Published in final edited form as:

Genes Brain Behav. 2015 July ; 14(6): 454–465. doi:10.1111/gbb.12229.

Putative calcium-binding domains of the *Caenorhabditis elegans* BK channel are dispensable for intoxication and ethanol activation

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Abstract

Alcohol modulates the highly conserved, voltage- and calcium-activated potassium (BK) channel, which contributes to alcohol-mediated behaviors in species from worms to humans. Previous studies have shown that the calcium-sensitive domains, RCK1 and the Ca²⁺ bowl, are required for ethanol activation of the mammalian BK channel *in vitro*. In the nematode *Caenorhabditis elegans*, ethanol activates the BK channel *in vivo*, and deletion of the worm BK channel, SLO-1, confers strong resistance to intoxication. To determine if the conserved RCK1 and calcium bowl domains were also critical for intoxication and basal BK channel-dependent behaviors in *C. elegans*, we generated transgenic worms that express mutated SLO-1 channels predicted to have the RCK1, Ca²⁺ bowl or both domains rendered insensitive to calcium. As expected, mutating these domains inhibited basal function of SLO-1 *in vivo* as neck and body curvature of these mutants mimicked that of the BK null mutant. Unexpectedly, however, mutating these domains singly or together in SLO-1 had no effect on intoxication in *C. elegans*. Consistent with these behavioral results, we found that ethanol activated the SLO-1 channel *in vitro* with or without these domains. By contrast, in agreement with previous *in vitro* findings, *C. elegans* harboring a human BK channel with mutated calcium-sensing domains displayed resistance to intoxication. Thus, for the worm SLO-1 channel, the putative calcium-sensitive domains are critical for basal *in vivo* function but unnecessary for *in vivo* ethanol action.

Keywords

Alcohol; behavior; BK channel; *Caenorhabditis elegans*; ethanol; SLO-1

Ethanol elicits intoxication in part by acting on membrane-bound ion channels (Harris *et al.* 2009; Morozova *et al.* 2012). The large-conductance, voltage- and Ca²⁺-activated potassium (BK) channel has emerged as a key mediator of alcohol behaviors across species. Genetic alteration of BK channel function modulates acute behavioral tolerance in *Drosophila* and mice (Cowmeadow *et al.* 2005, 2006; Martin *et al.* 2008) as well as the escalation of ethanol

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consumption during withdrawal periods in mice (Kreifeldt *et al.* 2013). In the nematode *Caenorhabditis elegans*, knocking out the *slo-1* gene that encodes the BK channel produces strong resistance to intoxication (Davies *et al.* 2003). Conversely, worms that carry gain-of-function mutations in the BK channel are hypersensitive to intoxication (Davies *et al.* 2003), and alcohol triggers dyskinesia in humans with a gain-of-function mutation in the BK channel (Du *et al.* 2005).

Clinically relevant concentrations of alcohol activate the BK channel in different species, suggesting a conserved mechanism of activation. Examples include homologues of the BK channel in *C. elegans* (Davies *et al.* 2003), mice (Dopico *et al.* 1998; Liu *et al.* 2008) and humans (Yuan *et al.* 2008). Although the molecular interactions between ethanol and the BK channel remain largely unknown, channel activation occurs in reconstituted lipid bilayers, suggesting a direct ethanol action on the channel that does not require diffusible second messengers or accessory subunits (Chu *et al.* 1998; Dopico, 2003).

Ethanol modulates the mammalian BK channel *in vitro* independent of voltage and magnesium gating (Liu *et al.* 2008). However, Ca^{2+} must be present intracellularly for ethanol to activate the channel (Liu *et al.* 2008). Consistent with this finding, abolishing Ca^{2+} sensitivity of the two calcium-binding domains, RCK1 and the Ca^{2+} bowl, via mutation of negatively charged aspartate residues prevents ethanol action on the mammalian BK channel *in vitro* (Liu *et al.* 2008). The importance of these domains in the behavioral responses to ethanol remains to be tested.

We recently determined that mutating residue T381 of the worm BK channel, SLO-1, conveyed dramatic resistance to intoxication (Davis *et al.* 2014). In addition, we transgenically expressed the human BK channel in a *slo-1(null)* mutant background, and found that the equivalent residue of the mammalian BK channel (T352) is critical for ethanol activation of the BK channel and intoxication in *C. elegans*. This conserved residue is positioned 15 residues upstream of the two critical RCK1 Ca^{2+} -sensitive aspartate residues in mouse and human BK channels. These residues are conserved in the worm BK channel. Thus, disruption of RCK1 by the T381 mutation might account for the resistance to intoxication, as RCK1 disruption is predicted to restrict ethanol action on the channel in mammalian *in vitro* studies (Liu *et al.* 2008). However, the T381/352I mutation that abolished behavioral ethanol sensitivity did not alter other worm behaviors that are dependent on BK channel function (Davis *et al.* 2014). This contrasts with RCK1 mutations restricting channel gating in mammalian BK channels (Cui *et al.* 2009). Therefore, it remains to be seen if RCK1 and the Ca^{2+} bowl of the worm BK channel must remain intact for behavioral intoxication to occur in the worm.

Here, we investigate the function of the putative Ca^{2+} -sensitive domains of the worm BK channel in mediating ethanol intoxication and basal BK channel-dependent behaviors. Both the RCK1 and Ca^{2+} bowl domains appear to be important for basal behaviors in *C. elegans*, but are surprisingly unnecessary for intoxication and activation by ethanol *in vitro*. By contrast, *C. elegans* harboring a human BK channel with mutated calcium-sensing domains displayed resistance to intoxication.

Materials and methods

Animals

Caenorhabditis elegans strains were grown at 20°C and fed the OP50 bacterial strain on seeded nematode growth media (NGM) agar plates as described previously (Brenner 1974). Worms cultured on plates contaminated with fungi or other bacteria were excluded. The reference wild-type (WT) strain was N2 Bristol. The reference *slo-1(null)* strain was NM1968, harboring the previously characterized null allele, *js379* (Wang *et al.* 2001).

