Published in final edited form as:

Neuroscience. 2010 October 27; 170(3): 692–702. doi:10.1016/j.neuroscience.2010.07.060.

Cloning and Distribution of Ca²⁺-activated K⁺ channels in Lobster *Panulirus interruptus*

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Abstract

Large conductance Ca²⁺-activated potassium (BK) channels play important roles in controlling neuronal excitability. We cloned the *PISlo* gene encoding BK channels from the spiny lobster, *Panulirus interruptus*. This gene shows 81–98% sequence identity to *Slo* genes previously found in other organisms. We isolated a number of splice variants of the *PISlo* cDNA within *Panulirus interruptus* nervous tissue. Sequence analysis indicated that there are at least 7 alternative splice sites in *PISlo*, each with multiple alternative segments. Using immunohistochemistry, we found that the PISlo proteins are distributed in the synaptic neuropil, axon and soma of STG neurons.

Keywords

BK channel; Alternative splicing; Distribution; STG; Lobster

Large conductance Ca²⁺-activated potassium (BK) channels play important roles in controlling neuronal excitability. Because they are activated by both depolarization and increased intracellular Ca²⁺, they establish a link between intracellular signaling pathways and neuronal electrical signals (Vergara et al., 1998, Magleby, 2003, Latorre and Brauchi, 2006, Salkoff et al., 2006). BK channels shape neuronal activity in a number of ways, including action potential termination (Lovell and McCobb, 2001), adjusting firing frequency and spike frequency adaptation, and terminating bursting or bistable firing (Kiehn and Harris-Warrick, 1992, Lara et al., 1999). They also regulate neurotransmitter release as an important negative-feedback system for Ca²⁺ entry in nerve terminals, through a physical interaction with voltage-gated calcium channels (Roberts et al., 1990, Robitaille et al., 1993b, Hu et al., 2001, Raffaelli et al., 2004, Berkefeld et al., 2006).

The α -subunit of the BK channel is encoded by the *slo1* gene, which is conserved among species (Fettiplace and Fuchs, 1999, Lu et al., 2006, Fodor and Aldrich, 2009). It contains seven transmembrane domains (S0–S6) with the N-terminus facing the extracellular side, two “regulator of conductance of potassium” (RCK) domains, a calcium-sensing “calcium bowl,” four intracellular hydrophobic domains (S7–S10), and an intracellular C-terminus (Ghatta et al., 2006, Latorre and Brauchi, 2006, Salkoff et al., 2006, Cui et al., 2009). A relatively conserved “core” consists of the transmembrane segments (N-terminus to S8), and the conserved “tail” region consists of the S9 to C-terminus region. These two components

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are separated by a non-conserved linker region (between S8 and S9), which shows significant differences in length between species (Ghatta et al., 2006, Latorre and Brauchi, 2006, Salkoff et al., 2006, Fodor and Aldrich, 2009). BK channels are subject to direct regulation by auxiliary β -subunits (Petrik and Brenner, 2007, Torres et al., 2007), posttranslational modification (Chung et al., 1991, Reinhart et al., 1991), heteromultimer formation (Joiner et al., 1998) and significant alternative splicing of the main α subunit (Atkinson et al., 1991, Butler et al., 1993, Tseng-Crank et al., 1994, Rosenblatt et al., 1997, Derst et al., 2003).

Across species, *slo1* has between 3 and 10 alternative splicing sites, resulting in thousands of potential transcript variants (Adelman et al., 1992, Butler et al., 1993, Fodor and Aldrich, 2009). In humans, alternatively spliced transcripts are differentially expressed throughout the brain, demonstrating the functional relevance of the variability on a physiological level (Tseng-Crank et al., 1994). The variable functional properties of alternative BK channels, and their many physiological roles in different networks, make examining BK channels within a well-understood neural network especially interesting (Turrigiano et al., 1995).

The stomatogastric ganglion (STG) of crustaceans is a powerful model system for dissecting the cellular mechanisms of rhythmic pattern generation in neuronal networks. The 14 neuron pyloric network within the spiny lobster (*Panulirus interruptus*) STG is a simple, well studied network with six major cell types that generates a rhythmic bursting motor pattern. BK channels could play an important role in regulating the pyloric rhythm, as they have previously been noted to terminate the plateau phase of bursting crab stomatogastric neurons (Kiehn and Harris-Warrick, 1992) as well as help to terminate oscillations in pituitary cells (Tsaneva-Atanasova et al., 2007). Although in the vertebrate nervous system, BK channels are found in axon terminals, somata and dendrites of many neurons, the cellular localization of BK channels in lobster neurons has not been reported. In this manuscript we report the initial cloning and alternative splicing of PISlo from *P. interruptus*, as well as the anatomical distribution of these channels within the STG.

