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Alternatively spliced C-terminal domains regulate the surface expression of large conductance calcium-activated potassium (BK_{Ca}) channels

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Abstract

The *Slo1* gene, also known as *KCNMA1*, encodes the pore-forming subunits of large-conductance Ca²⁺-activated K⁺ (BK_{Ca}) channels. Products of this gene are widely expressed in vertebrate tissues, and occur in a large number (≥ 20) of alternatively spliced variants that vary in their gating properties, susceptibility to modulation, and trafficking to the plasma membrane. Motifs in the large cytoplasmic C-terminal are especially important in determining the functional properties of BK_{Ca} channels. Here we report that chick ciliary ganglion neurons express transcripts and proteins of two Slo1 splice variants that differ at the extreme C-terminal. We refer to these variants as VEDEC and QEDRL (or QEERL for the orthologous mammalian versions), after the five terminal amino acid residues in each isoform. Individual ciliary ganglion neurons preferentially express these variants in different subcellular compartments. Moreover, QEERL channels show markedly higher levels of constitutive expression on the plasma membrane than VEDEC channels in HEK293T and NG108-15 cells. However, growth factor treatment can stimulate surface expression of VEDEC channels to levels comparable to those seen with QEERL. In addition, we show that co-expression of a soluble protein comprised of VEDEC C-terminal tail residues markedly increases cell surface expression of full-length VEDEC channels, suggesting that this region binds to proteins that cause retention of the these channels in intracellular stores.

Keywords

Slo1; traffic; ciliary ganglion; PDZ; neuregulin

The *Slo1* gene, also known as *KCNMA1*, encodes the principal pore-forming α -subunits of large-conductance Ca²⁺-activated K⁺ (BK_{Ca}) channels. This gene is expressed in a large number (≥ 20) of variants associated with alternative splicing at ten or more different sites (Meera et al, 2001; Shipston, 2001). Most of the splice sites are located in the large cytoplasmic domain in the C-terminal half of the channel molecule, which is also the region that contains multiple Ca²⁺ binding sites (Xia et al., 2002; Zeng et al., 2005; Krishnamoorthy et al., 2005). Alternative splicing in these regions yields variants with significant differences in BK_{Ca} gating properties and kinetics, especially with respect to the voltage- and Ca²⁺-dependence of gating (e.g. Adelman et al., 1992; Saito et al., 1997; Chen et al., 2005). Spatial gradients in the expression of different BK_{Ca} isoforms have been implicated in the initial steps of auditory

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processing (Fettiplace and Fuchs, 1999), and splicing can markedly alter the sensitivity BK_{Ca} to modulation by phosphorylation (Tian et al., 2001) and other processes (Erxleben et al., 2002). Moreover, in certain peripheral tissues the relative expression of BK_{Ca} splice variants can be regulated by endocrine status (Xie and McCobb, 1998;Lai and McCobb, 2002,2006;Holdiman et al., 2002).

In addition to determining channel gating properties, alternative splicing also affects the way in which the surface expression of BK_{Ca} channels is regulated. For example, one relatively rare BK_{Ca} variant contains a 33-residue insert close to the membrane-spanning S1 domain that includes a hydrophobic retention/retrieval motif with the sequence CVLF, (Zarei et al. 2001, 2004). Channels containing this motif are retained in the endoplasmic reticulum (ER), and indeed, can cause other co-expressed splice variants of Slo1 to remain in intracellular compartments, possibly by formation of heteromeric complexes. Most BK_{Ca} channels contain a C-terminal ER export signal (DLIFCL), and a nearly adjacent sorting motif (NAGQSRA) that is required for selective expression of Slo1 on the apical surface of polarized epithelial cells (Wang et al., 2003;Kwon and Guggino, 2004). The importance of C-terminal motifs for trafficking of BK_{Ca} channels is underscored by the fact that sequence variations, mutations, and naturally occurring truncations in this region can prevent channel expression on the cell surface (Bravo-Zehnder et al., 2000;Wang et al., 2003;Chen et al., 2005). Moreover, the C-terminal of Slo1 represents a rich area for protein-protein interactions, including interactions with proteins that potentially regulate their trafficking (Park et al., 2004;Brainard et al., 2005), modulation (Alioua et al., 2002;Ling et al 2004;Tian et al., 2006;Ma et al., 2007), and degradation (Jo et al., 2005).

