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EXPRESSION, PURIFICATION AND FUNCTIONAL RECONSTITUTION OF SLACK SODIUM-ACTIVATED POTASSIUM CHANNELS

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Abstract

The *slack* (*slo2.2*) gene codes for a potassium-channel alpha-subunit of the 6TM voltage-gated channel family. Expression of *slack* results in Na⁺-activated potassium channel activity in various cell types. In this paper we describe the purification and reconstitution of Slack protein, and show that the Slack alpha-subunit alone is sufficient for potassium channel activity activated by sodium ions as assayed in planar bilayer membranes and in membrane vesicles.

First identified by Kameyama et al. (Kameyama et al., 1984) in cardiac myocytes, sodium-activated potassium channels have been found in many cell types. In neurons (Bhattacharjee & Kaczmarek, 2005; Budelli et al., 2009; Yang, Desai & Kaczmarek, 2007) they control bursting and the adaptation of firing rates of action potentials; they may also be involved in protection from ischemia (Ruffin et al., 2008). Na⁺-activated K⁺ channels are found as well in diverse tissues such as kidney (Paulais, Lachheb & Teulon, 2006) and smooth muscle (Kim et al., 2007; Zhang & Paterson, 2007). Sodium-activated K⁺ channels can be formed from Slack (also called Slo2.2) subunits (Joiner et al., 1998; Yuan et al., 2003), and also from the related gene product Slick (Slo2.1) (Bhattacharjee et al., 2003). Slack and Slick are expressed widely and in varying proportions in the central nervous system (Bhattacharjee, Gan & Kaczmarek, 2002; Bhattacharjee et al., 2005). The longer splice variant of Slack, Slack-B (Brown et al., 2008) forms heteromeric channels with distinct properties when co-expressed with Slack (Chen et al., 2009). Slack-A, on the other hand, has a shorter N-terminal sequence and does not co-assemble with Slick subunits.

The structure and function of Slack channels is gradually becoming clear. A site of Na⁺ sensing has been identified in the large intracellular C-terminal region of Slack (Zhang et al., 2010). The C-terminal region forms a “gating ring” whose X-ray structure, solved at low resolution, is quite similar to that of the gating ring of the BK (Slo1) Ca²⁺-activated K⁺ channel. This similarity was sufficient to allow the molecular replacement procedure to be employed in deducing the quaternary structure of the BK gating ring (Yuan et al., 2010). Slack channels are modulated by phosphorylation (Santi et al., 2006) and participate in protein-protein interactions (Uchino et al., 2003) including the RNA-binding protein FMRP and others which depend on the state of activation of the channel (Brown et al., 2010; Fleming & Kaczmarek, 2009). A goal in our laboratory is to study Slack channels and their protein complexes by cryo-EM methods (Cong & Ludtke, 2010; Wang & Sigworth, 2009). We therefore sought to establish a system for expression, purification and reconstitution of Slack protein. Here we describe these methods as well as the results (Yuan et al., 2003) from functional assays of the reconstituted Na⁺-activated K⁺ channels.

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EXPERIMENTAL PROCEDURES

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The Slack-B cDNA sequence (1237 a.a.) has a short alternatively-spliced N-terminal region. A FLAG epitope tag was inserted at the C-terminus through ligation of a construct in the pcDNA3 vector (Joiner et al., 1998). Another construct was made in the pEGFP-C1 vector (Clontech) so that the EGFP sequence was fused to the N-terminal of Slack-B. All the constructs were confirmed by restriction digestion and sequencing

Establishing Slack stable cell lines

Constructs were transfected into HEK293 cells using Superfect (Qiagen) or Lipofectamine2000 (Invitrogen). Cells were cultured in a low-sodium medium containing 500 ml DMEM (Gibco) plus 250 ml Leibovitz's L-15, 15 ml 0.5 M HEPES, and 235ml H₂O, pH 7.3. Stable cell lines were selected by G418 sulfate (Gibco) at 600 µg/ml. Anti-FLAG western blotting was used to confirm the presence of Slack protein in monoclonal stable cell lines, and patch clamp recordings were made from these lines.

