmed by DNA sequencing. The N-terminal amino acid sequence was MSYY**HHHHHH**DYDIPTTENLTFQGAMDPEFKG**ALIIPVTMEVP**+. Six histidines (bold) were introduced and two amino acid residues, methionine and aspartate were deleted from the N-terminus of *hSlo*. The residues between the His-tag and *hSlo* comprise a spacer region and a protease cleavage site, although the tag was not removed. Conditions used for the growth of *Sf*9 insect cells and production and propagation of recombinant baculovirus were similar to those described previously [15]. Briefly, recombinant bacmid (recombinant baculovirus shuttle vector) was produced in *E. coli* by site-specific transposition using the Bac-to-Bac expression system [16]. Generation of recombinant bacmid was confirmed by PCR analysis. Purified bacmid DNA was used to infect *Sf*9 insect cells to obtain high titre stocks of *hSlo*-HT recombinant baculovirus. *Sf*9 cells were grown at 25 °C in Grace's insect medium supplemented with 10% foetal bovine serum, 2% yeastolate, 3.3 mg/ml lactalbumin containing 100 μg/ml streptomycin/penicillin, pH is 6.2. 10^5 cells were infected with *hSlo*-HT recombinant baculovirus in a 96-well plate and incubated at 25 °C for 40 h. The cells were harvested, washed twice in phosphate buffered saline (PBS), and protein fractions separated by PAGE electrophoresis (4–12% Bis–Tris gel). BK channel expression in *Sf*9 cells was verified by Western blot analysis using an anti-BK_{Ca} channel antibody 1:2000, peroxidase-conjugated goat anti-rabbit antibody 1:1000, and visualised using chemiluminescence. Cell cultures were upscaled to 75 cm^2 in adherent culture flasks for visualisation using Coomassie stain.

2.3. Isolation and purification of hSlo-HT

For protein production, cells were grown in spinner culture flasks to a density of at least 1×10^6 cells/ml and infected with *hSlo*-HT recombinant baculovirus at a multiplicity of infection (MOI) of 1. Virus-infected *Sf*9 cells were harvested 40 h post-infection by centrifugation at $1000 \times g$ for 10 min at 4 °C and then resuspended in 50 mM sodium phosphate (300 mM NaCl, pH 7.0) with complete protease inhibitor. The protease inhibitor was necessary because the α-subunit is known to be prone to protease degradation [17]. The cell suspension was sonicated for a total of 40 s in 5 s bursts with 5 s intervals between bursts using a Vibra Cell sonicator (Pierce). The suspension included 10% glycerol and 10 mM KCl and was cooled on ice during

sonication. The suspension was then centrifuged at 100,000 × *g* for 90 min at 4 °C and the pellet containing the membrane fraction collected. This was solubilised by resuspending in 50 mM sodium phosphate buffer, 300 mM NaCl, 2% Triton X-100 (v/v), 10 mM KCl, pH 7.0, containing protease inhibitors and incubating for 16 h before centrifugation at 160,000 × *g* for 1 h at 4 °C. The supernatant was collected and assayed by SDS-PAGE followed by Coomassie staining.

The solubilised membrane fractions were mixed with cobalt affinity resin and gently agitated for 20 min at room temperature on a platform shaker. The mixture was then centrifuged at 3000 rpm in a bench-top centrifuge for 10 min, the supernatant removed, and the protein-bound resin washed twice with 10 bed volumes of buffer A (50 mM sodium phosphate, 300 mM NaCl, with complete protease inhibitor, 10% glycerol (v/v), 10 mM KCl and 0.1% Triton X-100 (v/v), pH 7.0). The resin was transferred to a 2 ml gravity flow column and washed with a further five bed volumes of buffer A followed by a further wash with buffer A containing 10 mM imidazole. *hSlo*-HT protein was then eluted from the resin with 150 mM imidazole in buffer A pH 7.2, and the eluate collected in 500 μl fractions. BK protein was assayed for purity by gradient SDS-PAGE (4–12%) and detected using Coomassie blue stain. Fractions containing *hSlo*-HT were pooled and thoroughly dialyzed against a solution containing 50 mM sodium phosphate, 10 mM NaCl, 0.01% Triton X-100 (v/v) and 10 mM KCl, pH 7.0, using SpectraPro Float-A-Lyzer 100,000 Da cut-off dialysis tubing to remove imidazole.

