Published in final edited form as:

FEBS Lett. 2009 October 6; 583(19): 3109–3114. doi:10.1016/j.febslet.2009.08.039.

The $\beta 1$ -subunit of Na^+/K^+ -ATPase interacts with BK_{Ca} channels and affects their steady-state expression on the cell surface

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Abstract

Large conductance Ca^{2+} -activated K^+ channels (BK_{Ca}) encoded by the *Slo1* gene play a role in the physiological regulation of many cell types. Here, we show that the $\beta 1$ subunit of Na^+/K^+ -ATPase ($\text{NK}\beta 1$) interacts with the cytoplasmic COOH-terminal region of *Slo1* proteins. Reduced expression of endogenous $\text{NK}\beta 1$ markedly inhibits evoked BK_{Ca} currents with no apparent effect on their gating. In addition, $\text{NK}\beta 1$ down-regulated cells show decreased density of *Slo1* subunits on the cell surface.

Keywords

potassium channel; trafficking; ion transporter

Introduction

Large conductance Ca^{2+} -activated K^+ channels (BK_{Ca} channels) regulate physiology in a wide range of tissues [1]. BK_{Ca} channels can be preferentially targeted to different portions of polarized cells. For example, in hippocampal neurons, BK_{Ca} channels extensively localize to axons and presynaptic terminals [2-3] whereas in cerebellar Purkinje cells, they are observed primarily in the somato-dendritic region [3]. Similarly, in epithelial cells, BK_{Ca} channels can be preferentially expressed on either the apical or basolateral surface [4-5]. The pore-forming subunits of BK_{Ca} channels are encoded by the *Slo1* gene, also known as *KCNMA1*. Alternative splicing and protein-protein interactions at the COOH-terminal tail region contribute significantly to regulation of BK_{Ca} channels [8-26].

The Na^+/K^+ -ATPase is a ubiquitously expressed member of the P-type ATPase superfamily. The functional enzyme is a heterodimer comprised of a catalytic α -subunit as well as one of at least three highly conserved glycoprotein β -subunits [27-29]. Stoichiometrically unequal amounts of α - and β - Na^+/K^+ -ATPase subunits are present in some tissues [30-32], and the two classes of subunits are subjected to different modes of degradation [33]. This raises the possibility that some Na^+/K^+ -ATPase β -subunits may be free to interact with other proteins. More recent studies suggest that these subunits contribute to cell adhesion and formation of tight junctions [34-35].

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We now report that Slo1 proteins biochemically interact with NK β 1 and that this interaction plays a role in regulating the steady-state expression of BK_{Ca} channels on the cell surface.

Methods

Plasmids and antibodies

An expression plasmid encoding NH₂-terminal (ectofacial) Myc-tagged mouse Slo1 (VEDEC isoform) was provided by Dr. Min Li (Johns Hopkins University, Baltimore, MD). Other plasmids, including pGBKT7-Slo1G785-A985 and different pGEXKG-Slo1 constructs were created using PCR and confirmed by sequencing. Antibodies used in this work include: anti-Myc (9B11, Cell Signaling Technology, Inc); anti-Myc (06-549, Upstate Biotechnology, Inc); anti-NK β 1 (ab33144, Abcam Inc. Cambridge, MA), anti- α -Na⁺/K⁺-ATPase (anti-NK α 1) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and anti-Slo1 (APC-107, Alomone Laboratories, Jerusalem, Israel).

Yeast two-hybrid screen

Yeast two-hybrid screens of an embryonic day 9 (E9) chick ciliary ganglion (CG) cDNA library were carried out using the Matchmaker™ system (BD Biosciences, San Jose, CA) according to the manufacturer's instructions, as described in detail previously [10,24]. The bait construct was comprised of amino acids G785-A985 of mouse Slo1. NK β 1 emerged repeatedly in this screen.