Transgenesis

The background for all transgenic worms generated in this study used the *slo-1(null)* strain NM1968 with allele *js379*. Multi-site GATEWAY technology (Invitrogen, Carlsbad, CA, USA) was used to construct plasmids. For WT transgenes, 2001 bp of the native *slo-1* promoter (*Pslo-1*) and the traditional *unc-54* UTR were used in combination with cDNA for *slo-1* isoform A tagged on the intracellular C terminal with the red fluorophore *mCherry* (Wang *et al.* 2001). Mutant versions were constructed using site-directed mutagenesis on this WT plasmid as described below. All WT and mutant *slo-1* plasmids were injected at 20 ng/μl. The co-injection reporter PCFJ90 [*Pmyo-2::mCherry*] (1.25 ng/μl) was used to ensure proper transformation. To control for variation in transgenesis, two independent strains were isolated for each transgenic experiment. Strains include: JPS344: *slo-1(js379); vxEx344[Pslo-1::slo-1::mCherry+Pmyo-2::mCherry]*, JPS345: *slo-1(js379); vxEx345[Pslo-1::slo-1::mCherry+Pmyo-2::mCherry]*, JPS360: *slo-1(js379); vxEx360[Pslo-1::slo-1(D391/396A)::mCherry+Pmyo-2::mCherry]*, JPS361: *slo-1(js379); vxEx361[Pslo-1::slo-1(D391/396A)::mCherry+Pmyo-2::mCherry]*, JPS350: *slo-1(js379); vxEx350[Pslo-1::slo-1(5D5N)::mCherry+Pmyo-2::mCherry]*, JPS351: *slo-1(js379); vxEx351[Pslo-1::slo-1(5D5N)::mCherry+Pmyo-2::mCherry]*, JPS358: *slo-1(js379); vxEx358[Pslo-1::slo-1(D391/396A+5D5N)::mCherry+Pmyo-2::mCherry]* and JPS359: *slo-1(js379); vxEx359[Pslo-1::slo-1(D391/396A+5D5N)::mCherry+Pmyo-2::mCherry]*. Note that D391/396A signifies mutation of the RCK1 domain, and 5D5N signifies mutation of the five positive aspartate residues of the Ca²⁺ bowl to neutral asparagines (Fig. 3). For the control transgenic strain, the plasmid PCFJ150 (the GATEWAY destination vector used to construct the transgenic worms) was injected at a concentration of 20 ng/μl, along with 1.25 ng/μl of PCFJ90 plasmid to generate strain JPS383: *vxEx383[Pmyo-2::mCherry]*.

Site-directed mutagenesis

QuikChange II XL mutagenesis kit (Stratagene, La Jolla, CA, USA) was used. The primers 5'-ccattttcttcaaaatttctacacgagaaccgtgatgacgtg ga-3' and 5'-ccattttcttcaagatttctacacgaggaccgtgatgacgtgga-3' were used to generate two A->G mutations in the *slo-1* gene that resulted in D391A and D396A amino acid substitutions. The primers 5'-atgtgcaattctctgaccagaacaacaacaataccggacaccg-3' and 5'-atgtgcaattctctgaccaggacgacgacgacgatccggacaccg-3' were used to induce five A > G mutations in the *slo-1* gene that resulted in 5D5N substitutions in the Ca²⁺ bowl. All plasmids were confirmed by sequencing the full cDNA.

Neck curvature assay

All behavioral assays were performed on age-matched day 1 adults. The movement of single worms was digitally recorded at 30 frames/second for 5–10 min and analyzed as described previously after 20 min on ethanol or control plates (Pierce-Shimomura *et al.* 2008). Briefly, custom-written software automatically recognizes the worm from each frame and assigns 13 points spaced equally along the midline of the body from the head to the tail. The neck angle was defined as the angle formed by the most anterior three points when the head of the worm was in the ventral-most position while crawling on an unseeded NGM agar plate. The average value was computed by taking three to six independent neck curvature images of at least 10 individuals from each genotype.

Overall body curvature assay

Worm movement was recorded as described in the neck posture assay. The 13 points spaced equally along the midline of the body from the head to the tail were used to gather 11 body angles. To compare total body posture, we computed a single body curvature metric defined as the absolute sum of all angles. The average value was computed by taking three to six independent neck curvature images of at least 10 individuals from each genotype.

Egg-laying response to ethanol

Plastic Petri plates (6-cm diameter) filled with NGM agar (12 ml) were seeded with *Escherichia coli* at least 20 h before the assay and stored at 4°C for not more than 2 weeks. Plates were brought to room temperature (20°C) 1 h before testing. Ethanol plates were prepared by adding 200-proof ethanol (Sigma Aldrich, St. Louis, MI, USA) beneath the agar 30 min before the assay to allow ethanol time to soak into the agar. For 600-mm plates, 420 µl was added. At the start of the assay, 10 young adult worms were placed on a control plate containing no ethanol. After 1 h, worms were then transferred to an ethanol plate for another hour. The number of eggs laid by the 10 worms was counted on each plate. Average egg-laying frequencies per worm were determined for untreated and ethanol-treated conditions.

Locomotion response to ethanol

For all SLO-1 strains, locomotor ability was assayed using the net distance traveled from a central point. Plates were prepared as described above, using 600-mm EtOH plates seeded with 200 µl of OP50. At the start of the assay, five young adult worms were placed in the center of a seeded plate containing no ethanol. Their position was marked after 5 min. The worms then spent 20 min on an ethanol plate prior to measuring the distance traveled in 5 min on a fresh seeded ethanol plate. For each worm, the distance from the center (mm) was recorded as a measure of worm locomotion for untreated and ethanol-treated conditions.

Because worms expressing HSLO(DM) moved poorly, to gain power, all double domain mutant strains' locomotor abilities were also assayed by tracking the total distance traveled. Crawl behavior was assayed on 6-cm diameter plastic Petri dishes filled with unseeded NGM agar (12 ml). Ethanol plates (400 mm) were infused with 280 µl of 200-proof ethanol (Sigma Aldrich) 30 min before the start of the assay. Copper rings embedded in the agar corralled worms for ease of videoing behavior. Before starting the assay, all worms were cleaned of bacteria by allowing them to crawl on an unseeded plate until they left no tracks.

Next, 15 worms were placed in each copper ring and ethanol naïve crawl behavior was recorded for 2 min (2 frames/second). The worms were then moved to an ethanol-infused plate for 20 min, after which ethanol-treated behavior was recorded for 2 min. The distance each worm crawled per minute was measured using a semi-automated procedure in Imagepro v7.1 (Media Cybernetics, Rockville, MD, USA). Crawl speed of ethanol-treated worms was normalized to the group mean ethanol naïve crawl speed, and data are presented as normalized mean $\pm$ SEM.