Materials and Methods

RNA Isolation

Total RNA was isolated from lobster nervous system (brain, abdominal, & thoracic ganglia) using the RNeasy-4PCR kit (Ambion, Austin, TX). Contaminating DNA in the total RNA was removed by treatment with DNase (Ambion, Austin, TX) at 37°C for 30 minutes. The quality of the RNA was confirmed by RNA gel electrophoresis. The results reported here are from two independent RNA isolations.

Reverse-transcription PCR (RT-PCR) and Degenerate PCR

Total RNA was reverse transcribed to the first cDNA strand using SuperScript[™] III RT (Invitrogen, Carlsbad, CA) with an oligo dT primer. Primers pairs Slo5/8 & Slo7/6 (see table of primer sequences below), based on highly conserved regions of crab, *Drosophila*, and cockroach *slo* genes, were used to successfully amplify 2kb and 860 bp bands respectively using Platinum Taq DNA Polymerase High Fidelity (Invitrogen). All fragments were cloned into pGEM-T Easy vector (Promega) and sequenced for accuracy.

5' and 3' Rapid Amplification of cDNA Ends (5' and 3' RACE)

Pislo specific primers sloGSP1/sloNGSP1 and sloee8/slo3fov3 (see table of primer sequences below) were designed for the 5' RACE and 3' RACE reactions respectively based on the 2kb and 860bp fragments obtained from the degenerate PCRs. 5' and 3' RACE procedures were carried out according to the protocols in the 3' and 5' RACE Systems for

Rapid Amplification of cDNA Ends kits (Gibco-BRL, UK). The RACE products were subcloned into pGEM-T Easy Vector (Promega, Madison, WI) and sequenced.

End-to-End PCR

After the 5' and 3' terminal sequences were obtained by the RACE procedures, two reactions were performed to obtain the complete open reading frame (ORF) for *Plslo*. Primer pair SloEE1/SloEERev1 was designed outside the ORF to amplify our specific cDNA of interest. After obtaining the expected band at ~3.55kb, primers SloEE2 and SloEERev2 were designed to be nested within the first sequence flanking the complete ORF (Platinum TAQ Polymerase, Invitrogen). The products were cloned into pDrive vector (Invitrogen) and sequenced. Thirteen unique full length clones were identified in the end-to-end PCR.

Alternate Splice Variant Identification

The amino-acid sequences from the 13 full length clones were aligned using DNASTAR Lasergene MegAlign 8 software (Clustal W method), revealing seven sites of variability; these were sequentially named X1–X7 based on their relative proximity to the N-terminus. Primer pairs were designed (X1–X7 Forward/Reverse) flanking each site; these regions were amplified (Platinum TAQ Polymerase, Invitrogen), and sequenced. No primers pairs could isolate X2 independently, and thus X2 was combined with the X3 sequence to obtain the hybrid splice identification site X2/3.

Primer	Sequence
Slo5:	GGAGCGCCAACACAACACAACAAAT
Slo8:	TGGAGGCTCGAAGGGGCATAACTAAG
Slo6:	CGGGTGGGTTGGTTATTACATA
Slo7:	CTGCGGGCATCCAACCTCCAC
sloGSP1:	CACGCATAATATTCGAGCATCCTCAGC
sloNGSP1:	GCAAACACAGCCAATCCGACGAGG
sloee8:	CACACTTAGTCCTCTTGGGTCAC
slo3fov3:	ATTCCGCGACACATCAAGTTC
SloEE1:	AGTTACGTTGGATGTCCCGAGA
SloEERev1:	GACCATCCGGAACACAACAG
SloEE2:	TGAAGTGTTAACGATGTCAGACGAT
SloEERev2:	CAGCGCTGGACTCTTTAGATGTAGGA
X1-Forward	CTCCGGCCAGACCACAACAG
X1-Reverse	ATCTCCAGCATGAACCACAGC
X2/3-Forward	ACTTTGTTAATGCTCATCCTCTATCA
X2/3-Reverse	ACATCATCCCCACGTTTCCAGT
X3-Forward	GTGAGGACGTGGATGTAGAAGTTGTG
X4-Forward	AAAAATGAAGAAGGGACTGATAGC
X4-Reverse	GATTGTAACCCTGACTTGGAGA
X5-Forward	CTAGCAGCAGTTAAAAAGAATGGT
X5-Reverse	TGGGTCAGCAAAGAGGCATAC
X6-Forward	ATGATGATCCAACGCTAGCAGACAAA
X6-Reverse	TCATAGGGACGTTAGCACCATAGACA
X7-Forward	CCGTTGTCAAGTGGGTCAGATTT
X7-Reverse	TGTACTCCAGGCCAGGGTCAA

Western blot

Total protein extracts from lobster nervous tissue were transferred from a sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) to a polyvinylidene difluoride (PVDF) membrane using the Mini-Trans-Blot system (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% powdered milk in 1X Tris-buffered saline (TBS) for 30min at RT, and then incubated with the Rabbit anti-pslo primary antibody (1:1000, a gift from Dr. D. Wicher at the Max Planck Institute for Chemical Ecology in Germany) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. The blot was developed using the enhanced chemiluminescence system (Amersham). To demonstrate that the bands observed on the blots were specific for lobster slo channels, we performed a control using preimmune serum. There was no immunostaining in this treatment.