Co-immunoprecipitation, cell surface biotinylation, and GST pull-down assays

CG were excised from E9 chick embryos and lysed in PBST (phosphate-buffered saline with 1% Triton) containing protease inhibitors (P2714, Sigma-Aldrich, Inc.). The extracts were centrifuged briefly, and the supernatants were incubated with anti-Slo1 or anti-NK β 1, and precipitates were isolated using Protein A/G PLUS-Agarose beads (sc-2003, Santa Cruz Biotechnology, Inc.). Beads were washed in PBST, SDS-sample buffer was added and samples were boiled for 5 min and then loaded onto gels. SDS-PAGE separation, immunoblot analysis, cell-surface biotinylation assays, and GST pull-down assays were performed as described previously [10,19,24].

Cell culture and transfection

HEK293T cells were grown and transiently co-transfected with plasmids encoding Myc-Slo1 and si-NK β 1 (sc-36008, Santa Cruz Biotechnology, Inc.) using Lipofectamine 2000™ (Invitrogen) as described previously [10,19,24]. Control cells were co-transfected with Myc-Slo1 and non-specific siRNA (sc-37007, Santa Cruz Biotechnology, Inc.). Cells were used for physiology or biochemistry 48 hours after transfection. Neurons from E9 chick CG were dissociated and cultured as described previously [36-37].

Confocal microscopy

E9 CG neurons were maintained in culture for 3 hours before fixation in 4% paraformaldehyde for 10 min. Preparations were rinsed and incubated with rabbit anti-Slo1 (1:500 dilution) and mouse anti-NK β 1 (1:500 dilution) overnight at 4°C. This was followed by incubation with secondary antibodies and images were collected as described previously [10].

Electrophysiology and statistics

Inside-out patch recordings were made at room temperature (22° C) from HEK293T cells transiently co-expressing si-NK β 1 or non-specific siRNA, along with Myc-Slo1 and green fluorescent protein (GFP), which was used to allow identification of transfected cells during recording. Protocols for recording and analysis were described previously [10,24]. Student's

unpaired *t*-test was used to compare experimental groups to a single control group with $\alpha = 0.05$.

Results

We conducted a yeast two-hybrid screen of a chick CG cDNA library using as bait a conserved cytoplasmic domain (G785-A985) of mouse Slo1 (Fig. 1A). Based on the sequences of cDNAs that we isolated in these screens, the portion of NK β 1 that interacts with Slo1 channels includes residues between 228 to 299 in the extracellular COOH-terminal region of NK β 1 (Fig. 1B).

This interaction was also detected by co-immunoprecipitation from E9 chick CG extracts. NK β 1 could be detected in immunoprecipitates prepared using antibodies against Slo1 channels (Fig. 1C, left). Conversely, Slo1 could be detected when immunoprecipitation was carried out using anti-NK β 1 (Fig. 1C, right), whereas neither protein could be detected when immunoprecipitation was carried out with IgG. The Slo1-NK β 1 interaction was also seen using GST pull-down assays. Thus, GST-Slo1(G785-A985) could precipitate NK β 1 from CG extracts, whereas GST alone was ineffective (Fig. 2A). Additional GST fusion proteins, including GST-Slo1(L985-Q1108) were also able to precipitate NK β 1 from CG extracts (Fig. 2B). This construct comprises the most distal COOH-terminal portion of Slo1. It contains a caveolin-binding motif [15], and sorting motifs required for the apical expression of BK $_{Ca}$ channels in certain epithelial cells [38]. In addition, we made four smaller GST fusion constructs of the bait region used in the initial screens. We detected NK β 1 interactions with GST-Slo1(T884-N936) and GST-Slo1(I937-A985). However, GST-Slo1(G785-L843) and GST-Slo1(Q844-I883) were unable to pull NK β 1 out of CG lysates (Fig. 2C). We carried out a similar set of analyses in HEK293T cells and obtained the same interaction pattern (data not shown).

We also observed partial co-localization of NK β 1 and Slo1 by confocal microscopy in primary cultures of CG neurons (Fig. 3). Signals from anti-Slo1 and anti-NK β 1 show extensive overlap, especially in intracellular compartments of somatic areas. However, Slo1 signal in neurites does not appear to co-localize with NK β 1. Limits of resolution make it difficult to definitively observe co-localization at the cell surface.