Confocal microscopy

Day 1 adult worms were mounted on 2% agarose pads with 1 mM sodium azide as anesthetic and imaged with a Zeiss laser-scanning microscope (LSM710) using Zen (black edition) acquisition software (Carl Zeiss, Oberkochen, Germany). GFP fluorescence and phase contrast images were collected using a 488 laser, and mCherry fluorescence was collected using a 561 laser. At $\times 20$, slices were taken every 1.7 μ using a 1.7- μ pinhole. At $\times 63$ (water immersion objective), slices were taken every 0.8 μ using a 0.9- μ pinhole.

Electrophysiology

Wild-type and double-mutant (DM) SLO-1a were expressed heterologously in oocytes for electrophysiological recordings. Briefly, *Xba*I linearized DNA (addgene plasmid mg180) was transcribed *in vitro* (mMessage mMachine kit, Life Technologies, Grand Island, NY, USA). Oocytes were injected with 5–20 ng of DNA. Electrodes (8–12 MOhms) were filled with, in mM: 136 KOH, 2 KCl, 2 MgCl₂, 20 HEPES, pH 7.2 with methanesulfonic acid. Inside-out patches were recorded in a bath containing, in mM: 132 KOH, 6 KCl, 20 HEPES, 5 HEDTA, 0.005 free Ca²⁺ from 2.644 CaCl₂, pH 7.2 with methanesulfonic acid. Electrophysiological recordings from patches containing one to a few channels were made with an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA, USA; filtered at 5 kHz) at 50 kHz sampling using custom macros in Igorpro v6.0 (Wavemetrics, Lake Oswego, OR, USA). The probability of opening (P_o) was averaged over multiple 3-second traces during 5 min immediately prior to 50 mM ethanol application, and for ~5 min of peak increase in P_o after ethanol application (QuB, Buffalo, NY, USA). For WT SLO-1 channels, the P_o is reported for 80 mV; for SLO-1(DM) channels, P_o is reported for 100–150 mV because the DM channel was not sufficiently open at 80 mV.

Statistical analysis

Sigmaplot 12.5 was used for all statistical analyses to determine significance ($P < 0.05$, two-tailed) between two or more groups. If the groups being compared passed the Shapiro–Wilk normality test, they were analyzed using standard *t*- or analysis of variance (anova) tests where appropriate. If needed, *post hoc* multiple comparisons were performed using the Holm–Sidak method. If the groups being compared did not pass the Shapiro–Wilk normality test, the groups were analyzed using the Mann–Whitney rank sum test (unpaired) or Wilcoxon signed-rank *t*-test (paired) or Kruskal–Wallis anova on ranks where appropriate. If needed, *post hoc* multiple comparisons were performed with the Dunn’s method. The ethanol-induced change in probability of channel opening relative to baseline was tested using a *z*-test.

Results

Putative Ca²⁺-sensing domains in the worm BK channel are not required for intoxication

Following acute ethanol exposure, *C. elegans* displays intoxication by decreasing rates of egg laying and locomotion. Similar to previous reports (Davies *et al.* 2003; Davis *et al.* 2014), we found that *slo-1(null)* mutant worms were markedly resistant to the inhibitory effects of ethanol on egg laying (Fig. 1; Kruskal–Wallis anova, genotype, $H = 111.19$, $df = 10$, $P < 0.001$; Dunn's multiple comparison, *slo-1(null)* vs. WT, $P < 0.05$; $n = 32–43$). In addition, *slo-1(null)* mutant worms were strongly resistant to the inhibitory effects of ethanol on locomotion (Fig. 2; Kruskal–Wallis anova, genotype, $H = 36.65$, $df = 10$, $P < 0.001$; Dunn's multiple comparison, *slo-1(null)* vs. WT, $P < 0.05$; $n = 17$). Sensitivity to ethanol for egg laying could be restored in the *slo-1(null)* mutant by transgenically expressing *slo-1* cDNA under the endogenous *Pslo-1* promoter for two independently derived strains (#1 and #2) (Fig. 1; Dunn's multiple comparison, *slo-1(null)* vs. *slo-1(+)*#1, n.s.; *slo-1(null)* vs. *slo-1(+)*#2, n.s.; $n = 12–43$). However, the slightly lower level of egg laying exhibited by the *slo-1(null)* was not rescued. Additionally, the inhibitory effects of ethanol on locomotion were restored to WT levels after rescue with WT *slo-1* for both strains. (Fig. 2; Dunn's multiple comparison, *slo-1(null)* vs. *slo-1(+)*#1, n.s.; *slo-1(null)* vs. *slo-1(+)*#2, n.s.; $n = 17–20$). By contrast, a control fluorescent reporter transgene (*Co. inj*) failed to rescue ethanol sensitivity to egg laying (Fig. 1; Dunn's multiple comparison, *slo-1(null)* vs. *Co. inj*, $P < 0.05$; $n = 6–43$) and locomotion (Fig. 2; Dunn's multiple comparison, *slo-1(null)* vs. *Co. inj*, $P < 0.05$; $n = 17–19$).

The Ca²⁺-sensitive domains of the mammalian BK channel were previously shown to be important for ethanol action on channel gating (Liu *et al.* 2008). To test the importance of these two domains, RCK1 and the Ca²⁺ bowl, in the worm BK channel *in vivo*, we attempted to rescue intoxication behaviors in the *slo-1(null)* mutant with versions of the SLO-1 channel containing missense mutations of aspartate residues in these conserved putative Ca²⁺-binding domains (underlined in Fig. 3). As above, we used the native *slo-1* promoter to express the transgenes and generated two independently derived strains for each transgene to control for variation in transgenesis.

First, we tested the role of the RCK1 domain *in vivo* by generating transgenic strains expressing *slo-1(RCK1)*, which contained two mutations (D391A and D396A, equivalent to mouse D362A and D367A, respectively), in a *slo-1(null)* mutant background. These mutations were previously shown to strongly reduce Ca²⁺ sensitivity of the mammalian BK channel and RCK-type domains in other proteins (Cui *et al.* 2009; Smith *et al.* 2013). Two transgenic strains (#1 and #2) were generated to control for variation in transgenesis. We found that ethanol sensitivity of egg laying and locomotion in both transgenic *slo-1(RCK1)* mutant strains resembled WT levels for both egg laying (Fig. 1; Dunn's multiple comparison, *slo-1(null)* vs. *slo-1(RCK1)*#1, n.s.; *slo-1(null)* vs. *slo-1(RCK1)*#2, n.s.; $n = 6–43$) and locomotion (Fig. 2; Dunn's multiple comparison, *slo-1(null)* vs. *slo-1(RCK1)*#1, n.s.; *slo-1(null)* vs. *slo-1(RCK1)*#2, n.s.; $n = 17–20$). The *slo-1(RCK1)* rescue strains displayed a slightly lower displacement than the background strain. This may reflect a

consequence of slight overexpression of *slo-1* observed in previous studies (Davies *et al.* 2003).