Protein purification

Cells were harvested from 10–20 dishes (150 mm diameter, approximately 0.1 g cells per dish) in cell storage buffer (10ml/g cells) containing in mM: 10 Tris, 5 KCl, 1 MgCl₂, 1EGTA, 1:100 diluted protease inhibitor cocktail (PI; Sigma P 8340), and 5 EDTA. All buffers and recording solutions were titrated to pH 7.4. Cells were stored at –80°C, and upon thawing were broken using a Dounce Tissue Grinder on ice. After centrifugation at 2,000 × g for 15minues at 4 °C, the supernatant was collected; then after centrifugation at 141,000 × g for 1 hour at 4 °C the membrane pellet was collected and resuspended in membrane storage buffer (0.5 ml/g cells) containing in mM: 250 sucrose, 5 KCl, 10 Tris, 1:100 diluted PI and 5 EDTA. The membrane preparation was stored at –80°C

Membranes were incubated in solubilization buffer (1.5 ml/g cells) containing 150 KCl, 50 Tris, 5 EDTA, 1:100 PI and either 16mM Cymal-5 (Anatrace) or 10–16 mM dodecylmaltoiside (Calbiochem) for 2 hours at 4°C with rotation. After removing the insoluble material by centrifugation (16,000 × g for 20 minutes at 4 °C) an equal volume of the solubilization buffer but without detergent was added, and subsequently anti-FLAG affinity beads (0.3 ml beads/g cells, Sigma A2220). The mixture was rotated at 4°C for 2 hours and the beads were collected in a 10 ml column, washed 3 times with equal volumes of wash buffer (same as the solubilization buffer but with only 5 mM detergent) and protein was eluted with three 150 µl applications at 20 minute intervals of wash buffer with added 500µg/ml FLAG peptide. A spin column (Bio-Rad, 732-6204) was used to extract the eluate. The final protein concentration was approximately 70 ng/µl as quantified by fluorescence of the GFP-fusion protein and was kept at 4° C and used immediately for assays or reconstitution. For gel electrophoresis, protein was incubated in 8M urea buffer overnight at room temperature and run on 8% polyacrylamide gels.

Slack channel reconstitution

Slack protein was reconstituted into liposomes containing POPC, POPE and POPS at molar ratios of 7:2:1. The final protein to lipid ratio was about 0.3 (should be 0.3, as our concentration was over estimated before using GFP calibration) Slack tetramer per 50,000 lipid molecules for 40 nm liposome.

Mixed lipids, 3.6, 1, and 0.8 mg of POPC, POPE, and POPS, were dried by argon and vortexed with 450 µl of reconstitution buffer (150 KCl, 30 TRIS, 5 EGTA, pH 7.4) for 15 minutes followed by 10 freeze-thaw cycles using liquid nitrogen and a 40°C water bath and

60 seconds sonication. Detergent (Cymal-5 or DM) was added to a 3:1 detergent to lipid ratio, vortexed for 30 minutes and sonicated for 30 seconds, then left on ice for about 3 hours. The final lipid concentration was 12.8 mM.

Slack protein was concentrated 3:1 (Vivaspin 0.5 ml, 100 kDa cutoff). Of the ~200 μ l of protein solution, 100 μ l was mixed with 100 μ l solubilized lipids with rotator at 4°C for 3 hours. The mixture was then loaded into dialysis tubing Spectra/Por Dialysis Membrane, MWCO: 25 kDa, and followed by 36 hr of dialysis at 4 °C in 1L of buffer (450 KCl, 4 N-methyl D-glucamine (NMDG), 20 TRIS, pH 7.4) to form lipid vesicles. A Nycodenz (NYC) discontinuous gradient was used to collect vesicles containing protein. Initial gradient layers were 100 μ l 40% NYC, 200 μ l 30% (containing 100 μ l protein liposomes), and 200 μ l each for 20%, 15%, 3%, and 1% NYC concentration. After 3 hours at $214,000 \times g$ a liposome band was visible at the top of 15% NYC layer and was collected. The presence of Slack protein in vesicles was confirmed by Western blotting with the anti-Flag M2 antibody (Sigma).