An alternate method using Ni^{2+} affinity resin was also used to purify *hSlo*-HT following the protocol we reported previously for purification of functional human voltage-gated sodium channels [15].

2.4. Preparation of liposomes

A phospholipid solution was prepared by evaporating solutions containing: 50 mg PE, 20 mg PS, 10 mg PC, and 10 mg CH in a small glass tube in a stream of N_2 gas. These lipids were then dispersed in 9 ml of reconstitution buffer (15 mM HEPES, 0.5 mM EGTA, 300 mM NaCl, 200 mM sucrose, pH adjusted to 7.4 with KOH) by sonicating twice for 20 s with a 1 min intervening pause on ice and then kept on ice. To 900 μl of this suspension, 90 μg Triton X-100 detergent and 900 μl solution containing resuspended *hSlo*-HT protein were added, and kept on ice for 20 min, frozen and thawed twice in a dry-ice/ethanol bath, centrifuged for 30 min at 100,000 × *g* and the pellet resuspended in 900 μl of reconstitution buffer. Aliquots of the resuspended liposomes were frozen at −80 °C. Before use the aliquots were thawed and sonicated for 10 s.

2.5. Electrophysiology

2.5.1. Patch-clamp

Infected cells from 96-well plates were also used in patch-clamp experiments. Macroscopic currents were recorded from inside-out membrane patches. The internal solution contained (mM): 140 $KMeSO_3$, 2 KCl, 20 HEPES, 5 HEDTA and 3.65 $CaCl_2$ to give 10 μM free calcium, pH 7.2. The external solution contained (mM): 140 $KMeSO_3$, 2 KCl, 20 HEPES, 2 $MgCl_2$, pH 7.2. Pipettes were made from borosilicate glass using a two-stage microelectrode puller (List-Medical, Germany) and had resistances of 3–5 MΩ when filled with external solution. Voltage ramps were applied to voltage-clamped cells from a holding potential of −80 mV. Currents were recorded with an EPC-9 amplifier and PC-based pulse Version 8.53 data collection software. Collected data were analysed using Pulse Tools 8.67 (HEKA, Germany) and Sigma Plot 9.01 software (Richmond, USA). Data from macroscopic recordings were filtered at 5 kHz and sampled at 20 μs intervals.

2.5.2. Planar bilayer lipid membranes

Planar bilayer lipid membranes (pBLMs) were formed by painting a phospholipid mixture over the aperture of a Warner Instruments (www.warneronline.com) perfusion bilayer chamber model BCH-P fitted with a 1 ml Delrin perfusion cup with a 200 μm aperture. Both chambers of this apparatus contained the internal solution used for patch-clamp experiments but at 100 μM $CaCl_2$ without HEDTA, because this concentration does not require a chelator [18]. The phospholipid solutions used to form a BLM contained 50 mg/ml PC and 20 mg/ml CH dissolved in *n*-octane. High resistance BLMs were formed (>2 GΩ). Voltages were applied and currents measured by a HEKA List EPC7 or EPC9 patch-clamp amplifier (www.heka.com) interfaced to a personal computer. HEKA Pulse was used to apply voltages and record data from trains of voltage pulses and ramps; Bruxton Acquire (www.bruxton.com) was used to record continuous single channel data that was filtered at 5 kHz and sampled at 100 μs intervals.

2.6. Reconstitution into pBLMs

Following formation of a pBLM, 50 μl of proteoliposome suspension was added to the chamber containing the reference electrode (*cis*) with further additions of 20 μl if no channel activity was seen within 30 min. An osmotic gradient was used to facilitate fusion [9]. Successful reconstitution commonly occurred within a few minutes of adding proteoliposomes to the chamber as determined by the distinctive transitions in current as the channels opened and closed.