We have recently presented evidence indicating that the trafficking of Slo1 channels to the cell surface is regulated by multiple neural growth factors in developing ciliary neurons of the chick ciliary ganglion (Lhuillier and Dryer, 2002;Chae et al., 2005a,b). Specifically, we have observed that functional BK_{Ca} channels are expressed at a detectable but low level on the surface of ciliary ganglion neurons prior to the developmental stages at which they form synapses with target tissues in the eye (Dourado and Dryer, 1992). Interactions between ciliary neurons and their target tissues, as well as with afferent preganglionic inputs, allows for access to two different growth factors, transforming growth factor- β 1 (TGF β 1) and β -neuregulin-1 (NRG1), respectively. These growth factors synergistically stimulate trafficking of BK_{Ca} channels to the plasma membrane of ciliary neurons, a phenomenon that is observed both in vivo and in vitro (Cameron et al., 1998,2001;Chae et al., 2005 a,b).

In the present study, we report that chick ciliary ganglion neurons express transcripts and proteins of two Slo1 variants that differ at the extreme C-terminal. We refer to these variants as VEDEC and QEERL (or QEDRL for the avian version), after the five terminal amino acid residues in each isoform. Both isoforms contain the ER export and sorting motifs mentioned above (Kwon and Guggino, 2004), but neither contain the CVLF motif in the S0 domain or anywhere else in the molecule (Zarei et al., 2004). Individual ciliary ganglion neurons express both isoforms, but the two variants appear to be preferentially expressed in different subcellular compartments. More importantly, we show in heterologous expression systems that QEERL channels are constitutively expressed at higher steady-state levels in the plasma membrane than VEDEC channels. However, growth factor treatment can stimulate surface expression of VEDEC channels to levels comparable to those of QEERL. In addition, we show that co-expression of a soluble protein comprised of VEDEC C-terminal tail residues markedly increases cell surface expression of full-length VEDEC channels, suggesting that this region binds to proteins that cause retention of the VEDEC channels in intracellular stores.

Experimental procedures

Cell culture and transfection

Embryonic day 9 (E9) or E13 ciliary ganglion neurons (from eggs obtained from Spafas, Peoria, IL, USA) were dissociated and plated onto poly-D-lysine-coated coverslips or dishes as described previously (Cameron et al. 1998; Lhuillier and Dryer 2000, 2002). HEK293T (human embryonic kidney) and NG108-15 (neuroblastoma × glioma hybrid) cells were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10 % heat-inactivated fetal bovine serum (FBS) at 37 °C in a 5 % CO₂ incubator. HAT media supplement (Sigma) was added to NG108-15 cells. Cells were transiently transfected in 10 cm dishes (for biochemistry) or 24-well plates (for electrophysiology) using Lipofectamine-2000TM (Invitrogen) in serum-reduced medium (Opti-MEM, Invitrogen) following the manufacturer's instructions. The DNA concentration in the transfection medium was 1 µg/ml of each plasmid. Cells were used for electrophysiological, immunochemical or biochemical studies 24–48 h after transfection. In experiments involving growth factors, a recombinant form of human NRG1 comprised of amino acids 177–246 of the EGF domain of β-isoforms NRG1 (R and D Systems, Minneapolis, MN) was added (1 nM) 1 hr prior to assays of surface expression.

Plasmid constructs and RT-PCR

Expression plasmids encoding N-terminal (ectofacial) myc-tagged VEDEC and QEERL isoforms of Slo1 were kindly provided by Dr. Min Li of the Department of Neuroscience at Johns Hopkins University. Constructs encoding a green fluorescent protein (GFP)-VEDEC C-terminal (1129–1170) fusion protein (V-tail) and a GFP-QEERL C-terminal (1096–1118) fusion protein (Q-tail) were generated by subcloning PCR products into pcDNA3.1/NT-GFP-TOPO vector (Invitrogen). The fidelity of all constructs was confirmed by sequencing. For RT-PCR, total mRNA was isolated from embryonic day 9 chick ciliary ganglion neurons using the RNeasy kit (Qiagen, Valencia, CA). Reverse transcription of 1 µg of DNase I-treated total RNA was carried out using 10 U/µl Invitrogen Superscript 3TM primed with 2.5 µM oligo-dT at 50° for 1 hr. PCR was carried out using the following primer pairs: 5'AAG-ATC-TAT-TTT-GTA-AAG-CAC-TGA 3' and 5'ATC-TAG-ATC-AAA-GTC-TAT-CTT-CCT-GCA-C3' (for VEDEC) and 5'AAG-ATC-TAT-TTT-GTA-AAG-CAC-TGA 3' and 5' AAG-TCT-ATC-TTC-CTG-CAC-ATA3' (for QEDRL). PCR occurred in a 20 µl reaction mixture containing 1 mM MgCl₂, 300 µM dNTPs, 200 µM of each primer, 200 ng of cDNA; and 0.1 U of Amplitaq GoldTM (Applied Biosystems, Foster City, CA) buffered to pH 8.0 in 15 mM Tris HCl. The cycling parameters were: denaturation at 95° for 4 min, followed by 30 cycles of 95° for 30 s; annealing at 57° for 20 s; and extension at 72° for 1 min. A final extension at 72° for 1 min occurred after the last cycle.