Patch clamp and bilayer experiments

Inside-out patch clamp recordings were carried out with the EPC-9 patch clamp amplifier and PULSE acquisition program (HEKA Instruments). The SF-77B step perfusion system (Warner Instruments) controlled by PULSE was used for fast internal solution perfusion. For Na^+ dose-response measurements the pipette solution contained (mM): 160 KCl, 1MgCl₂, 140 NMG Aspartate, 1 EGTA, 10 HEPES; while the bath (internal) solution contained 50 K⁺, 100 Cl⁻, 200 Aspartate⁻, 10 HEPES and variable concentrations of Na^+ and NMDG⁺ summing to 250 mM.

Bilayer experiments were carried out using reconstituted vesicles obtained as above with a Bilayer Recording System (Warner Instruments). Lipids (Avanti, 25mg/ml) POPE: POPG = 18 μ l:6 μ l or POPE: POPC = 16 μ l:4 μ l were dried by argon and washed with an equal volume of pentane. Hexadecane (Sigma) was then added to yield a final lipid concentration of 10 μ g/ μ l. Two different initial solution configurations were used for our experiments in this paper. Cis and trans buffers were 350KCl, 30NaCl, 10MOPS and 100 KCl, 6 HEPES, 0.6 EGTA, or alternatively 100 KCl, 150 NaCl, 5 MOPS and 20 KCl, 30 NaCl, 5 MOPS respectively. After formation of the bilayer, Slack protein vesicles were added to the cis chamber; then after the appearance of channel events the cis solution was exchanged to one with lower osmolality. An EPC-9 patch amplifier and PULSE software were used for recording.

JC-1 fluorescence measurements

The fluorescent dye JC-1 (Molecular Probes) was used for monitoring changes in vesicle membrane potential. Slack protein was purified in the presence of Cymal-5 and reconstituted into liposomes with dialysis against 1000ml of Reconstitution Buffer (150 KCl, 30 TRIS, 5 EGTA, pH 7.4) for 36 h at 4°C with one buffer exchange. Vesicles were collected after centrifugation at $214,000 \times g$ and 10 μ l of vesicles were resuspended in 1 ml buffer containing 3 mM K⁺ (150 NMG-Cl, 10 HEPES, 1 EGTA, 3 KOH, pH 7.4). After taking baseline spectra with a FluoroMax-3 spectrofluorimeter, 3 μ M JC-1 was added to the cuvette. The excitation wavelength was 465 nm, and emission at wavelengths of 530 (JC-1 monomer) and 590 nm (JC-1 aggregate) was recorded. The emission ratio at 590 and 530 nm was used as an indicator of membrane potential.

Cryo-EM imaging

Reconstituted vesicle suspension, 3–4 μ l having an approximate concentration of 1.9 mg/ml lipid, was applied to a home-made holey carbon film (Chester et al., 2007), blotted

manually, and plunge-frozen in liquid ethane. Imaging was performed at liquid N₂ temperature in a Tecnai F20 microscope.

RESULTS

Making stable cells lines expressing Slack-GFP and Slack

Transient transfection of a SlackB construct with an N-terminal GFP fusion into HEK293 cells yielded clear membrane-associated fluorescence (Fig. 1A) as well as Na⁺-activated K⁺ currents as measured in whole-cell and inside-out patch recordings. However, when we attempted to grow these cells in either normal medium or with selection, the cells survived for no more than 3–4 days after transfection. We noticed in whole-cell recordings from transfected cells the activation of K⁺ current by extracellular Na⁺, and reasoned that either an intrinsic external Na⁺ sensitivity or a Na⁺ leak that increases internal Na⁺ is allowing Slack channels to be activated, yielding excessive K⁺ conductance. We modified the growth medium which is based on DMEM to reduce the Na⁺ concentration from 155 to 112 mM, and found that this medium allowed growth even under selection with G-418. After successfully establishing the SlackB-GFP stable cell line, we also made stable cell lines expressing SlackB alone. Data shown in the rest of this paper were obtained from these “native” SlackB channels.

Stabilized Slack on HEK293 cell preserves its Na⁺ gating properties

In a whole-cell recording, rupture of the patch membrane allows Na⁺ to diffuse into the cell, yielding an increase in current as Na⁺ equilibrates in a few seconds (Fig. 1B).