Immunocytochemistry

Ganglia were fixed in 3.5% Paraformaldehyde for 80–100 min and washed 8 times with 1X phosphate-buffered saline containing 0.3% Triton X-100 (PBST). For whole mounts, ganglia were blocked in 1XPBST containing 0.5% BSA, 5% goat serum for 4hr at RT and then incubated with primary antibody (rabbit anti-pslo antibody, 1:1000; mouse anti-synapsin monoclonal antibody, 3c11, 1:50, The Developmental Studies Hybridoma Bank at the University of Iowa) overnight at 4°C. Ganglia were rinsed at least 6 times in PBST over 2 hours and then incubated with secondary antibody (goat anti-rabbit Alexa 488, goat anti-mouse Alexa 635) for 2hr at RT. Ganglia were washed 6 times again in PBST over 2 hours and then mounted with VECTASHIELD Fluorescent Mounting Media (Vector Laboratories). For cryostat sections, fixed ganglia were cryoprotected in 30% sucrose in 0.1M PBS overnight and then sectioned via a cryostat. The thickness of the sections was 20µm. After sectioning, the slides were thoroughly air dried and stored at 4°C until staining. For neurobiotin/streptavidin co-labeling, neurons were microinjected with 4% neurobiotin and then cultured at 14°C for 1hr. Alexa 635-conjugated streptavidin was added during the secondary antibody incubation step. Specimens were scanned with a Leica TCS SP2 confocal microscope.

Results

Cloning of *PISlo*, the large conductance, Ca^{2+} -activated potassium channel gene from *Panulirus interruptus*

Based on the previously identified partial crab *slo* sequence and the full length sequences of *slo* from *Drosophila* and cockroach, degenerate primers were designed to amplify lobster homologous sequences at the conserved S2–S8 and S9–S10 regions. We obtained two fragments (2kb and 860bp) from these degenerate PCRs. Sequencing revealed that the deduced amino acid sequence of the 2kb fragment shares 94.6% amino acid identity to the S2–S8 region of the crab *slo* protein, and the 860bp fragment shared 96% amino acid identity to the S9–S10 region of crab *slo*. Therefore we considered that we had cloned the S2–S8 and S9–S10 regions of *PISlo*.

These two sequences were then used to design primers sloGSP1/sloNGSP1 and sloee8/slo3fov3 to amplify the 5' and 3' end of *PISlo* cDNA using 5' and 3'RACE methods respectively. The 5'RACE reaction yielded a 1,099bp fragment containing 133bp of 5'UTR, the start codon with a contiguous consensus Kozak sequence and the S0–S7 region, while the 3'RACE experiment provided a 1.5kb fragment containing the sequence beyond S10, the stop codon and 1323bp of 3' UTR. With this information, two end-to-end PCR primer pairs (SloEE1/SloEERev1 and SloEE2/SloEERev2) were designed flanking the entire open reading frame (ORF), which were used to obtain full length *PISlo* sequences.

In all, 21 single clones from these reactions were sequenced. This revealed 13 distinct, full length splice variants of *PISlo*. Of the eight other clones, two were copies of clones A49 and C35, and six were sequences containing nonsense mutations. We do not know whether these were PCR-induced mutations or true mutations. The ORF lengths of the 13 full-length splice variants ranged from 3,324 to 3,495bp, coding for proteins of 1,107–1,164 amino acid residues with a calculated molecular mass of 123.6–130 kDa (Table 1). The encoded proteins showed all the typical features of a BK channel α subunit, slo (Figure 1B): (1) 7 transmembrane domains (S0–S6); (2) a pore region (P) containing the potassium channel selectivity filter motif GYG; (3) a large cytoplasmic c-terminus containing 4 hydrophobic domains (S7–S10); (4) the Ca^{2+} bowl, the putative Ca^{2+} binding domain; (5) two regulators of conductance of K^{+} domain, RCKs. These *PISlo* splice variants shared very high homology with the crab *slo* partial gene (GenBank accession number DQ103256) (96.1–97.7% amino acid identity); 83.1–85.1% identity with cockroach *pslo* (AF452164) and 80.8–83.4% identity with the *Drosophila* homologue, *slowpoke* (DROKCHAN). Alignment of the amino acid sequences for the 13 splice variants showed strong conservation among six of the seven transmembrane domains, the four cytoplasmic hydrophobic domains, and the calcium bowl regions.