Electrophysiology

Plasmids encoding GFP or GFP-fusion proteins were co-transfected with either VEDEC or QEERL expression vectors, and whole cell recordings were made from fluorescent HEK293T cells using standard methods similar to those described previously (Chae et al., 2005a, 2005b; Kim et al., 2007). Because HEK293T cells do not express endogenous voltage-activated Ca²⁺ currents, we designed pipette solutions to provide sufficient intracellular Ca²⁺ for activation of BK_{Ca} channels by depolarizing step pulses (Ramanathan et al., 2000), while keeping the resulting macroscopic currents small enough to avoid saturation of the patch clamp amplifier or significant series resistance errors. The latter was accomplished by reducing the concentration of permeant ions by ~60-fold while still maintaining a physiological E_K of -80 mV. Specifically, the bathing solution contained (in mM): NaCl 150, KCl 0.08, MgCl₂ 0.8, CaCl₂ 5.4, glucose 10, HEPES 10, and the pH was adjusted to 7.4 with NaOH. The pipette solution contained (in mM): NaCl 145, KCl 2, MgCl₂ 6.2, CaCl₂ 5 µM, pH 7.2. The free concentration of Ca²⁺ in this solution was checked using an Orion 97-20 calcium electrode

(Thermo Fisher Scientific, Waltham, MA) calibrated using commercial solution standards obtained from World Precision Instruments (Sarasota, FL). Whole-cell currents were not observed when recording pipettes contained 0 CaCl₂ and 10 mM EGTA (data not shown). Recording electrodes were made from thin borosilicate glass and fire-polished. They had resistances of 3–4 MΩ when filled with pipette saline and it was possible to compensate up to 85% of this without introducing oscillations into the current output of the patch clamp amplifier (Axopatch 1D, Axon Instruments). All physiological experiments were conducted at room temperature. Currents in HEK293T cells were evoked by a series of eight 450-ms depolarizing steps (from -25 mV to +80 mV in 15 mV increments) from a holding potential of -60 mV, digitized, and analyzed off-line using PClamp™ v9.0 software (Axon Instruments). Activation curves were constructed by plotting fractional activation (the normalized conductance $G/G_{\max}$) against command potential and fitting the resulting curves with the Boltzmann function:

$$G/G_{\max} = [1 + \exp(-(V - V_{1/2})q/RT)]^{-1}$$

where G is conductance at the command potential V , $G_{\max}$ is the maximal conductance, $V_{1/2}$ is the voltage of half-maximal activation, q is a slope constant, and F , R , and T have their usual significance. Fitting was done by non-linear least squares using Origin v7.0 software (Northampton, MA). Current-voltage from the same data set are also shown with superimposed spline curves that do not have theoretical significance. In previous studies we have demonstrated that iberiotoxin causes a complete block of macroscopic currents in HEK293T cells transfected with these constructs and examined using these voltage-clamp protocols (Kim et al., 2007).