Second, we tested the role of the Ca^{2+} bowl domain *in vivo* by generating transgenic strains expressing *slo-1(Ca²⁺ bowl)*, which contained mutations that neutralize the five negatively charged aspartate residues (D969–973, equivalent to mouse D897–901) to asparagine, in a *slo-1(null)* mutant background. These 5D5N mutations were previously shown to abolish Ca^{2+} sensitivity of the Ca^{2+} bowl in the mammalian and invertebrate BK channel (Bian *et al.* 2001; Schreiber & Salkoff 1997). Two transgenic strains (#1 and #2) were generated to control for variation in transgenesis. Similar to the results with the *slo-1(RCK1)* mutants, the two different *slo-1(Ca²⁺ bowl)* mutants displayed WT ethanol sensitivity of egg laying (Fig. 1; Dunn's multiple comparison, *slo-1(null)* vs. *slo-1(Ca²⁺ bowl)#1*, n.s.; *slo-1(null)* vs. *slo-1(5D5N)#2*, n.s.; $n = 6-43$) and locomotion (Fig. 2; Dunn's multiple comparison, *slo-1(null)* vs. *slo-1(Ca²⁺ bowl)#1*, n.s.; *slo-1(null)* vs. *slo-1(5D5N)#2*, n.s.; $n = 17-20$).

Our success in restoring ethanol sensitivity with SLO-1(RCK1) and SLO-1(Ca^{2+} bowl) mutant channels suggested that each putative Ca^{2+} -sensitive domain may act singly to mediate intoxication in *C. elegans*. Alternatively, but in contrast to the mammalian model of ethanol action on BK channels, neither domain may be required for intoxication. To test this idea, we expressed a double-mutant *slo-1(DM)* transgene, which contained both sets of calcium-domain mutations, in a *slo-1(null)* mutant background. These combined mutations were shown to eliminate all Ca^{2+} sensitivities (within the physiological Ca^{2+} concentration range) of the mammalian BK channel *in vitro* (Xia *et al.* 2002). Surprisingly, we found that ethanol sensitivity was rescued for two *slo-1(DM)* mutant strains, as measured by egg laying (Fig. 1; DM; Dunn's multiple comparison, *slo-1(null)* vs. *slo-1(DM)#1*, n.s.; *slo-1(null)* vs. *slo-1(DM)#2*, n.s.; $n = 6-43$) and locomotion (Fig. 2; Dunn's multiple comparison, *slo-1(null)* vs. *slo-1(DM)#1*, n.s.; *slo-1(null)* vs. *slo-1(DM)#2*, n.s.; $n = 17-19$).

Ca^{2+} -sensing domains in the human BK channel are required for intoxication

Our unexpected results described above showing that the putative calcium domains of the worm BK channel are dispensable for intoxication made us question whether the human BK channel with mutations in the calcium-binding domains can support intoxication if expressed in *C. elegans*. To test this possibility, we generated transgenic strains in a *slo-1(null)* background that expressed either the WT human BK channel (*hslo*) or the human BK channel with D362/367A+5D5N mutations (DM) to abolish calcium sensing in both RCK1 and Ca^{2+} bowl domains. Both transgenic channels were tagged with the fluorophore mCherry to observe expression. Consistent with our previous results (Davis *et al.* 2014), we found that the WT HSLO channel rescued ethanol sensitivity of the *slo-1(null)* mutant for egg laying (Fig. 4a,b; Kruskal–Wallis anova, genotype, $H = 30.183$, $df = 5$, $P < 0.01$; Dunn's multiple comparison, *slo-1(null)* vs. *hslo#1* $P < 0.05$; *slo-1(null)* vs. *hslo#2*, $t_{(19)} = 2.69$, $P < 0.05$; $n = 6-12$). However, in contrast to our findings described above with the worm SLO-1 channel, we found that the HSLO(DM) channel failed to rescue intoxication for egg laying (Fig. 4a,b; Kruskal–Wallis anova, genotype, $df = 5$, $P > 0.05$; *slo-1(null)* vs. *hslo(DM)#1* and *slo-1(null)* vs. *hslo(DM)#2*; $n = 16-122$). Identical results were found for locomotion (Fig. 4c,d). We compared the speed of worms rather than the displacement because *hslo*

expression interfered with this latter measure. Expression of WT HSLO rescued ethanol sensitivity for crawling speed but expression of HSLO(DM) did not (Fig. 4c,d; Kruskal–Wallis anova, genotype, $H = 99.092$, $df = 5$, $P < 0.001$; Dunn's multiple comparison, *slo-1(null)* vs. *hslo(+)*#1 and *slo-1(null)* vs. *hslo(+)*#2, $P < 0.05$; *slo-1(null)* vs. *hslo(DM)*#1 and *slo-1(null)* vs. *hslo(DM)*#2; $n = 16$ –122). The lack of rescue could not be easily explained by a failure of expression because these transgenic worms showed similar levels of mCherry-tagged HSLO and HSLO(DM) expression when evaluated with confocal microscopy (Fig. 5).

Putative Ca^{2+} -sensing domains in the worm BK channel are together necessary and functionally redundant for basal behaviors