3. Results and discussion

3.1. Expression of His-tagged hSlo in insect cells

Expression of *hSlo* channels with an N-terminal His-tag (*hSlo*-HT) in *Sf*9 insect cells was confirmed by immunodetection. A Western blot of cells expressing *hSlo*-HT is shown in Fig. 1A. After 40 h of infection, a prominent band was detected at 120 kDa, the position expected for the α-subunit of the BK ion channel protein, with no discernable corresponding band for uninfected controls (Fig. 1A). When the culture

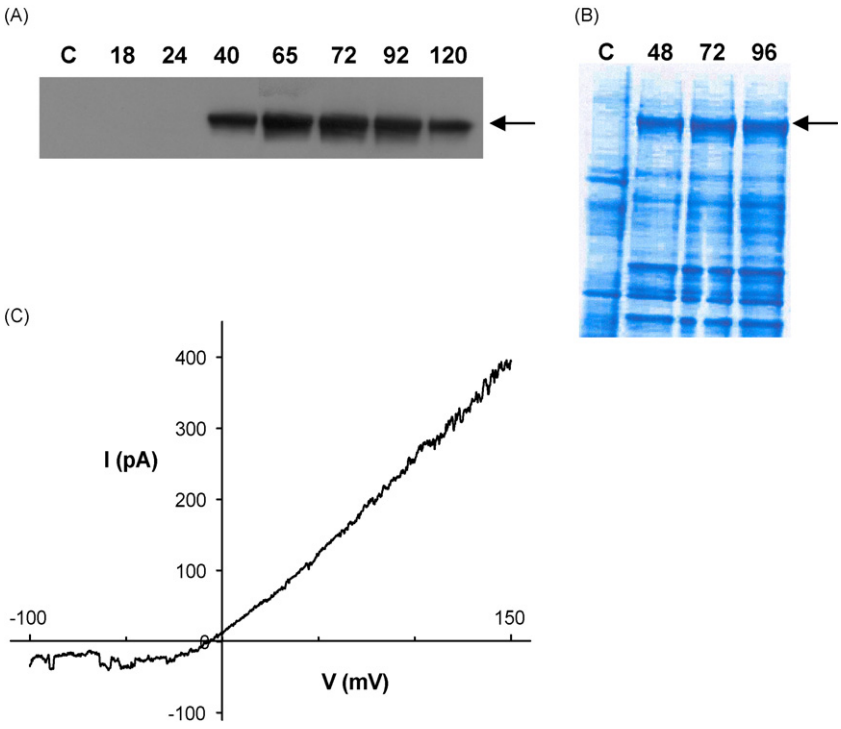

Fig. 1. Expression of functional *hSlo*-HT in *Sf*9 insect cells. The presence of BK channel protein in *Sf*9 cells infected with *hSlo*-HT recombinant baculovirus was analysed by separation on a 4–12% SDS-PAGE gel followed by detection using (A) anti-BK_{Ca} channel antibody as shown in a Western blot at different post-infection times from 18 h to 120 h using cells plated on a 96-well plate. (B) Coomassie blue stain using cells infected in flat flasks and harvested at 48–96 h post-infection. Uninfected cells were used as controls. The position of a 120 kDa band is indicated, the expected position for BK channel α-subunit monomers. (C) Patch-clamp current trace from an inside-out membrane patch. Channels were activated by a voltage ramp applied from −100 mV to +150 mV over 50 ms in the presence of 10 μM intracellular free calcium, where pipette and bath solutions contained 140 mM K^+.

volume was upscaled to 75 cm^2 and grown in adherent culture flasks, a 120 kDa band was visible on a Coomassie-stained gel at 48 h, 72 h or 96 h post-infection, but not in uninfected cells (Fig. 1B).

To assess whether the *hSlo* channels remained functional after addition of the His-tag, patch-clamp experiments were conducted. A current trace from an inside-out membrane patch from a cell expressing *hSlo*-HT is shown in Fig. 1C. This recording shows the current in response to a ramp voltage applied from −100 mV to +150 mV over 50 ms. The outwardly rectifying current response was typical of that expected for *hSlo* channels in the presence of 10 μM intracellular free calcium [19]. These results showed that the BK ion channel protein was expressed in *Sf*9 insect cells and that it formed functional ion channels after addition of the His-tag.

The data in Fig. 1 was obtained for *Sf*9 cells grown as adherent cultures. In subsequent work where larger quantities of protein were required for isolation and purification, the cells were grown in spinner culture flasks of 50–250 ml. To further upscale the culture volume for protein production, 500 ml spinner culture vessels were used to infect cells. To optimise protein yield for subsequent purification, an assay was carried out to determine the multiplicity of infection (MOI, number of infectious viruses per cell) and post-infection time for harvest of infected *Sf*9 insect cells grown in spinner cultures that gave optimal protein expression. The optimal growth period was 40 h at the MOI of 2.