Between the 13 *Panulirus* clones, there were in-frame variants at seven different locations, each with multiple variants indicating that they were alternative splicing sites. These sites were designated X1 to X7 based on their relative proximity to the N-terminus (Figure 1A and 1B). Five of the seven splice sites (X3–X7) are located downstream of the transmembrane regions and are thus cytoplasmic. X1 is in the extracellular S1–S2 linker, while X2 begins at the S6 transmembrane domain and extends to the very beginning of the cytoplasmic region (Figure 1).

In the *Drosophila dslo* and cockroach *pslo* genes, five alternative splice sites have been described, each with multiple alternative segments (Adelman et al., 1992, Derst et al., 2003, Fodor and Aldrich, 2009). These five splice sites are all in the C-terminus of *dslo* and *pslo*. The location and alternative sequences of their sites 1, 2 and 5 are conserved with the X2, X3 and X5 sites of *PISlo*, respectively. The location of their site 4 is conserved with the X4 site of *PISlo*, but the alternative sequences in this site are different between species; *PISlo* does not appear to have an alternative site corresponding to the site 3 of *dslo* and *pslo*, and these genes do not have alternative sites corresponding to the lobster sites X1, X6 and X7.

We further analyzed the alternate exons at each of the seven *PISlo* alternative splicing sites, using a new RNA preparation to verify the alternative splice segments identified in our first screen and to seek additional splice segments. PCRs from experiments using primer pairs flanking each of the seven splice sites (Figure 2A) showed different size bands when run on a 3.5% agarose gel, and most primer pairs amplified multiple bands at a splice site (Figure 2B). Based on the variation observed at each splice site in the full length clones, we expected to obtain varying length products from each single site amplification reaction, representing the splice variant(s) for that site. Any sequences that varied by less than 10 nucleotides generally appeared as a single band with a slight smear due to an inability to properly separate them on the 3.5% agarose gel. Each band was therefore isolated, cloned and sequenced to identify additional alternative sequences that had not been identified in our 13 full length clones. In total, 30 different splice sequences were identified across the seven sites (Table 1, Figure 1): these included all 22 alternative sequences that had been observed in our full length clones, and 8 novel sequences that had not been previously identified.

Each full length clone was composed of conserved regions and a specific sequence in each splice site. Using the splice sequences at each splice site identified by letter in Figure 1, we determined the composition of each full length sequence (Table 1).

Localization of PISlo channels in the lobster stomatogastric ganglion

$I_{K(Ca)}$ is present in all six pyloric cell types, based on electrophysiological measurements (Kloppenburg et al., 1999; J. Peck, L. Kadiri and R. Harris-Warrick, unpublished data), and the slo gene is expressed in all examined crab STG cell types including LP, IC and PD (Schulz et al., 2007, Goaillard et al., 2009). However, it is not known where PISlo channels are expressed within the STG. We used immunostaining and confocal microscopy to investigate the distribution of PISlo channels in the lobster STG. The antibody we used was a gift from Dr. D Wicher at the Max Planck Institute for Chemical Ecology in Germany. This antibody was raised in rabbit against a synthetic peptide of the C-terminal sequence of pSlo from cockroach. This peptide sequence is not in an alternative splice site and thus is identical in all of our 13 full-length PISlo splice variants. PISlo has the identical amino acid sequence except for the last 3 amino acids (83% identity to the corresponding pSlo sequence). Western blots of lobster nervous system total protein were used to test antibody specificity (Figure 3A). Anti-pSlo recognized four bands at approximately 250, 123~128, 100 and 87 kDa, while the preimmune serum did not label any bands (Figure 3A). The strongest band (123~128kDa) is in the deduced molecular weight range of the 13 PISlo splice variants. As a negative control, we performed Western blots on proteins isolated from *Xenopus* oocytes, which do not express *Slo* or $I_{K(Ca)}$; no bands were seen on these blots, suggesting that our antibody specifically recognizes the Slo proteins.