Immunoblot analyses and cell surface biotinylation

For immunoblot analyses of VEDEC and QEDRL expression in native cells, E9 CG neurons were dissociated and plated onto poly-D-lysine-coated dishes. Affinity purified rabbit antibodies used in these experiments were raised against the C-terminals of the avian Slo1 isoforms. The peptides used to prepare these antibodies were: THMRPNRTKTRDSREKQKYVQEERL (avian QEDRL) and ANQINQYKSTSSLIPPIREVEDEC (avian VEDEC). These peptides were conjugated to keyhole limpet hemocyanin prior to immunization. Some of the characteristics of these antibodies are shown in Fig. 1. Experiments on HEK293T cells used a commercially available mouse anti-myc (antibody 9B11, Cell Signaling Technology, Danvers, MA) against the ectofacial N-terminal myc tags on the Slo1 proteins encoded by our expression vectors. Cells were grown on 100 mm plates for 24–48 h after transfection and in some cases were treated with growth factors (10 nM NRG1 for 1 h or 1 nM TGFβ1 for 3 h). For all immunoblot analysis, cells were washed in ice-cold PBS and lysed in Laemmli buffer, and samples were boiled for 5 min at 95°C and separated by SDS-PAGE on 9% gels. Proteins were transferred to nitrocellulose filters by wet transfer (1 hr) on ice. Blots were blocked with 5% nonfat dried milk dissolved in TBST buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, washed three times with TBST buffer, incubated with the primary anti-myc (1:1000) overnight at 4 °C, washed again with TBST, and the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) (Pierce Biotechnology, Rockford, IL) for 2 h at room temperature. The proteins were visualized using a chemiluminescent substrate (Super Signal West Pico, Pierce Biotechnology). Cell-surface biotinylation was carried out by treating intact HEK293T cells on 10 cm dishes with sulfo-*N*-hydroxysuccinimidobiotin (Pierce Biotechnology) (1 mg/ml in PBS buffer) for 1 h on ice with gentle shaking. Cold PBS buffer containing 100 mM glycine was then added to stop the reaction. After an additional 20 min of incubation, the cells were collected by centrifugation and lysed by trituration in 500 μl PBS containing 0.5% Triton X-100 (PBST). A portion of the cell lysate (100 μl) was reserved. The biotinylated proteins from the cell surface were recovered

from the remainder of the lysates by incubation with 30 μ l of immobilized streptavidin-agarose beads (Pierce Biotechnology). Beads were collected by centrifugation, washed, and bound proteins were eluted from the beads by boiling in 30 μ l of Laemmli buffer, all of which was loaded and separated on SDS-PAGE. Slo1 isoforms were then quantified by immunoblot analysis using antibody 9B11 directed against the myc tags. Samples (30 μ l) of the initial cell lysates were also separated and analyzed in this way for comparison of total Slo1 expression. All blots shown are representative of at least three repetitions of each experiment.

Confocal microscopy

HEK293T cells were grown on poly-D-lysine-coated glass coverslips and cells were transiently transfected with the myc-tagged VEDEC or myc-tagged QEERL expression vectors. Cells were subsequently exposed to FITC-conjugated mouse anti-myc (Abcam) (1:500) in Optimem™ medium for 1 hr at 37°, washed in PBS, and fixed by 30 min exposure to 4% paraformaldehyde in PBS. Cells were then rinsed in PBS, blocked with 10% normal goat serum, and then permeabilized in PBS containing 0.5% Triton X-100 (PBST). They were then incubated with myc antibody 9B11 (1:1000) for 1 h and then exposed to Alexa-568-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) (1:1000) for 1h. The cells were then rinsed in PBS and mounted using Vectashield (Vector Laboratories, Burlingame, CA). E9 ciliary ganglion neurons were fixed in 4% paraformaldehyde, blocked, and permeabilized in PBST as described above, and exposed to the isoform-specific VEDEC or QEDRL antibodies described above (both at 1:1000). After rinsing, cells were exposed to FITC-conjugated goat anti-rabbit, rinsed, and mounted in Vectashield. All images were collected on an Olympus FV-1000 inverted stage confocal microscope using a Plan Apo N 60X 1.42NA oil-immersion objective. Green fluorescence (from FITC) was evoked using an excitation wavelength of 495 nm while monitoring emission at 519 nm. Red fluorescence (from Alexa-568) was evoked by excitation at 580 nm and emission was monitored at 620 nm.

Statistics

Quantitative data are presented as mean $\pm$ S.E.M. Each group in a bar graph was comprised of 10-24 cells. Data were analyzed by one-way ANOVA followed by *post hoc* analysis using Tukey's honest significant difference test for unequal sample size, with $p < 0.05$ regarded as significant.