3.2. Purification of hSlo using metal affinity chromatography

The presence of *hSlo*-HT protein through successive stages of purification was monitored by Western blots. Cells were harvested by centrifugation, lysed by sonication and the supernatant collected. To determine the optimal conditions for solubilisation, the cell pellets were treated with either 2% Tween 20 (v/v) or 2% Triton X-100 (v/v) for 16 h, centrifuged and the supernatants and the cell pellets collected. Fig. 2A shows the resultant bands for supernatants at successive stages, and using different detergents to extract the protein: untreated cells (lane 1), after sonication (lane 2), treatment with Tween 20 (lane 3), and treatment with Triton X-100 (lane 4). In the supernatant from the Tween 20 treatment the 120 kDa band expected for the α-subunit of the *hSlo*-HT protein was reduced in intensity and several lower molecular weight bands were also observed. Protein obtained after treatment with Triton X-100 (lane 4), was free of bands other than that at 120 kDa. These results suggest that Triton X-100 solubilised the *hSlo*-HT protein more efficiently than Tween 20, and that less protein degradation occurred, therefore this detergent was selected for further studies.

hSlo-HT protein was purified by IMAC using a cobalt affinity resin (Talon™, Clontech). The solubilised cell extract was gently agitated for 20 min with Talon™ resin (previously equilibrated with extraction/wash buffer augmented with 0.02% Triton X-100 (v/v), 10 mM KCl and protease inhibitor). The resin was

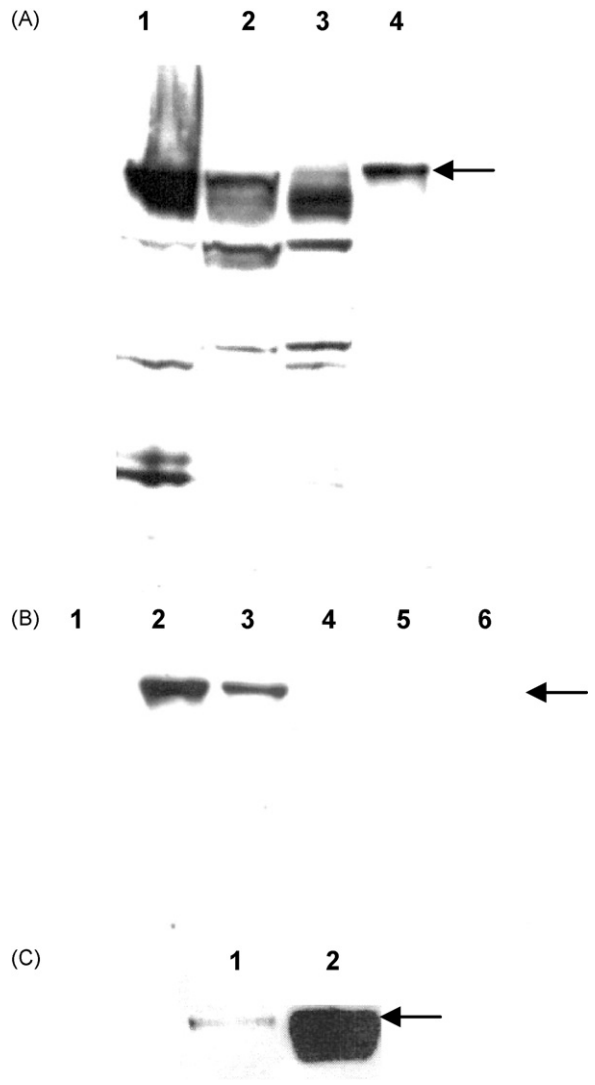

Fig. 2. Western blot of histidine-tagged BK channel protein at successive stages of purification, separated by SDS-PAGE and blotted with anti-BK channel antibody. (A) Protein from untreated cells (lane 1), or treated using sonication (lane 2), 2% Tween 20 (lane 3), or 2% Triton X-100 (lane 4). (B) Collected fractions eluted from Co^{2+} affinity resin. (C) Dialysed protein used for production of liposomes: supernatant (lane 1), and liposome pellet (lane 2) following ultracentrifugation for use in planar lipid bilayer experiments. The arrow indicates a band of 120 kDa.

then rinsed twice with the extraction/wash buffer and transferred to a gravity column. The protein was eluted with 150 mM imidazole in extraction/wash buffer and 500 μl fractions collected. Aliquots of the fraction were run on an SDS-PAGE gel and blotted with anti-BK antibody. Results are shown in Fig. 2B. The bulk of the *hSlo*-HT protein was found in fraction 2 with a smaller amount in fraction 3. There was no protein detected in fraction 1 or fractions 4–6. Fractions containing *hSlo*-HT were collected and pooled for dialysis.