Our results described above suggested that mutations in the putative Ca^{2+} -sensitive domains of the worm BK channel may not compromise channel function as severely as shown for mammalian channels *in vitro*. To test this possibility, we assessed the performance of two basal behaviors that depend on the SLO-1 channel and presumably the two putative calcium domains. First, we tested neck curvature. The *slo-1(null)* mutant worms exhibit a 'crooked neck' characterized by an abnormally greater degree of bending of the worm's head (Fig. 6; Kim *et al.* 2009). We found that the crooked-neck phenotype of the *slo-1(null)* mutant was rescued with a WT *slo-1(+)* transgene in two strains (Fig. 6b; Holm–Sidak multiple comparison, *slo-1(null)* vs. *slo-1(+)*#1, $t_{(51)} = 1.32$, n.s.; *slo-1(null)* vs. *slo-1(+)*#2, $t_{(49)} = 0.46$, n.s.; $n = 15$ –36). By contrast, a control fluorescent reporter transgene did not rescue this phenotype (Fig. 6b; Holm–Sidak multiple comparison, *slo-1(null)* vs. *Co. inj.*, $t_{(67)} = 6.80$, $P < 0.001$; $n = 33$ –36). The crooked-neck curvature was also fully or partially rescued with *slo-1* transgenes containing mutations in either the RCK1 domain (Fig. 6b; Holm–Sidak multiple comparison, *slo-1(null)* vs. *slo-1(RCK1)*#1, $t_{(63)} = 0.61$, n.s.; *slo-1(null)* vs. *slo-1(RCK1)*#2, $t_{(50)} = 0.63$, n.s.; $n = 16$ –36) or the Ca^{2+} bowl (Fig. 6; Holm–Sidak multiple comparison, *slo-1(null)* vs. *slo-1(Ca²⁺ bowl)*#1, $t_{(51)} = 1.95$, n.s.; *slo-1(null)* vs. *slo-1(Ca²⁺ bowl)*#2, $t_{(59)} = 1.31$, n.s.; $n = 17$ –36). Interestingly, neck curvature was not rescued with the *slo-1(DM)* transgene (Fig. 6; Holm–Sidak multiple comparison, *slo-1(null)* vs. *slo-1(DM)*, $t_{(50)} = 3.02$, $P < 0.05$; *slo-1(null)* vs. *slo-1(DM)*#2, $t_{(49)} = 3.12$, $P < 0.05$; $n = 15$ –36). Thus, the basal function of the worm BK channel is severely compromised *in vivo* only when both of these putative Ca^{2+} -sensitive domains are mutated.

Second, as an independent measure of basal BK channel function, we tested body curvature. Body curvature requires a distinct combination of neurons and muscles from neck curvature (Kim *et al.* 2009). Quantitative assessment of curvature showed that six different body positions showed greater curvature in *slo-1(null)* vs. WT (dorsal and ventral were assigned positive and negative values; Fig. 7a; paired *t*-test, point 5, $t_{(84)} = 2.70$, $P < 0.01$; points 1,4,8,9 and 11, Mann–Whitney rank sum test, $U = 323$ –664, $P < 0.05$ – $P < 0.001$; $n = 36$ –50). To conveniently compare overall body curvature of individual worms, we computed a single body curvature metric as the sum of the absolute value of the 11 angles along the midline of the worm. The *slo-1(null)* mutant strain displayed significantly higher body curvature when compared with WT (Fig. 7b; Mann–Whitney rank sum test, $U = 119$, $P < 0.001$; $n = 36$ –50). We used the body curvature metric as another measure to quantitatively

compare the degree of rescue for each transgenic strain vs. WT and *slo-1(null)* mutant strains.

As observed above for neck curvature, body curvature of the *slo-1(null)* mutant was rescued with WT as well as with the RCK1 or Ca²⁺ bowl single-mutant versions of SLO-1 (Fig. 7c; Kruskal–Wallis anova, genotype, $H = 152.68$, $df = 10$, $P < 0.001$; Dunn's multiple comparison, *slo-1(null)*, $t_{(84)} = 7.40$, $P < 0.05$; *slo-1(null)* vs. *slo-1(+)*#1, $t_{(51)} = 2.60$, n.s.; *slo-1(null)* vs. *slo-1(+)*#2, $t_{(49)} = 1.15$, n.s.; *slo-1(null)* vs. *slo-1(RCK1)*#1, $t_{(63)} = 1.38$, n.s.; *slo-1(null)* vs. *slo-1(RCK2)*#2, $t_{(50)} = 0.40$, n.s.; *slo-1(null)* vs. *slo-1(Ca²⁺ bowl)*#1, $t_{(51)} = 2.56$, n.s.; *slo-1(Ca²⁺ bowl)*#2, $t_{(59)} = 1.23$, n.s.; $n = 15$ –50). By contrast, we found that SLO-1 with both Ca²⁺-sensitive domains mutated failed to rescue body curvature for *slo-1(null)* (Fig. 7c; Dunn's multiple comparison, *slo-1(null)* vs. *slo-1(DM)*#1, $t_{(64)} = 0.78$, n.s.; *slo-1(null)* vs. *slo-1(DM)*#2, $t_{(63)} = 1.25$, n.s.; *slo-1(null)* vs. *Co. inj* $t_{(67)} = 7.49$, $P < 0.05$; $n = 15$ –50). This indicates that basal BK channel function was severely compromised *in vivo* only after mutation of both domains.

To test whether the Ca²⁺-sensitive domains are also dually required for basal *in vivo* function of the human BK channel, we measured neck curvature for human BK channel transgenic strains. As expected, we rescued neck curvature in *slo-1(null)* with WT HSLO. The degree of rescue was partial or full depending on the independently derived transgenic strain (Fig. 7; Kruskal–Wallis anova, genotype, $H = 55.053$, $df = 6$, $P < 0.001$; Dunn's multiple comparison, *slo-1(null)* vs. *hslo(+)*#1, $t_{(58)} = 2.60$, n.s.; *slo-1(null)* vs. *hslo(+)*#2, $t_{(19)} = 1.67$, n.s.; $n = 23$ –36). By contrast, we found that HSLO with both Ca²⁺-sensitive domains mutated failed to rescue neck curvature in two strains (Fig. 7; Dunn's multiple comparison, *slo-1(null)+hslo(DM)*#1, $t_{(54)} = 3.97$, $P < 0.05$; *slo-1(null)* vs. *hslo(DM)*#2, $t_{(56)} = 4.69$, $P < 0.05$; $n = 19$ –36). These results are consistent with a conserved synergistic role for the two Ca²⁺-sensitive domains in basal *in vivo* behavior. Due to incomplete rescue in whole body curvature of strains expressing the WT human BK channel, we did not examine differences in *hslo(+)* and *hslo(DM)* strains compared with WT worms.

Putative Ca²⁺-sensing domains in the worm BK channel are not required for activation by ethanol

Our behavioral analyses suggested that basal behaviors depend on the two putative calcium domains. Perhaps, unlike the mammalian BK channel's dependence on the calcium-binding domains for ethanol sensitivity, ethanol action on the worm BK channel can occur independent of a functional RCK1 and Ca²⁺ bowl? To test this idea, we performed patch-clamp electrophysiology on SLO-1 channels expressed heterologously in oocytes. We assayed both WT and DM mutant versions of the channel. We found that mutation of the two putative calcium-binding domains increased the voltage required for activation (Fig. 8a), consistent with the expected synergistic role of intracellular calcium and membrane voltage in channel gating. Application of 50-mm ethanol raised open probability for both the WT and DM SLO-1 channels (Fig. 8b). In both cases, four of the five patches showed an increase in open probability (P_o) after ethanol application. On average, the SLO-1(WT) channel increased P_o by 2.2 ± 0.57 ($P < 0.01$) and the SLO-1(DM) channel increased P_o by 2.4 ± 0.67 ($P < 0.005$). Thus, the behavioral intoxication observed for the SLO-1(DM) may

be simply explained by a retained ability for direct activation of the SLO-1(DM) channel by ethanol.