Results

Two C-terminal Slo1 splice variants are expressed in embryonic chick ciliary ganglion neurons

Searches of sequence databases indicate that vertebrate Slo1 variants can be divided into three classes (for purposes of this study) based on the sequence at the C-terminal end of the molecules. These include channels that end in QEERL (or QEDRL in birds), as well as channels that end in either VEDEC or EMVYR (which are the same in mammals and birds) (Fig. 1A). We observed by RT-PCR that transcripts encoding QEDRL and VEDEC variants of Slo1 are expressed in E9 chick ciliary ganglion neurons (Fig. 1B). These products were cloned and sequenced, and the sequences are shown in Fig. 1A from the point at which they diverge. We prepared and extensively characterized isoform-specific antibodies against peptides comprised of 24 unique residues present in each avian variant, shown underlined in Fig. 1A. The VEDEC antibody recognizes proteins of the appropriate molecular weights (for Slo1 monomers and dimers) in HEK293T cells expressing the VEDEC isoform, but does not yield signal from cells expressing an empty vector or from cells expressing the QEERL isoform (Fig. 1C, *top*). Conversely, the QEDRL antibody recognizes Slo1 monomers and dimers in HEK293T cells expressing mammalian QEERL channels, but not in cells expressing an empty vector or VEDEC (Fig. 1C, *middle*). For comparison, an immunoblot analysis was performed in parallel

using an antibody against the N-terminal (ectofacial) myc tags of Slo1 channels in HEK293T cells expressing either the VEDEC or QEERL isoforms (Fig. 1C, *bottom*). The same monomer or dimer bands were labeled. Immunoblot analysis using these isoform-specific antibodies confirms that VEDEC and QEDRL isoforms of Slo1 are expressed in the chick ciliary ganglion (Fig. 1D, *top*). Moreover, both of these antibodies yielded robust confocal immunofluorescence signal from dissociated E13 ciliary ganglion neurons (Fig. 1D, *bottom*). Both Slo1 isoforms were detected in all of the neurons in the cultures, regardless of cell size. However, the distribution of these channels within cells is not identical, as the QEDRL channels appear to be expressed at a higher density than VEDEC channels around the periphery of the cells. It is interesting to note the presence of some nuclear staining with the VEDEC antibody, as we described earlier in ciliary ganglion neurons using three different Slo1 antibodies that would not distinguish between VEDEC and QEERL (Lhuillier and Dryer, 2002). These data indicate that both isoforms are expressed in native neuronal systems, and they strongly suggest that trafficking of these channels is regulated differently.

Regulation of the two C-terminal splice variants in heterologous expression systems

To examine surface expression of these Slo1 variants in more detail, we utilized HEK293T cells that are more amenable to biochemical analysis. These cells were transiently transfected with mammalian expression constructs that encode mammalian Slo1 VEDEC and QEERL channels with N-terminal (exofacial) myc tags that allow for monitoring of channel movement. Placing a myc tag in this position has been previously shown to have minimal effects on gating and trafficking of BK_{Ca} channels (Meera et al., 1997; Bravo-Zehnder et al., 2000). We examined the expression of these constructs in HEK293T cells by confocal microscopy and cell-surface biotinylation assays using anti-myc antibodies (Fig. 2) and whole-cell recordings (Fig. 3). In confocal experiments (Fig. 2A), surface channels in intact cells were labeled with FITC-conjugated anti-myc antibody. The cells were then fixed, blocked, permeabilized, and labeled with a non conjugated anti-myc to obtain signal from intracellular channels using an Alexa-568-conjugated secondary antibody. We observed that QEERL channels can be easily detected both on the surface (*green*) and in intracellular pools (*red*) of HEK293T cells (Fig. 2A, *top*). By contrast, VEDEC channels are robustly expressed inside the cell, but are only weakly detected at the cell surface. Note that we used identical procedures, myc antibodies and laser excitation intensities to determine the distribution of both isoforms. The only difference was the C-terminal sequence of the expressed Slo1 variants. A similar pattern is observed with cell-surface biotinylation assays in which channel detection in immunoblots was carried out using anti-myc (Fig. 2B). We observed that steady-state surface expression of QEERL channels was much greater than that of VEDEC channels when total Slo1 protein levels were present at comparable levels in HEK293T cells. Interestingly, we observed that surface expression of the VEDEC isoform was increased after treating HEK293T cells with 10 nM NRG1 for 1 hr.