In order to examine the functional significance of the Slo1-NK β 1 interaction, we used an siRNA targeting NK β 1 transcripts (si-NK β 1) to reduce the endogenous expression of NK β 1 protein in HEK293T cells (Fig. 4). Treatment with si-NK β 1 had no effect on the expression of Na $^{+}$ /K $^{+}$ -ATPase α -subunits (NK α) compared to controls (Fig. 4A). To determine the functional implications of Slo1-NK β 1 interactions, inside-out patch recordings were made from HEK293T cells transiently co-transfected with si-NK β 1 or non-specific siRNA together with Myc-tagged Slo1. Large BK $_{Ca}$ currents were observed in patches excised from Slo1-expressing cells treated with non-specific siRNA. However, we observed significantly reduced currents at all membrane potentials in cells treated with si-NK β 1 (Fig. 5A, B). There was no difference in the voltage-dependence of BK $_{Ca}$ activation (Fig. 5D) or activation or deactivation kinetics due to NK β 1 siRNA treatment (Fig. 5C). We also examined the effects of si-NK β 1 or non-specific siRNAs on the distribution of Myc-Slo1 protein using a cell-surface biotinylation assay. These analyses revealed a reduced surface expression of Slo1 in si-NK β 1-transfected cells in comparison to control cells (Fig. 6A, top). There was no change in the protein expression levels of Slo1 as revealed by immunoblot analyses of the whole cell lysates (Fig. 6A, bottom). Quantitative analysis of the cell surface assays showed a significant reduction in the signal derived from the cell surface in NK β 1 down-regulated cells (Fig. 6B). Probing the same blots with anti-NK β 1 antibody showed a corresponding reduction in the cell surface expression of NK β 1.

Discussion

The functional expression of BK_{Ca} channels on the cell membrane is a highly regulated process in many tissues. Here we show that NKβ1 is an interaction partner of Slo1. The region of NKβ1 that interacts with Slo1 is highly conserved in all known Na⁺/K⁺-ATPase and H⁺/K⁺-ATPase β-subunits [27]. Down-regulation of NKβ1 expression caused a marked reduction in the surface expression of Slo1 in HEK293T cells. The effects of NKβ1 on the cell surface expression of BK_{Ca} channels may be related to other physiological effects recently ascribed to NKβ1. For example, NKβ1 plays a major role in the localization of tight junction-associated proteins in certain cell types and in the development of cell polarity [43–45]. In morphologically polarized cells such as neurons and epithelial cells, the interaction between Slo1 and NKβ1 may be responsible for targeting of BK_{Ca} channels to specific regions on the plasma membrane. There is evidence that formation of tight junctions in epithelia is sensitive to ionic conditions, including localized increases in intracellular Na⁺ concentration that lead to alterations in the subjacent cytoskeleton [44]. It is possible that co-localization of Slo1 channels with Na⁺/K⁺-ATPases contributes to local ionic homeostasis and maintenance of tight-junction integrity. Moreover, in cardiac myocytes and other tissues, NKβ1 regulates the concentration of Na⁺/K⁺-ATPases in caveolae [46] where these enzymes are pre-assembled with their partners [47]. Since NKβ1 is a ubiquitous protein, its ability to target proteins to caveolae may be a generalized mechanism that affects additional proteins, including BK_{Ca} channels [15–16].

Is NKβ1 an independent mediator of physiological regulation inside cells? Unequal amounts of α- and β-ATPase proteins are observed in many tissues [48]. In addition, mature β-subunits can be recovered independent of α-subunits, at least in the case of H⁺/K⁺-ATPases [29]. We have observed that Slo1 and NKα subunits interact with different regions on NKβ1 (see [27] for NKα binding sites), and it is possible that NKβ1 can interact with Slo1 and NKα simultaneously. We cannot exclude that the interaction between NKβ1 and BK_{Ca} channels may be facilitated by the presence of NKα subunits, or that the Slo1-NKβ1 interaction occurs within a larger complex formed between Na⁺/K⁺-ATPase, BK_{Ca} channels, and other proteins.