Discussion

The RCK1 and the Ca^{2+} bowl domains are critical for normal calcium sensitivity in mammalian BK channels (Cui *et al.* 2009; Smith *et al.* 2013; Xia *et al.* 2002). In this study, we examined the functional role of these two conserved intra-cellular domains of the worm BK channel, SLO-1, in basal and ethanol-mediated behaviors. Our most salient finding was that these purported Ca^{2+} -sensing residues of the worm BK channel were critical for normal *in vivo* basal function, but not essential for *in vivo* action of ethanol on the channel. Supporting these findings, electrophysiological recordings of heterologously expressed SLO-1 channels showed ethanol sensitivity of channel gating when these domains were intact or mutated. Thus, our results support the idea that ethanol influences gating of the worm BK channel in the absence of intact RCK1 and Ca^{2+} bowl domains both *in vivo* and *in vitro*.

We found that the RCK1 and Ca^{2+} bowl domains were necessary for proper SLO-1 channel function *in vivo*. Introducing mutations in both of these domains in the worm and human BK channel perturbed basal behaviors (neck and/or body curvature) that depend on proper function of SLO-1 in the worm. Xia *et al.* (2002) previously reported that mutating both RCK1 (D362/367A) and the Ca^{2+} bowl (5D5N) in heterologously expressed mammalian BK channels dramatically shifted the voltage range of activation to non-physiological levels (e.g. voltage of half-activation $>+120$ mV). Thus, mutating the same negatively charged intracellular aspartate residues knocked out *in vivo* function of the SLO-1 channel and impaired *in vitro* function of mammalian BK channels. Interestingly, we also found that mutating both of these domains necessitated higher voltages for activating SLO-1 channels during our electrophysiological recordings *in vitro*. This suggested that, as for mammalian channels, calcium sensitivity of the RCK1 and Ca^{2+} bowl domains supports channel activity in a physiological voltage range for worm BK channels.

Although knocking out both domains was deleterious, we found that leaving either the RCK1 or Ca^{2+} bowl domain intact recapitulated WT worm behavior, suggesting relatively normal *in vivo* SLO-1 function. For mammalian BK channels, these domains contribute to Ca^{2+} sensitivity via distinct mechanisms (Cui *et al.* 2009). It is unlikely that the loss of either domain completely eliminated Ca^{2+} sensitivity of the SLO-1 channels expressed *in vivo*. Nonetheless, neutralizing the aspartate residues in either RCK1 or the Ca^{2+} bowl reduces Ca^{2+} sensitivity of mammalian BK channels recorded *in vitro*. This reduction in Ca^{2+} sensitivity, at least with Ca^{2+} bowl mutation, is shared between species, including *Drosophila*, mouse and human BK channels (Bian *et al.* 2001; Savalli *et al.* 2012; Zeng *et al.* 2005). Given the likely reduction in Ca^{2+} sensitivity for the single Ca^{2+} -binding domain mutants, then, why did such a change in channel properties leave SLO-1-dependent behaviors intact? One possibility is that the transgenic strains can successfully compensate for the reduced function of BK channels with neutralized aspartate residues in either domain, but not for both. For example, increased expression of partially functional BK channels may be able to rescue posture in the transgenic rescue strains. Another possibility

is that because WT invertebrate BK channels differ in Ca^{2+} sensitivity with mammalian channels (Bian *et al.* 2001; Johnson *et al.* 2011), the loss of a single Ca^{2+} -binding domain does not dramatically alter channel function under physiological conditions. Specifically, Johnson *et al.* (2011) found that WT SLO-1 channels showed less activation at low intracellular calcium concentrations than mammalian BK channels (Xia *et al.* 2002). Thus, while Ca^{2+} sensitivity may be less important for normal physiological function for worm than mammalian BK channels, our results support an important physiological role for these conserved domains from worms to humans.

Previous electrophysiological recordings of heterologously expressed mammalian BK channels indicated that calcium binding to RCK1 influenced ethanol-induced changes in gating (Liu *et al.* 2008). While neither mutation of the RCK1 domain nor of the Ca^{2+} bowl alone disrupted ethanol-induced changes in gating at intracellular Ca^{2+} levels below 100 μM , RCK1 domain mutation reduced ethanol-induced changes at higher Ca^{2+} concentrations (Liu *et al.* 2008). Thus, Ca^{2+} binding to the Ca^{2+} bowl appears to be dispensable for both ethanol modulation of human BK channel gating *in vitro* (Liu *et al.* 2008) and for intoxication behavior linked to ethanol modification of SLO-1 channel gating *in vivo* shown here. In contrast, a comparison with our present findings indicates a potential divergence in sensitivity to RCK1 mutation for ethanol-induced changes in HSLO and SLO-1 gating. RCK1 mutation in human BK channels inhibited ethanol-induced changes in channel gating *in vitro* (Liu *et al.* 2008), but we found the same mutation in SLO-1 left behavioral intoxication intact *in vivo*. Such differences could arise from inherent differences in channel biophysics, but differences in the lipid environment, local intracellular Ca^{2+} levels and/or the presence of endogenous binding partners may instead alter SLO-1 ethanol sensitivity *in vivo*.

Our findings more strongly support the idea that combined RCK1 and Ca^{2+} bowl mutation in the human and worm BK channel differentially regulate ethanol sensitivity of channel gating. We found that our transgenic strains expressing the SLO-1 channel containing mutations in both domains exhibited normal intoxication. Moreover, our electrophysiological recordings showed that SLO-1 channels with both RCK1 and Ca^{2+} bowl mutations retained sensitivity to ethanol *in vitro*. By contrast, transgenic strains expressing the human BK channel with both calcium-sensing domains mutated failed to respond to ethanol *in vivo*. This latter finding is consistent with previous electrophysiological recordings, which showed that ethanol failed to modulate the mammalian BK channel when both the RCK1 and Ca^{2+} bowl domains were neutralized (Liu *et al.* 2008). Taken together, these findings suggest that combined neutralization of RCK1 and Ca^{2+} bowl interferes with the ability of ethanol to bind or influence channel gating in the worm BK channel, but not the human BK channel.