These data are supported by the results of whole-cell recordings from transfected HEK293T cells (Fig. 3). In these experiments, recording electrodes were filled with a saline containing 5 μ M free Ca²⁺ so as to allow for robust activation of BK_{Ca} channels by step commands within a range comparable to that observed by other workers in excised patches (Ramanathan et al., 2000). We observed that currents evoked in cells expressing QEERL channels were always much larger than in cells expressing VEDEC channels (Fig. 3), although they became active with a similar voltage-dependence (Fig. 3C). Macroscopic currents with both isoforms were completely blocked by exposure of HEK293T cells to 10 nM iberiotoxin (data not shown). It is interesting to note, however, that exposure of VEDEC-transfected HEK293T cells to 10 nM NRG1 for 1 hr causes a significant ($P < 0.05$) increase in mean current amplitude, up to levels observed in cells expressing QEERL channels (Fig. 3D). NRG1 did not evoke a statistically significant increase in the amplitude of macroscopic currents in cells expressing QEERL channels. HEK293T cells are derived from renal epithelial cell precursors, but are highly de-

differentiated. Therefore, we carried out a similar set of experiments in NG108-15 cells, a neural-derived cell line, and observed a similar pattern (Fig. 4). Specifically, we observed substantially higher constitutive cell surface expression of QEERL channels in transfected NG108-15 cells examined by cell surface biotinylation assays (Fig. 4A), as well as more than five-fold greater currents through QEERL channels in whole-cell recordings using the same protocols that we used for HEK293T cells (Fig. 4B). Therefore, the qualitative differences in steady-state surface expression of the two BK_{Ca} isoforms occur in multiple cellular contexts (see also Ma et al. 2007).

One possible explanation for the different behavior of the two Slo1 variants is that motifs unique to the VEDEC isoform bind to proteins that in some way suppress its steady-state expression on the plasma membrane, thereby causing it to accumulate preferentially in intracellular stores. This hypothesis predicts that expression of a short construct comprised of only the C-terminal portions of VEDEC should compete with co-expressed full-length VEDEC channels resulting in an increase in the constitutive trafficking of the full-length proteins. To test this prediction, we prepared a GFP fusion protein with a C-terminal comprised of residues 1129-1170 of the human VEDEC isoform, which we hereafter refer to as the V-tail. In addition, we prepared a GFP fusion protein that has a C-terminal comprised of residues 1096-1118 of human QEERL channels, which we refer to as the Q-tail. We expressed full-length myc-tagged VEDEC or QEERL channels in HEK293T cells, either by themselves, or along with either the V-tail or the Q-tail, and monitored channel expression by cell-surface biotinylation assays (using an antibody against the myc tag of the full-length channels) (Fig. 5A). We also monitored surface expression with whole-cell recordings from transfected fluorescent cells (Fig. 5B). The results agree with the prediction of the hypothesis. Specifically, we observed that HEK293T cells have a significantly ($P < 0.05$) higher surface expression of full-length VEDEC channels in cells co-expressing the V-tail than in cells expressing the full-length VEDEC by itself or in combination with the Q-tail. By contrast, the effect of the Q-tail protein on surface expression of VEDEC channels as monitored by electrophysiology was not statistically significant. Moreover, the V-tail and Q-tail proteins had no effect on surface expression of QEERL channels, which was robust in all conditions. We know that cells were expressing the tail constructs because they carried GFP tags, and cells chosen for electrophysiology had green fluorescence of comparable intensity.

Discussion

In this study, we have shown that alternatively spliced motifs at the extreme C-terminals of the Slo1 subunits of BK_{Ca} channels regulate their steady-state expression in the plasma membrane of multiple cell types with distinctly different embryonic origins. A different group recently observed a similar pattern in different cells (Ma et al., 2007). Our data further support a model in which motifs in the VEDEC forms of Slo1 channels bind to proteins that cause their retention in intracellular stores. Both of these Slo1 isoforms are expressed in native chick ciliary ganglion neurons.

In previous studies we have shown that the largest increase in the normal developmental expression of functional plasma membrane BK_{Ca} channels in CG neurons coincides with the formation of synapses with target tissues in the eye, long after the cells become excitable (Dourado and Dryer, 1992). Prior to that stage, BK_{Ca} channels are detectable in most cells but are present at a very low level on the cell surface. The large increase in surface BK_{Ca} that occurs after E8 is induced by cell-cell interactions during development. Thus, ciliary neurons that develop *in vivo* in the absence of their normal target tissues, or their afferent preganglionic inputs, fail to express normal levels of BK_{Ca} channels on their cell surface (Dourado et al., 1994). More recently, we have shown that these inductive effects are mediated by target-derived TGFβ1 (Cameron et al., 1998) acting synergistically with NRG1 that is secreted at

least in part from preganglionic nerve terminals that originate in cells of the Edinger-Westphal nucleus (Cameron et al., 2001). The effects of NRG1 and TGF β 1 on ciliary neurons are exerted on preexisting BK_{Ca} channels (Subramony et al., 1996; Subramony and Dryer, 1997; Cameron et al., 1998; Lhuillier and Dryer, 2000), and these growth factors stimulate trafficking of BK_{Ca} channels to the plasma membrane (Lhuillier and Dryer, 2002; Chae et al., 2005a, 2005b).