To evaluate the sodium dependency of the expressed channels, we used inside-out patches with a fixed gradient for potassium ([K]_i = 50 mM, [K]_o = 160 mM) and chloride ([Cl]_i, [Cl]_o = 100, 162 mM) having the equilibrium potentials E_K = +30 mV and E_{Cl} = −12 mV. The bath (intracellular) Na⁺ concentration was varied while keeping the sum of Na⁺ and N-methylglucamine (NMG⁺) concentrations equal to 250 mM. Figure 1C shows the currents evoked by voltage ramps as the bath Na⁺ concentration was changed with a rapid perfusion system. The currents increased steeply with Na⁺ concentration but the reversal potentials remained very close to E_K, regardless of the Na⁺ concentration.

Lithium opens Slack channels, but is about 6 times less potent as sodium

Zhang et al. (Zhang et al., 2010) have shown that, unlike larger ions, Li⁺ is able to activate Slack channels expressed in *Xenopus* oocytes, although at lower potency. We obtained dose-response relationships with internal potassium, lithium, cesium, and ammonium ions in inside-out patches from our cells. Responses at −80 mV are plotted in Fig. 2A, with the responses from each patch normalized to the response of the same patch to 250 mM internal Na⁺. At a concentration of 1M, cesium and ammonium produced less than 0.6% of maximal activation. 1M Li⁺ was capable of inducing a nearly saturating current, but its affinity was about 6 times lower than that of Na⁺. The sodium dose-response was fitted with K_{1/2} = 54 mM with the Hill slope $n_H = 2.4$. When the Li⁺ data were fitted with the constraint $n_H = 2.4$, the estimated K_{1/2} was 294 mM. Fig. 2B shows the nearly identical currents evoked by a voltage ramp in the presence of 50mM Na⁺ or 250 mM Li⁺ in the internal solution.

Purification and reconstitution

The expression of Slack protein is only a few micrograms per 150 mm dish of cells; however for some purposes such as single-particle cryo-EM studies quantities of ~10 μg protein is sufficient. The membrane fraction isolated from cells was solubilized in dodecylmaltoside or Cymal-5. Purification made use of the C-terminal FLAG tag on the

expressed protein, making use of an anti-FLAG affinity column and elution with FLAG peptide. The resulting protein runs at the expected size of 136 kDa on an SDS-PAGE gel (Fig. 3A).

The protein was reconstituted into membranes by first mixing with detergent-solubilized lipids and then removing the detergent by dialysis. The vesicle fraction was enriched by density-gradient centrifugation, and cryo-EM revealed that the resulting vesicles were unilamellar and 25–50 nm in size (Fig. 3B). Successful reconstitution of the protein was assayed by re-solubilizing the vesicles and running an SDS-PAGE gel. Western blotting with anti FLAG showed the recovery of the 136 kDa protein band (Fig. 3C).

Assay for functional channels after reconstitution

Reconstituted vesicles were fused with planar bilayers for single-channel recordings. No channel currents were seen in the absence of Na^+ on the cis side (from which the vesicles were added), but channel activity is reversibly seen when a solution containing 100 mM Na^+ is perfused (Fig. 4A). Under the recording conditions (100 mM K^+ on the trans side) the single channel conductance was 270 pS.

Fig. 4C shows another bilayer recording with 100 mM K^+ in the cis solution and 20 mM K^+ trans. The recording from a voltage ramp (bottom trace) shows a reversal potential of -45mV , while the theoretical E_K was -42mV . The single channel conductance was 244 pS under these conditions.