Despite potential differences in sensitivity to Ca^{2+} gating, the high level of BK channel sequence conservation across species suggests that ethanol modulation occurs through a largely conserved mechanism. The BK channel is highly conserved with 58% protein sequence similarity between the worm and mammalian BK channels (Wang *et al.* 2001). Only two residues in the RCK1 calcium-sensing domain are highly dissimilar between the worm and mammalian channel. Conservation in the Ca^{2+} bowl is even higher, with only one

amino acid differing in this region. Our recent work established the importance of a conserved residue in the 5' region to the RCK1 domain in the human and worm BK channels' responses to ethanol. Mutating a conserved threonine (T381) to an isoleucine in SLO-1 produced mutant worms that were extremely resistant to intoxication, but otherwise normal for other behaviors dependent on BK channel function (Davis *et al.* 2014). Likewise, transgenic expression of the human BK channel containing the corresponding mutation (T352I) produced a similar resistance to intoxication. Moreover, this T352I mutant human channel was not potentiated by ethanol *in vitro* (Davis *et al.* 2014).

While high sequence conservation and a shared critical residue for ethanol sensitivity suggest a largely conserved mechanism for ethanol modulation, divergent residues may modify aspects of BK channel ethanol modulation across species. One interesting possibility is that a differential requirement for Ca²⁺ gating in ethanol binding or signal transduction underlies the potential ethanol modulation differences between the worm and mammalian BK channels discussed above. For example, in the absence of Ca²⁺ gating, the binding pocket for ethanol may be occluded in the human but not in the worm BK channel. Alternately, ethanol may be able to bind in both channels without Ca²⁺ gating, but signal transduction of the binding event may be restricted in the human channel. A recent report by Bukiya *et al.* (2014) established a putative ethanol-binding site involving residue K361 of the mouse BK channel. While this is at the opposite end of the same alpha helix as T352, a key residue for ethanol modulation in both worm and human BK channels, K361, is the one of the few residues in the RCK1 domain not conserved from the human to worm BK channel. Future studies employing *in vitro* patch-clamp and *in vivo* behavioral analysis will investigate if this residue contributes to the differential sensitivity to RCK1 and Ca²⁺ bowl mutation in the worm and mammalian BK channel response to ethanol. Additionally, exploring other RCK1 domain residue differences between the worm and mammalian channel may lead to novel insights into channel kinetics and ethanol activation of the BK channel.

Acknowledgments

Support for this study was provided by an NRSA award F31AA021641 to S. J. D by NIAAA, as well as the Waggoner Center, ABMRF, NIAAA R03AA020195 and R01AA020992 to J.T.P.-S. We thank the *Caenorhabditis* Genetic Center (funded by the NIH) Hoky Kim for reagents, J. Mayfield for critical editing and anonymous reviewers for constructive comments. The authors declare no competing financial interests

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Figure 1. Ca^{2+} -sensing domains of the *Caenorhabditis elegans* BK channel are not critical for ethanol-induced reduction in egg laying

(a) Intoxication sensitivity as determined by egg laying in control vs. ethanol plates for various strains: WT strain N2 ($N = 41$), *slo-1*(null) strain NM1968 ($N = 30$), *slo-1*(+) strains rescued with WT *slo-1* cDNA (#1 $N = 12$, #2 $N = 22$), *slo-1*(*RCK1*) strains rescued with *slo-1*(D3912/396A) mutant transgene (#1 $N = 6$, #2 $N = 11$), *slo-1*(Ca^{2+} bowl) strains rescued with *slo-1*(*5D5N*) transgene (#1 $N = 6$, #2 $N = 8$), *slo-1*(DM) strains rescued with *slo-1* transgenes that contained both sets of Ca^{2+} -domain mutations (#1 $N = 16$, #2 $N = 6$) and the co-injection control strain ($N = 6$) (N represents an assay with 10 worms each). All groups, except for the co-injection control, displayed a significant reduction in egg laying on ethanol plates compared with control plates (paired Wilcoxon signed rank t -test, $t = 2.00$, n.s.; $n = 6$; all other groups paired t -test/Wilcoxon signed rank t -test, $P < 0.05$; $n = 6-43$). Note that while the *slo-1*(null) strain displayed significant reduction in egg laying in the presence of ethanol, the effect was extremely small (mean values for control = 4.8 and ethanol = 4.1) compared with other strains. (b) Intoxication as determined by relative egg-laying sensitivity to ethanol in the strains in panel (a). The *slo-1*(null) and co-injection control strains were significantly less intoxicated than WT ($P < 0.05$). All Ca^{2+} -domain mutant BK channels rescued ethanol sensitivity when transgenically expressed in *slo-1*(null) worms. In both panels, bars represent SEM. #1 and #2 refer to independently isolated strains expressing the same cDNA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2. Ca²⁺-sensing domains of the *Caenorhabditis elegans* BK channel are not critical for ethanol-induced reduction in locomotion

(a) Displacement from a central starting location on control and ethanol plates for various strains: WT strain N2 ($n = 17$), *slo-1*(null) strain NM1968 ($n = 19$) and *slo-1* rescue strains (#1 $n = 18$, #2 $n = 20$), *slo-1* (RCK1) rescue strains (#1 $n = 20$, #2 $n = 20$), *slo-1*(Ca²⁺ bowl) rescue strains (#1 $n = 19$, #2 $n = 20$), *slo-1*(DM) rescue strains (#1 $n = 19$, #2 $n = 18$) (n represents number of worms). Abbreviated names for the strains are the same as in Fig. 1. Intoxication as measured by locomotion was rescued in all versions of *slo-1* transgenes regardless of whether one or both Ca²⁺-sensitive domains were mutated (paired t -test/ Wilcoxon signed rank t -test, $P < 0.05$; $n = 17$ –20). The only resistant strains were the *slo-1*(null) mutant (paired t -test, $t_{(34)} = 1.69$, n.s., $n = 17$) and the co-injection control (paired t -test, $t_{(36)} = 1.38$, n.s., $n = 19$) (b) Intoxication as determined by relative displacement on control vs. ethanol plates. The *slo-1*(null) and co-injection control strains exhibited resistance to intoxication shown by significantly more relative movement compared with WT ($P < 0.05$).