Functional BK_{Ca} expression is also regulated by growth factors in other neuronal cell types, including developing chick sympathetic neurons (Raucher and Dryer, 1995) and spinal motoneurons (Martin-Caraballo and Dryer, 2002a, 2002b), although the relevant growth factors are different in those cases from the ones used by ciliary neurons. It is therefore possible that growth factor-evoked trafficking of BK_{Ca} channels is a phenomenon of general significance in the nervous system, and possibly in other tissues. These results imply the existence of mechanisms to retain the channels in various intracellular stores prior to growth factor stimulation. The behavior of VEDEC channels noted in this study provides one such mechanism.

Are there structural features in the two Slo1 isoforms that might provide a basis for their different behavior inside cells? One possibility is that the two isoforms appear to have different putative PDZ domain-binding motifs at their C-terminals. Thus, the last four residues in vertebrate VEDEC channels (E-D-E-C) correspond to a canonical type-III PDZ-binding domain (D/E-X-X-C) (Harris and Lim, 2001), similar to one conserved in several neuronal N-type Ca²⁺ channels (Maximov et al., 1999). Interestingly, the terminal E/D-X-W-C motif of the neuronal Ca²⁺ channels binds to adaptor proteins such as Mint-1 that are essential for channel targeting to developing hippocampal synapses (Maximov et al. 1999; Maximov and Bezprozvanny, 2002). By contrast, vertebrate QEERL isoforms end in the residues E-D/E-R-L, which correspond to a canonical type-I PDZ-binding domain motif (Harris and Lim, 2001). This sequence is quite similar to the C-terminal of the CFTR transporter, which ends in E-T-R-L, and which is necessary for CFTR localization in apical membranes of polarized epithelial cells (Moyer et al., 1999; Milewski et al., 2001). It is also similar to the C-terminal of Na⁺- and Cl⁻-activated Slo2.1 and Slo2.2 channels, which end in the residues E-T-Q-L (Bhattacharjee et al., 2003; Yuan et al., 2003), and which bind to the prototypical PDZ domain-containing protein SD-95 (Uchino et al., 2003). On this basis, one would predict that the C-terminals of QEERL and VEDEC channels would be likely to exhibit distinct protein-binding specificities that could cause the pattern observed in the present study.

Within ciliary neurons, QEDRL channels appear to be more heavily expressed in the cell periphery. We have previously shown that ciliary neurons express more than one functionally distinct intracellular pool of intracellular BK_{Ca} channels. Thus, NRG1 treatment preferentially mobilizes BK_{Ca} channels located in a post-Golgi compartment (Chae et al., 2005b) that may be associated with cortical F-actin (Chae and Dryer, 2005). By contrast, TGF β 1 seems to be unable to mobilize that pool, and instead causes mobilization of channels located in the ER and Golgi apparatus (Chae et al., 2005b). NRG1 is also able to mobilize channels in that pool, but only when it is present at considerably higher concentrations. It is possible that alternatively spliced sequence motifs at the C-terminal cause BK_{Ca} channels to preferentially move to different intracellular compartments. However, it bears noting that both Slo1 isoforms studied here are expressed in intracellular stores, so the differences between the isoforms may be quantitative rather than absolute.

In summary, we have shown that different C-terminal splice variants exhibit different trafficking behavior in two different heterologous expression systems and that both of these variants are expressed in a single native population of developing vertebrate neurons. These C-terminal variants may differentially bind to proteins that regulate their trafficking inside

cells, in particular by suppressing trafficking of VEDEC isoforms until they are released to the surface by activation of appropriate signal transduction cascades.

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Figure 1.