With this evidence that the anti-pslo antibody labels the intended PISlo proteins, we used this antibody to examine the channels' localization within the STG. In all, 19 preparations were stained; each preparation had about 10–13 somata strongly stained for PISlo. As shown in Figure 3B from a whole mount experiment, and in Figure 3C–3D from 20 μ m cryostat slices, the STG somata exhibit varying degrees of Slo-like immunoreactivity. Some neurons' cell membranes stained intensely and showed sharp rings of label around the edges of these neurons, while staining of other neurons was less intense and more punctate (Figure 3B and 3D). This likely reflects differences in the abundance of PISlo channels in the somatic cell membranes of different STG neurons. PISlo immunostaining is also visible in punctate distributions within the cytoplasm of the cell bodies (Figure 3B). This most likely represents staining in the endoplasmic reticulum, Golgi apparatus, and/or cargo vesicles. PISlo-like immunostaining is not restricted to the somata, but is also present in the initial segment of neurites (Figure 3C, arrow and 3D). The immunolabeling was eliminated when the primary antibody was pre-absorbed with the appropriate antigen peptides, or removed from the immunohistochemistry steps (Figures not shown). Derst C. et al. (2003) reported a similar immunostaining pattern in dorsal unpaired median (DUM) neurons and midline neurons in *Periplaneta* abdominal ganglia.

To view PISlo immunostaining in the coarse and fine neuropil, we injected neurobiotin into single pyloric neurons to label their fine neurite structure. We then performed immunohistochemical labeling in cryostat sections to assure adequate antibody penetration of the fine neuropil. Figure 3D shows cryostat sections from one STG, with neurobiotin/Streptavidin staining of one Pyloric Dilator (PD) neuron in red and PISlo staining in green. We found that punctate PISlo staining was concentrated primarily in the fine neuropil region (Figure 3D, stars and 3E), with little or no staining in the large branches of neurites in the coarse neuropil (Figure 3D, arrow). In the fine neuropil, the PD neurites terminate in large clusters of smaller terminals (Figure 3E). Punctate PISlo immunostaining overlapped with the neurobiotin staining in this region, indicating that PISlo channels are localized in the very high order branches of PD neuron (Figure 3E). Similar distribution patterns of PISlo channels in the coarse and fine neuropil have been found in all of the slices of four preparations from identified PD and LP neurons.

The fine neuropil region of the STG contains the fine higher branch order processes and the large majority of synapses (King, 1976, Bucher et al., 2007). Since PISlo staining was found at high density in the fine neuropil, we examined whether PISlo channels are localized in presynaptic terminals. We used an antibody to synapsin to label presynaptic sites in the STG neuropil. This antibody has already been demonstrated to label pre-synaptic terminals containing synaptic vesicles in other lobster species (Skiebe and Ganeshina, 2000, Skiebe and Wollenschlager, 2002). Our western blots showed that anti-synapsin recognized 2 bands with molecular weights of 70 and 80 kDa (data not shown), consistent with staining in the American lobster *Homarus americanus* (Bucher et al., 2007) and in *Drosophila* (Klagges et al., 1996). As seen in Figure 4B, strong synapsin-like immunostaining was restricted to the fine neuropil, though not all of the fine neuropil was strongly stained with synapsin. PISlo staining (fig. 4A and 4C) overlapped with synapsin-like staining. This indicates that PISlo channels are localized to the synaptic neuropil, suggesting, though not proving, that this staining is in the presynaptic terminal (n=9 preparations; see Discussion). There is also strong PISlo immunostaining in other regions in the fine neuropil where synapsin labeling is weak or absent (Figure 4C); these could be finer branches or/and fine terminals that do not carry chemical synapses. We could not distinguish these structures in our experiments.

BK channels have been visualized in axons of vertebrate Purkinje cells, where they have a punctate immunofluorescent appearance, which could represent single or clustered BK channels (Sausbier et al., 2006). We tested whether anti-Slo labels axons of STG neurons. Immunohistochemical staining in both whole mount STG and cryostat sections showed that PISlo channels are localized in STG axons (n=8 preparations). Fig. 5 is an optical section showing that axons in the Dorsal Ventricular Nerve (DVN) exiting the STG were stained intensively with anti-Slo antibody. Similar axonal staining was seen in the stomatogastric nerve (STN) that provides input to the STG from higher ganglia.

Discussion

Alternate Splicing of *PISlo* channels

In this study, we cloned the *PISlo* gene encoding a large conductance, calcium-activated potassium channel from the spiny lobster, *Panulirus interruptus* and showed that it shows very high sequence homology to *Slo* genes previously found in other organisms. We isolated a number of splice variants of the *PISlo* cDNA, and isolated complete sequences of 13 different channel transcripts within *Panulirus interruptus* nervous tissue. The PISlo protein is distributed in the synaptic neuropil and soma of STG neurons.

Extensive alternative splicing of the *slo* gene has been previously seen in several other species (Adelman et al., 1992, Tseng-Crank et al., 1994, Jones et al., 1999, Derst et al., 2003, Langer et al., 2003). Based on the 30 separate splice segments at 7 different splice sites so far identified in our study, there are at least 16,128 potential transcripts that could be created, assuming that each variant is independently expressed. Among the seven splicing sites, the X1 site, in the S1–S2 linker (Figure 1), has previously been reported only in vertebrate BK transcripts (Rosenblatt et al., 1997, Fettiplace and Fuchs, 1999, Fodor and Aldrich, 2009). Our study demonstrates that this splice site is also present in invertebrate *Slo* transcripts and thus is evolutionarily old. Although the position of this site is conserved in vertebrates and invertebrates, the sequences of the splice variants are totally different (Rosenblatt et al., 1997, Fodor and Aldrich, 2009).