The final step in the preparation was the reconstitution of the protein into liposomes using the procedure given in Section 2.4. Following separation of the liposomes and supernatant by centrifugation, incorporation of the protein into the liposomes was assessed by Western blot (Fig. 2C). The liposomes showed a strong band at 120 kDa whereas the corresponding band for the supernatant was weak, which indicated that the protein had combined with the lipid to produce proteoliposomes.

A variation of the above purification procedure, normally used to purify functional voltage-gated sodium ion channels, was also successfully used to purify His-tagged BK channels [15]. This method uses a nickel affinity column and Nonidet P-40 detergent, which is then removed by a Gel D column. A silver-stained SDS-PAGE gel of liposomes containing protein prepared in this manner showed a single band at 120 kDa (pellet) but not in the corresponding supernatant, confirming the formation of proteoliposomes (Fig. 3A). Western blot analysis of these samples (Fig. 3B) confirmed that these bands were BK channel protein.

3.3. hSlo-HT channel function in planar bilayer lipid membranes

When liposomes containing reconstituted *hSlo*-HT channels (Fig. 2C) were applied to planar lipid bilayers, channel activity was observed (Fig. 4). Channels were activated using 100 μM calcium added to both chambers and their activity recorded at voltages ranging from −60 mV to +80 mV. At this calcium concentration BK channels have a high open probability and were therefore open most of the time. The BK channel inhibitor paxilline [20] (1 μM) reduced the current response as shown in Fig. 4B and C. In the presence of paxilline the current level was reduced and single channel events were detected. The amplitude of a channel closing event in the presence of paxilline was ∼13 pA at −40 mV (Fig. 4B) giving a single channel conductance estimate of ∼325 pS, which lies in the range of that previously reported for *hSlo* (230–360 pS) [19,21,22]. After 28 min the current response was completely inhibited by paxilline (Fig. 4C), confirming the activity as being due to activation of BK channels. Similar channel activity was also observed upon reconstitution of the alternatively purified protein (Fig. 3) and was inhibited by paxilline (data not shown).

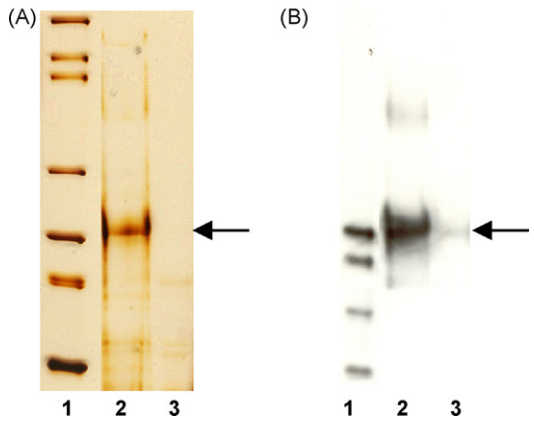

Fig. 3. SDS-PAGE analysis of *hSlo*-HT solubilised using Nonidet P-40, purified using nickel affinity resin, and detergent removed by affinity purification [15]. BK protein following reconstitution into liposomes was detected using (A) Silver-staining, and (B) Western blot using anti-BK_{Ca} channel antibody following ultracentrifugation of proteoliposomes for standard (lane 1), *hSlo*-HT liposome pellet (lane 2), and supernatant (lane 3). The arrow indicates a band of 120 kDa.