The possible existence of Slo1-NKβ1 interaction within a larger complex is intriguing in light of growing evidence that Na⁺/K⁺-ATPases can function as transducers for signaling pathways [49–50]. A number of studies point to the functional coupling between Na⁺/K⁺-ATPases and other ion channels [51–52] possibly including L-type Ca²⁺ channels [53]. Therefore, Na⁺/K⁺-ATPases may co-exist with a Ca_v-BK_{Ca} complex at the plasma membrane that plays a role in regulation intracellular Ca²⁺ levels. NKβ1 subunits may be important for formation or stabilization of such complexes.

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Abbreviations

CG	Ciliary ganglion
RCK	regulators of potassium conductance
NKβ1	$\text{Na}^{+}\text{-K}^{+}$ -ATPase β 1 subunit
NKα	$\text{Na}^{+}\text{-K}^{+}$ -ATPase α -subunit
BK$_{\text{Ca}}$	large-conductance Ca^{2+} -activated K^{+} channels

Fig. 1.

NK β 1 interacts with neuronal Slo1 channels. A, B Schematic representations of Slo1 and NK β 1 respectively. The bait fragment used in the yeast two-hybrid screen is highlighted in blue. C, Co-immunoprecipitation of NK β 1 and Slo1 from chick CG extracts. Initial immunoprecipitation was carried out using an antibody against Slo1 (left) or NK β 1 (right).

Fig. 2.

Interaction between NKβ1 and Slo1 occurs at multiple regions. A, GST pull-down assay carried out on chick CG lysates showing that a fusion protein containing the bait, GST-Slo1(G785-A985) binds to NKβ1. Additional fusion proteins from regions downstream and adjacent to the bait, GST-Slo1(L985-Q1108) also bind to NKβ1. B, Immunoblots of GST pull-down assays using smaller GST-fusion proteins from the bait region.

Fig. 3.

Co-localization pattern of NKβ1 and Slo1. Top panel shows co-localization of NKβ1 (red) and Slo1 (green) in E9 chick CG neurons. The regions enclosed by white squares are magnified in the bottom panels to show neurites and cell bodies.

Fig. 4. Reduction of endogenous NK β 1 protein expression by si-RNA in HEK293T cells. A, Representative immunoblots showing expression of NK β 1 and NK α in HEK293T cells transfected with either non-specific si-RNA or si-NK β 1. B, Densitometric analysis of four repetitions of this experiment (mean $\pm$ S.E.M.).

Fig. 5.

Inside-out patch recordings made from HEK293T cells expressing Slo1. A, Typical families of currents evoked by voltage steps (-80 mV to +80 mV) in inside-out patches from transfected HEK293T cells. The bath solution contained 20 μM Ca²⁺. No currents were detected in Ca²⁺-free bath solutions (data not shown). Note reduced current amplitudes in cell that received si-NKβ1 compared to cell treated with control siRNA. B, Quantitative analysis of peak currents obtained by a step to +80 mV shows significant reduction in mean current amplitude ($n = 8$) in si-NKβ1 treated cells. C, Currents evoked by a step to +80 mV in 20 μM Ca²⁺ normalized and superimposed to facilitate comparisons of activation and deactivation kinetics in a control cell (red trace) and a si-NKβ1 expressing cell (black trace). D, Normalized voltage activation curves

of BK_{Ca} currents recorded at 20 μ M Ca²⁺. Data points (mean $\pm$ S.E.M.) are shown with superimposed fitted Boltzmann curves. Eight cells were analyzed from each group. Treatment with siNK β 1 did not affect the voltage-dependence of BK_{Ca} activation.

Fig. 6. NKβ1 down-regulation reduces the cell surface expression of Slo1 channels in HEK293T cells. A, Cell-surface biotinylation assay showing cell surface and total expression of Myc-Slo1 in HEK293T cell transiently cotransfected with si-NKβ1 or non-specific siRNA. Top panel shows that si-NKβ1 cause a reduction in surface expression of Slo1 and total NKβ1 but no effect on total Slo1 expression. B, The bar graph shows relative surface expression (mean ± S.E.M.) of Slo1 calculated from blots of three separate cell surface biotinylation assays.