The X2 splice site is particularly interesting because of its location within the S6 transmembrane domain, extending to the starting region of the cytoplasmic “tail” region (between S6 and S7; Figure 1). The S6 transmembrane domain interacts with the S5 and p-loop to form the wall of the ion-conducting pore in potassium channels (Latorre and

Brauchi, 2006, Cui et al., 2009). Therefore, we could expect that alternative splicing in such an important region will have a profound impact on single-channel conductance and open probability. *In vitro* oocyte expression studies of the *Drosophila dslo* transcripts differing only at this site showed that they differ primarily in their unitary conductance (Lagrutta et al., 1994). In *PISlo*, the X2 site has six identified alternate splice segments, three of which are lacking a majority of the S6 transmembrane sequence and therefore probably cannot make a functional channel. Because these three variants were not found among our few full length clones, we do not know if they are fully transcribed, how long they survive, or whether they are translated.

The X3 site is another alternative splice site, located between the S6 and S7 regions. Fodor and Aldrich (2009) performed a manual curation analysis of Unigene clusters mapped to mouse, human, chicken, *Drosophila*, and *C. elegans* genomes. They found that the S6–S7 region is a hot spot of alternative splicing in BK transcripts across phyla. X3 is in the RCK1 domain. Jiang et al. (2002) proposed that four RCK1s form a gating ring with four RCK2 domains at the intracellular membrane surface. Binding of Ca^{2+} to the gating ring was proposed to expand the ring, pulling on the linkers to the S6 gates to open the channel (Jiang et al., 2002). Alternative splicing in this region (especially the 0AA variant X3d, which completely removes two α -helices and one β -strand) may change the structure of this intracellular gating ring, thus may cause functional changes in channel kinetics and calcium sensitivity. Although we found this 0AA variant by splice site-specific PCR, we failed to find it in any of our full-length clones.

Another hot spot for multiple alternative splicing is between S8 and S9 (Fodor and Aldrich, 2009). In this region, *PISlo* channels have two splice sites, X4 and X5; each site has multiple alternative segments. Alternative splicing in this region is common across phyla, but the exact sites and sequences are poorly conserved because the BK channel can tolerate insertions and variation in this interdomain region (Fodor and Aldrich, 2009). Splice variants in this region have been functionally characterized in vertebrate and invertebrate *Slo* channels (Lagrutta et al., 1994, Hanaoka et al., 1999, Ramanathan et al., 1999, Liu et al., 2002). For example, a 59 amino acid insertion between S8 and S9 in the rabbit gene *rbslo1* increased the Ca and voltage sensitivity (Hanaoka et al., 1999). A 61-amino acid exon in this region in chick *slo* cDNA encodes an $\text{I}_{\text{K}(\text{Ca})}$ that is kinetically distinct and more calcium-sensitive than the exonless form (Ramanathan et al., 1999). Therefore, we predict that splicing at the lobster X4 and X5 sites might change the biophysical properties of *PISlo* channels, perhaps by modulating the interaction between the RCK1 and RCK2 domains and the binding of calcium ions. Splicing in this region is also regulated by stress hormone (Xie and McCobb, 1998) and can change the susceptibility of the channel to regulation by phosphorylation (Tian et al., 2001, Chen et al., 2005).

The X6 splice site is located in the second RCK domain, near the Ca^{2+} -bowl, a putative intracellular calcium-sensing region. This site has either a 39aa long insertion or near complete removal of the splice sequence (1 aa or 2 aa). Such strong variation in sequence near the Ca^{2+} -sensing domain could be a potential mechanism for tuning the sensitivity to intracellular Ca^{2+} . A rat *Slo* variant with a 27 aa insertion in this region has faster activation kinetics and significantly different co-operative gating behavior compared with a variant lacking an insertion in this region (Ha et al., 2000).

We also found alternative splicing at site X7 in the 3'-terminal region of *PISlo*. Splicing in this site does not appear to influence the activation and inactivation properties of r*Slo* (Saito et al., 1997). Kim et al. (2007) proposed that the *Slo* COOH-terminals may function as PDZ domain binding motifs that could regulate the trafficking and steady-state surface expression of BK channels (Kim et al., 2007). Alternative splicing at the X7 site in *PISlo* could thus

modify gene expression and protein trafficking to the cell membrane. To confirm our predictions, more electrophysiological characterization of the splice variants will be necessary; our difficulty in successfully expressing PISlo in *Xenopus* oocytes or lobster neurons has to date made testing these predictions impossible.