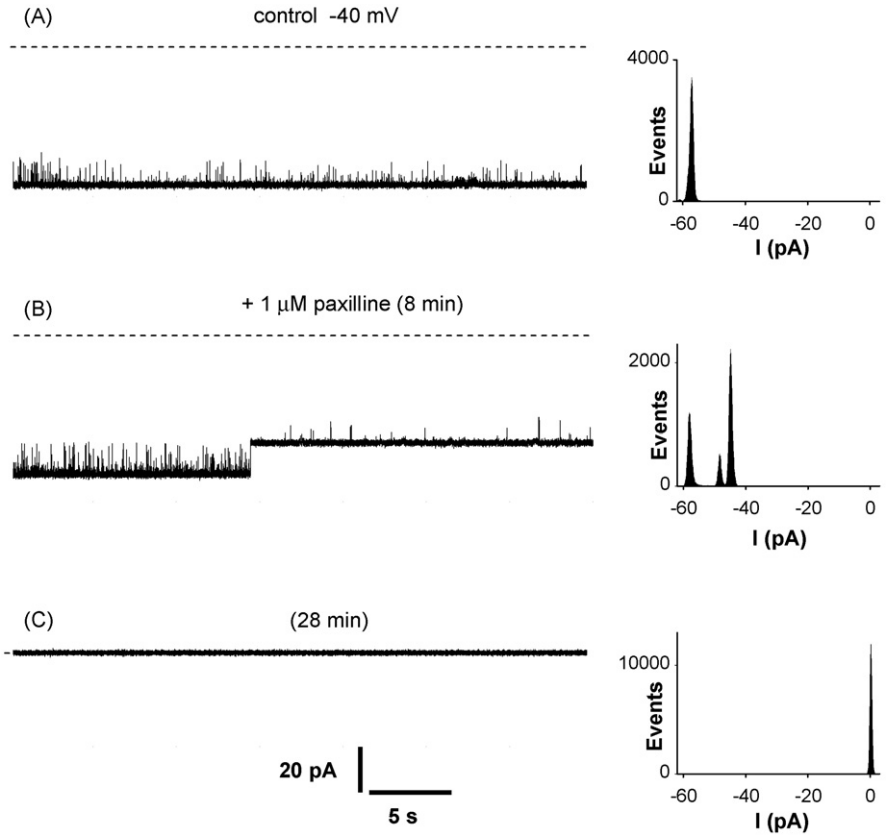

Fig. 4. Current recordings of *hSlo*-HT channel activity following purification and reconstitution into planar bilayer lipid membranes. The data shown in A–C are at an applied potential of −40 mV where both *cis* and *trans* chambers contained 140 mM K^+ for (A) control, (B) 8 min after addition of paxilline and (C) after a further 20 min. The broken line indicates the zero current level with channels opening downward. Data were filtered at 300 Hz. Histograms are from 2 min recordings.

Data on the current–voltage relationship are shown in Fig. 5. The solutions had a 2:1 (*cis:trans*) K^+ concentration gradient across the bilayer to determine whether ion flow through the channel is selective for potassium, and contained 100 μM calcium to activate BK channels. The current reversed at approximately +16.6 mV which is close to the calculated Nernst potential for potassium (+18.4 mV). The recorded K+ current was completely inhibited by 1 μM paxilline, as expected for BK channels.

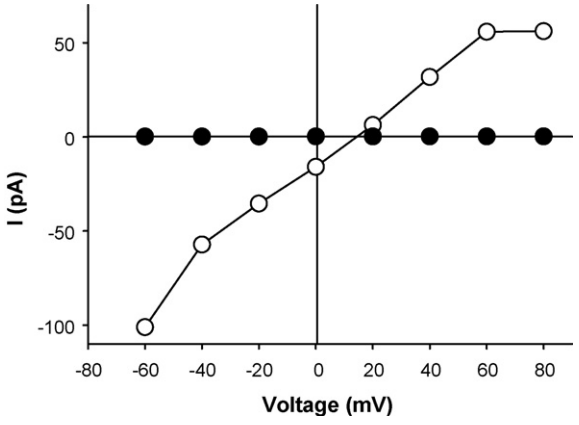

Fig. 5. The current–voltage relationship for *hSlo*-HT channels in pBLMs. The solutions had a 2:1 (*trans*:*cis*) K^+ concentration gradient across the bilayer, and contained 100 μM calcium. The open symbols are for control data; filled symbols are data following the addition of 1 μM paxilline.

4. Conclusion

The procedures outlined in this paper provide a method for expression of His-tagged BK ion channels in insect cells and their purification using IMAC under non-denaturing conditions such that their ion channel activity was retained. Our results show that a His-tag can be fused to the BK channel protein without loss of ion channel activity and therefore the protein can be used without removing the tag. BK channels purified using metal affinity chromatography, are therefore suitable for use in electrophysiology experiments using planar BLMs. As more robust artificial membrane systems are developed for research and to screen for potential pharmaceuticals the ability to upscale purification of ion channels such as BK in a functional form from heterologous expression systems will become increasingly important.

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