Multiple Slo1 variants are expressed in chick ciliary ganglion neurons. **A**, schematic diagram of Slo1 showing location of the exon considered in the present study (*red box*) and the sequences of the isoforms from the point at which they diverge. Synthetic peptides of the underlined residues were used to generate isoform-specific rabbit polyclonal antibodies. **B**, RT-PCR showing expression of VEDEC and QEERL transcripts in embryonic day 9 chick ciliary ganglion. **C**, characterization of isoform-specific Slo1 antibodies. Immunoblot analysis of HEK293T cells expressing an empty vector, myc-VEDEC, or myc-QEERL using the anti-VEDEC (*top*). Note signal from monomers and multimers in the cells expressing VEDEC, but lack of signal in the other lanes. Conversely, anti-QEERL yields signal only from cells expressing QEERL (*middle panel*). These blots can be compared to those in which Slo1

monomer and multimer signal was obtained using anti-myc, resulting in signal from cells expressing either VEDEC or QEERL (*bottom panel*). D. Immunoblot analysis and confocal immunofluorescence of ciliary ganglion neurons using isoform-specific antibodies against VEDEC and QEDRL. Both Slo1 isoforms are expressed in these cells, but QEDRL appears to be more heavily expressed in the periphery of the cells (*arrows*).

Figure 2.

Differences in steady-state surface expression of mammalian VEDEC and QEERL isoforms of Slo1 channels expressed in HEK293T cells. A, confocal immunofluorescence in HEK293T cells of surface (*green*) and intracellular (*red*) Slo1 channels in cells expressing VEDEC or QEERL isoforms, as indicated. These signals were obtained by applying different myc antibodies before and after cell fixation and permeabilization. The same laser excitation intensities were used to collect images from both groups of cells. Note that QEERL channels are robustly expressed on the cell surface and in intracellular pools, but VEDEC channels appear to be largely excluded from the cell surface. B, cell-surface biotinylation assay showing difference in steady-state surface expression of VEDEC and QEERL channels in transfected HEK293T cells. In addition, 3 hr treatment with 10 nM NRG1 evokes an increase in the surface

expression of VEDEC channels, but not QEERL channels. Detection of the two isoforms in these immunoblots was done using the same myc antibody. The high molecular weight (>200 kD) signal is derived from channel multimers.

Figure 3.

Whole-cell recordings of current through BK_{Ca} channels in HEK293T cells expressing mammalian VEDEC or QEERL. Expression constructs were the same as the ones used in Fig. 2. Recording electrodes contained 5 μM CaCl_2 to allow activation of surface channels by depolarizing voltage steps. A, examples of typical currents in HEK293T cells expressing VEDEC or QEERL channels as indicated. B, Current voltage relationship calculated from four repetitions of experiments like that shown in A for VEDEC channels (*squares*) or QEERL channels (*circles*). Data show mean $\pm$ s.e.m. Line is a spline curve through the points with no theoretical significance. Note substantially larger currents in cells expressing QEERL channels. C, Normalized conductance-voltage curve constructed from data like that shown in A indicating that differences in current amplitude cannot be attributed to differences in the voltage-dependence of the two Slo1 isoforms. Line through points is a fitted Boltzmann curve as described in the text. D, mean currents observed by step pulses to +50 mV in transfected HEK293T cells in the presence or absence of 10 nM NRG1 for 3 hr prior to recording. Asterisks indicate significant ($P < 0.05$) difference from untreated cells expressing VEDEC channels as revealed by one-way ANOVA and a post hoc test. Note that increase in mean current evoked

by NRG1 is statistically significant in cells expressing VEDEC channels but not in cells expressing QEERL. Each group contains 10-14 cells.

Figure 4.

Differences in steady-surface expression of VEDEC and QEERL channels in transiently transfected NG108-15 cells. A, cell surface biotinylation assay using antibody against the ectofacial myc tag. Note greater surface expression of QEERL isoform. Mean macroscopic currents $\pm$ s.e.m. determined from whole-cell recordings from transfected cells using protocols and conditions identical to those shown in the previous figure.

Figure 5.

Expression of a fusion protein containing the C-terminal residues of the VEDEC isoform (V-tail) increases surface expression of co-expressed full-length VEDEC channels in HEK293T cells. This construct has no effect on QEERL channels. Expression of a fusion protein containing the C-terminal residues of the QEERL form (Q-tail) has no effect on either full-length isoform. A, results from representative cell-surface biotinylation assay. Image exposures are optimized for the monomer band. B, data from whole-cell recordings using methods described in Fig. 2. Effects of V- and Q-tails on mean currents in QEERL-expressing cells are not statistically significant. Asterisk indicates $P < 0.05$.