Distribution of PISlo channels in the STG and its possible role in shaping the firing properties of PD neurons

BK channels are thought to play important functional roles in action potential repolarization (Adams et al., 1982, Elkins et al., 1986), secretion (Petersen and Maruyama, 1984), and determination of the resonant frequency (Ramanathan et al., 1999, Ramanathan et al., 2000). In the crab DG neuron, $I_{K(Ca)}$ plays a role to terminate a prolonged plateau potential and tonic firing when the neuron is in a bistable state (Kiehn and Harris-Warrick, 1992). Since neurons are highly specialized cells, precise localization of BK channels is critical for correctly performing their functions. Our Western blot result showed that the anti-pslo antibody we used in immunohistochemistry recognizes a major band of the right size (123–128 kDa) for PISlo proteins, as well as three minor bands. The largest band (250kDa) could be PISlo multimers; extremely hydrophobic proteins, such as BK channels containing multiple transmembrane and hydrophobic domains, are easy to multimerize during sample preparation, and they are normally found as tetramers forming the channel. Possible explanations for the 100 and 87kDa bands include alternative splice variants which haven't yet been identified in our study, truncated proteins or degraded protein. Using immunohistochemistry, we demonstrated that PISlo channels are located in the membranes of STG somata. We also found slo-like staining in the initial segment of the primary neurite. Although somatic BK channel expression can regulate the firing properties of vertebrate neurons (Pedarzani et al., 2000, Faber and Sah, 2002, Sun et al., 2003, Benhassine and Berger, 2005), the neuronal somata of STG neurons are electrically inexcitable, and action potentials arise far from the somata as their axons leave the ganglion. We also observed significant PISlo immunostaining in axons of STG neurons, suggesting that $I_{K(Ca)}$ helps to regulate spike propagation in these neurons.

PISlo immunostaining was also detected clustered in puncta in the fine neuropil region of the STG, where synaptic interactions occur. A punctate distribution of BK channels has been reported in cultured hippocampal pyramidal neurons, as well as dorsal unpaired median (DUM) neurons and midline neurons in the *Periplaneta* abdominal ganglia (Derst et al., 2003, Sailer et al., 2006). By co-staining with synapsin, a protein associated with synaptic vesicles in axonal terminals, we demonstrated that PISlo proteins were strongly co-localized in synapsin-staining fine neuropil. This suggests a presynaptic localization of PISlo channels in STG fine neuropil, but our light level microscopic analysis cannot prove that the BK channels are indeed on the pre-synaptic terminal as opposed to closely apposed post-synaptic structures. Presynaptic BK channels have been identified in glutamatergic hippocampal pyramidal neurons (Sailer et al., 2006), the neuromuscular junction (Robitaille et al., 1993a) and the rod photoreceptor synapse (Xu and Slaughter, 2005). Presynaptic BK channels have been found to colocalize with voltage-gated Ca^{2+} channels, where they modulate neurotransmitter release in many neurons (Roberts et al., 1990, Robitaille et al., 1993b, Hu et al., 2001, Raffaelli et al., 2004, Berkefeld et al., 2006). It will be interesting to examine whether PISlo channels colocalize with voltage-gated Ca^{2+} channels in STG synaptic terminals of STG. However, BK channels have also been reported in postsynaptic membranes in cultured hippocampal neurons and in mouse brain (Sailer et al., 2006, Sausbier et al., 2006). We observed that BK channels had a significantly wider distribution than synapsin staining in STG fine neuropil, suggesting a function not related to synaptic function.

We tried in many ways to express *PISlo* RNA to study its channel properties, both in *Xenopus* oocytes and in the Pyloric Dilator (PD) neuron in the STG. It proved unexpectedly difficult to obtain functional expression of any *PISlo* splice variant. Despite our previous success with expression of several other lobster ion channels (Baro et al., 1996, Kim et al., 1997, Kim et al., 1998, Baro et al., 2001, MacLean et al., 2003, Zhang et al., 2003, Zhang and Harris-Warrick, 2004, French et al., 2005, Ouyang et al., 2007), and after many modifications of experimental conditions, we failed to obtain channel expression in *Xenopus* oocytes. As a positive control, we obtained excellent expression of a mouse *slo* RNA. When microinjected into lobster PD neurons, only a minority (7 of 33) of the neurons showed an increase in *PISlo* expression, and the average increase in $I_{K(Ca)}$ measured from the soma of these neurons was only about 20%. We tried several different splice variants, and a number of modifications of our expression procedures, but without further success. Further research will be needed to improve *PISlo* expression in order to analyze the effect of the splice variants on the properties of pyloric neurons.