Bilayer experiments demonstrate the presence of individual Na^+ -activated channels but do not assay the population of reconstituted channels. We used the fluorescent dye JC-1 to measure Na^+ -activated membrane potential changes driven by a K^+ diffusion potential. JC1 in its monomeric form emits at 530 nm, but at high concentration it forms aggregates with a red-shifted fluorescence at 590 nm. As JC-1 is positively charged and membrane permeable, it serves as a “slow” voltage indicator. Based on quantitative fluorometry of the Slack-GFP fusion construct we were able to define a protein-to-lipid ratio in the reconstitution process of approximately one Slack tetramer to 50,000 lipid molecules. This ratio corresponds to approximately 0.3 tetrameric channels per 40 nm lipid vesicle, typical for the vesicle sizes we observed (Fig. 3B). Vesicles containing 160 mM K^+ were diluted into a solution with 3 mM K^+ . Addition of 30 mM NaCl to the outside solution resulted in a 6% increase in the ratio of intensity at 590 nm relative to 530 nm (Fig. 4D). This change reflects the establishment of a negative membrane potential in a fraction of vesicles. Subsequent addition of 5 μM valinomycin, which is expected to produce a K^+ diffusion potential in every vesicle, yielded a peak increase of 36% in the ratio. These results show that a substantial fraction of vesicles, roughly 16%, have a K^+ permeability that is activated by external Na^+ , presumably from inside-out Slack channels.

If we assume that channel reconstitution has no bias for orientation and assume a reconstituted density of roughly 0.3 tetrameric channels per vesicle, then the total yield of inside-out channels should be about 15%, consistent with the result from the JC-1 fluorescence measurement and implies a high specific activity of reconstituted channels.

DISCUSSION

The Slack sodium-activated potassium channel is a member of the six-transmembrane-segment ion channel superfamily which also includes the voltage-gated channels. Voltage-gating of Slack channels is very weak (Yuan et al., 2003), as seen in Fig. 2, which would be expected from the absence of charged residues in the primary voltage-sensing S4 helix (Joiner et al., 1998). However, Slack activity is very sensitive to Na^+ concentrations in the

range of tens of millimolar. This sensitivity is appropriate for a channel that responds to action-potential induced fluxes in neurons and muscle cells as well as providing Na⁺-activated fluxes in other cells. In this paper we describe the establishment of a stable cell line expressing Slack, and show that the Slack protein can be purified on an antibody-affinity column. The purified protein can be reconstituted to form Na⁺-activated and K⁺ selective channels as detected in a planar lipid bilayer assay. Also, using a flux assay based on a potential-sensitive dye, we found that the fraction of vesicle-enclosed volume that participated in Na⁺-activated K⁺ flux was essentially equal to number of vesicles containing outside-out Slack channels, assuming random orientation. Thus the size of the flux signal was consistent with a high specific activity of the channel protein.

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Figure 1. HEK 293 stable cell lines express functional Slack protein

(A) Fluorescence of HEK cells expressing the N-terminal EGFP-Slack fusion construct. (B-1) Whole cell recording made from a cell expressing wt-Slack with 30 mM Na⁺ in the pipette (pipette solution, in mM: 30 NaCl, 100 KCl, 5 EGTA, 10 HEPES; bath solution: 160 KCl, 1 EGTA, 10 HEPES, 1 MgCl₂). Shown are 22 current traces evoked by voltage ramps from -100 to +120 mV recorded immediately after establishing the whole-cell recording configuration; currents increased as Na⁺ diffused from the pipette into the cell. (B-2) Time course of the development of inward currents from the cell shown in B-1, measured at -60 mV. The time constant is about 6s. (C) An inside-out patch was perfused with various Na⁺ concentrations (0, 5, 10, 50, 100, 250 mM) while keeping the potassium and chloride gradients constant. The calculated equilibrium potential E_K was +30mV and E_{Cl} was -12 mV, while E_{Na} varied from 0 (at 5 mM internal Na⁺) to -100 mV (at 250 mM internal Na⁺). The measured reversal potential of the currents was near +30 mV, indicating that K⁺ conductance was predominant. Pipette solution, in mM: 160 KCl, 1 MgCl₂, 140 NMG-Aspartate, 1 EGTA, 10 HEPES; perfused solution (cytoplasmic side): 50 K⁺, 100 Cl⁻, 200 aspartate⁻, and [Na⁺]+[NMG⁺]=250).