In conclusion, the cloning and mapping of lobster *PISlo* channels will be a helpful tool for interpretation and understanding the function of BK channels in both the firing properties of neurons and their synaptic integration within neural networks.

Acknowledgments

This work was supported by NIH grant RO1 NS17323. We thank Dr. Bruce Johnson, Jack Peck, David McCobb, Guisheng Zhong, Nate Cramer, Lola Mukhamedieva and Mark Kvarta for valuable input and suggestions. We are grateful to Dr. D. Wicher for providing the anti-*pslo* antibody.

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Fig. 1. Alternative splicing of *PISlo*

(A) Schematic diagram of alternative splicing of the *PISlo* transcripts isolated from lobster nervous system. The seven transmembrane domains are shown as black boxes labeled S0–S6. The four hydrophobic domains in the C-terminus are shown as grey boxes labeled S7–S10. The pore forming region and the Ca²⁺ Bowl are labeled P and Ca respectively. Seven splicing sites, X1–X7, have been identified. (B) Amino acid sequences of clone L37 (GenBank accession number HM208587). This clone encodes a protein of 1133aa. The seven putative transmembrane segments, S0–S6, pore forming region (P), the calcium bowl domain, c-terminal hydrophobic segments, S7–S10 and the splice sites (X1–X7) are indicated. Two intracellular regulators of conductance of potassium domains (RCK1 and 2)

are italicized. At the splice sites, the original L37 sequence is shown as the top branch, while identified alternative sequences are shown underneath.

Fig. 2. RT-PCR analysis of *PISlo* splicing sites

(A) Location of PCR-primers in the *PISlo* mRNA coding region used for RT-PCR. (B) Agarose gel electrophoresis of RT-PCR products obtained with various primer pairs on RNA from lobster nervous system.

Fig. 3. PISlo channels are found in STG neurons

(A) Anti-Slo antibody specifically recognizes its respective protein. Western blot containing total protein extracts from the lobster nervous system was probed with rabbit anti-pSlo antibody or preimmune serum. The molecular weight standards are indicated. The arrow points to the position of the 123–128kDa PISlo proteins. (B) PISlo channels are found in the membranes of neuronal somata. Confocal optical section from whole-mount STG preparations stained with anti-pSlo antibody. Note that the neuronal somatic membrane stains intensely. (C) PISlo channels are found in the initial segment of the neurite. Cryostat section stained with anti-pSlo antibody. The arrow points to the initial segment of a neuronal neurite. (D) PISlo channels are localized in the fine neuropil of the PD neuron but not in the large branches of the neurites. One PD neuron was injected with neurobiotin. Cryostat sections were stained with anti-pSlo antibody and Streptavidin. The arrows indicate large branches of neurite; the * indicates the fine neuropil. (E) High magnification of the fine neuropils. Note that PISlo staining (green) overlapped with neurobiotin/Streptavidin (red) staining. Fig. 3Ea and 3Eb are from different ganglia. Fig 3Ec is the high magnification of the box area in Fig 3D.

Fig. 4. PISlo channels are localized in or near presynaptic terminals
(A and B) PISlo/Synapsin double staining of a cryostat section. PISlo staining appears green **(A)**. Synapsin staining appears red **(B)**. **(C)** Merged image. Bottom panels showed the boxed region in the top panels at 63X magnification. The arrows indicate the co-labeling regions.

Fig. 5. PISlo channels are found in STG axons

Horizontal optical section from the distal region of an anti-pSlo-stained Dorsal Ventricular Nerve (dvn) which exits from the STG.

Table 1

Splice site composition of the 13 full-length clones.

Clone	ORF Length (bp)	Amino acid	MW (kD)	X1	X2	X3	X4	X5	X6	X7
A18	3354	1117	124.9	c	c	c	f	c	b	b
A27	3324	1107	123.6	a	a	b	g	a	a	a
A49	3354	1117	124.8	c	a	b	f	c	b	b
B1	3336	1111	124.2	a	a	b	g	c	a	b
B45	3351	1116	124.7	d	a	b	f	c	b	b
C35	3333	1110	124.2	a	a	b	g	c	b	b
D2	3357	1118	125	c	a	b	f	c	a	b
E31	3396	1131	126.3	d	a	b	b	d	b	b
E52	3453	1150	128.2	b	a	b	g	b	c	a
H72	3327	1108	123.6	a	a	b	f	b	a	b
L15	3495	1164	130	a	a	a	f	d	c	b
L19	3354	1117	124.6	b	a	b	a	a	a	b
L37	3402	1133	126.1	a	a	a	a	a	a	a

Letters for each splicing site correspond to those described in Fig 1B. The total ORF nucleotide length, amino acid length, molecular weight are provided for each clone.