Figure 2. Dose-response of ion activation of Slack channels

(A) Inward currents activated by internal Na⁺ and Li⁺ were measured from an inside-out patch at -80 mV while keeping potassium and chloride gradient unchanged. K_d for Na⁺ was about 54 mM and the Hill coefficient $n_H = 2.4$ (pipette: 160 KCl, 1 MgCl₂, 140 NMG-Aspartate, 1 EGTA, 10 HEPES. Perfused (internal) solution: 50K⁺, 100Cl⁻, 200 aspartate⁻, and Na⁺+NMG⁺ = 250). Saturation was not attained in the lithium dose response but the dashed curve shows the same function as fitted for Na⁺, but with $K_d=295$ mM. Cs⁺, NH₄⁺ and K⁺ did not measurably open Slack channels at concentrations up to 1 M. (B) Very similar current traces are evoked by 50 mM Na⁺ (solid) or 250 mM Li⁺ (dotted curve). A voltage ramp from -100 to +100 mV was applied to an inside-out patch with intracellular solutions containing 50 mM K⁺, 100 Cl⁻, 200 aspartate⁻, and either 200 NMG⁺ + 50Na⁺ or 250 Li⁺ respectively. The pipette contained 160 mM K⁺ (160 KCl, 1 MgCl₂, 140 NMG-Aspartate, 1 EGTA, and 10 HEPES).

Figure 3. Slack protein purification and liposome reconstitution

(A) Coomassie-stained protein gel made with 8% polyacrylamide. Lane 1: cell lysate. Lane 2: affinity purified Slack protein. Lane 3: protein molecular weight markers. The purification yield was about 2 μ g protein per 150 mm dish from 0.1 g cells. The ~70kDa contaminant was not always present and appears to come from the antibody beads. (B) Examination of the reconstituted preparation by cryo-EM shows overwhelmingly unilamellar vesicles. The image shows the edge of a hole in the carbon film. The image was recorded with 2 μ m underfocus at 200 keV. (C) Western blot probed with anti-FLAG antibody. Lane 1: the Slack protein (monomer 136 kDa) as solubilized with Cymal-5 and purified; lane 2: protein extracted from liposomes after reconstitution. A 70 kDa band is again present in lane 1. It appears in control western blots and appears to come from the antibody beads; note that it is absent from lane 2.

Figure 4. Reconstituted Slack forms Na⁺-activated K⁺ channels

(A) Slack-containing vesicles formed from Cymal-5 solution were fused with a POPE:POPC = 4:1 bilayer membrane. Each sweep shows a recording at -40 mV followed by a step to -50 mV. After vesicles were added to the cis side and channel events were first observed, the hypertonic solution on the cis side (1 ml volume) was replaced by perfusion of 10 ml K-buffer: 160 KCl, 10 HEPES, 1 EGTA (top trace), followed 10 ml Na-buffer, 100 mM NaCl, 10 HEPES, 1 EGTA (middle trace) and then another 10 ml K-buffer (bottom trace). Solution on the trans side was 100 KCl, 6 HEPES and 0.6 EGTA. (B) The single channel conductance was calculated to be 270 pS from a linear fit of unitary current amplitudes from the experiment in A, with a reversal potential of +10 mV. This reversal potential differs from the theoretical E_K consistent with the possibility that the 160 K⁺ on the cis side was not completely replaced by perfusion. (C) Recording from a POPE:POPG = 3:1 bilayer in which the ion concentrations on both sides were fixed. Cis side: 100 KCl, 150 NaCl, 5 MOPS; trans side: 20 KCl, 30 NaCl, 5 MOPS. Vesicles were formed from the same lipid mixture using DM. Recordings at +20 and +60 mV show two active channels (openings are upward). Unitary current values (black dots) are plotted along with the current from a voltage ramp. The fitted line corresponds to a single-channel conductance of 244 pS and reversal potential -45 mV; the dashed line and blue dots indicate twice the conductance when two channels are active. The theoretical E_K was -42 mV under these conditions. (D) Flux assay for potassium transport. Reconstituted vesicles loaded with 150 mM K⁺ were diluted into a solution containing 3 K⁺ and 150 NMG⁺. The addition of 30 mM Na⁺ evoked a 5% increase in the ratio of fluorescence at 595 and 540 nm. Subsequent addition 5 μM valinomycin caused a further 36% increase in the ratio, consistent with about 15% of the liposomes containing channels that were activated by external Na⁺.