(a)	<u>RCK1</u>
Human	VCGHITLESVSNFLK <u>D</u> FLHKDRDDVNVEIVFLHNIS
Bovine	VCGHITLESVSNFLK <u>D</u> FLHKDRDDVNVEIVFLHNIS
Mouse	VCGHITLESVSNFLK <u>D</u> FLHKDRDDVNVEIVFLHNIS
<i>C. elegans</i>	VCGHITYDSVSHFLQ <u>D</u> FLHEDRDDVDVEVVFLHRVV
<i>Drosophila</i>	VCGHITYESVSHFLK <u>D</u> FLHEDREDVDVEVVFLHRKP
	***** :***. **:*****: **:***:***:*****.
(b)	<u>Calcium Bowl</u>
Human	ITELVNDTNVQFLDQ <u>DDDDDD</u> PDTELYLTQPFACGT
Bovine	ITELVNDTNVQFLDQ <u>DDDDDD</u> PDTELYLTQPFACGT
Mouse	ITELVNDTNVQFLDQ <u>DDDDDD</u> PDTELYLTQPFACGT
<i>C. elegans</i>	ITELVNDSNVQFLDQ <u>DDDDDD</u> PDTELYLTQPFACGT
<i>Drosophila</i>	ITELVNDSNVQFLDQ <u>DDDDDD</u> PDTELYLTQPFACGT
	*****. :*****

Figure 3. Primary sequence comparison of the RCK1 and Ca²⁺ bowl Ca²⁺-sensitive domains of the worm BK channel

(a) The RCK1 domain is highly conserved from *Caenorhabditis elegans* to humans. Both negatively charged aspartate (D) residues critical for RCK1 Ca²⁺ sensing (underlined) are completely conserved, while amino acids surrounding this region contain only one residue that is highly dissimilar between worm and human. (b) The region of the BK channel containing the Ca²⁺ bowl domain is highly conserved across species. All underlined aspartate (D) residues are conserved, while only one amino acid residue is not identical in the adjacent region. * = Residues are completely conserved, : = residues are strongly similar in properties (>0.5 in the Gonnet PAM 250 matrix) and. = residues are weakly similar (0.5 in the Gonnet PAM 250 matrix).

Figure 4. Ca^{2+} -sensing domains of the mammalian BK channel are critical for ethanol-induced reduction in egg laying and locomotion

Intoxication as determined by relative egg-laying (a,b) and locomotion (c,d) sensitivity to ethanol. The *slo-1*(null), *slo-1*(null)+*hslo*(DM) and co-injection control strains were significantly less intoxicated than *slo-1*(null)+*hslo*(+) strains ($P < 0.05$). Bars represent SEM. #1 and #2 refer to independently isolated strains expressing the same cDNA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5. Human BK channels expressed in *Caenorhabditis elegans* neurons

Confocal images show similar neuronal expression of either *slo-1(null)* mutant expressing mCherry-tagged WT (columns 1–3) or double calcium mutant (columns 4–6) human BK channels. Whole-worm images at $\times 20$ (columns 1 and 4) show the overlap between cholinergic neurons labeled with *punc-17::GFP* (top row) and mCherry-tagged HSLO (second row) along the ventral nerve cord. Yellow in the merged images (third and fourth rows) indicates colocalization. Higher resolution ($\times 63$) images of the tail (columns 2 and 5) and just posterior to the mCherry-labeled pharynx (columns 3 and 6) highlight neuronal expression of the human BK channel. Some of the mCherry colocalized in neurons in the tail and ventral nerve cord are also cholinergic (see merged). Scale bars upper left for $\times 20$ and $\times 63$.

Figure 6. Neck curvature depends on Ca²⁺-sensitive domains of the worm BK channel
 (a) Representative images of WT and *slo-1(null)* worms crawling on an agar surface. The three white circles near the head of the worm form the neck angle. Note the sharper 'crooked' neck of the *slo-1(null)* mutant worm. (b) Quantitative analysis shows neck curvature is sharper in *slo-1(null)* mutants ($n = 50$) than in WT worms. Expressing either *slo-1(+)*, *slo-1(RCK1)* or *slo-1(Ca²⁺ bowl)* transgenes in *slo-1(null)* worms fully or partially rescued WT-like neck curvature, suggesting that BK channel function was at least partially intact. In contrast, both *slo-1(DM)* strains had significantly greater neck curvature than WT. Both of these strains are not significantly different than *slo-1(null)*. The co-injection control also failed to rescue neck curvature. $n = 36$ for all groups. * $P < 0.05$ from WT, # $P < 0.05$ from *slo-1(null)*

Figure 7. Body curvature depends on Ca^{2+} -sensitive domains of the worm BK channel
 (a) Representative images of WT and *slo-1(null)* mutant worms crawling on an agar surface. The thirteen white points along the mid-body of each worm form 11 consecutive angles, which are quantified below the pictures. *Caenorhabditis elegans* crawls on its left or right side propagating bends to the dorsal (D) and ventral (V) sides from anterior (A) to posterior (P). (b) Quantitative analysis shows body curvature is higher in *slo-1(null)* mutants ($n = 50$) than in WT worms ($n = 36$). (c) Expressing either *slo-1(+)*, *slo-1(RCK1)* or *slo-1(Ca²⁺ bowl)* transgenes fully or partially rescued body curvature in *slo-1(null)*, suggesting that channel function is at least partially intact for each type of BK channel. In contrast, expressing a *slo-1(DM)* transgene failed to rescue body curvature. The co-injection control transgene also failed to rescue body curvature. * $P < 0.05$, *** $P < 0.001$ vs WT; # $P < 0.05$ vs *slo-1(null)*.

Figure 8. RCK1 and Ca^{2+} bowl domains are not required for ethanol to activate the worm BK channel

Representative patch-clamp recordings of currents mediated by SLO-1 channels expressed in oocytes. (a) Channel openings as a function of voltage with free intracellular calcium set to $5 \mu\text{M}$ showed greater channel activation for WT than mutant channels. Only one channel was observed in each patch at 150 mV, consistent with a single channel recording, at least for WT SLO-1. The P_o is given for a 3-second trace at each voltage. (b) Application of ethanol (50 mM) increased open probability for both WT and DM SLO-1 channels.

Membrane potential was held high enough to elicit basal channel opening for both WT SLO-1 ($V_m = +80 \text{ mV}$) and DM SLO-1 ($V_m = +150 \text{ mV}$) channels. Free intracellular calcium was set to $5 \mu\text{M}$. The mean P_o is given for 5–10 three-second traces recorded over 5 min before and during ethanol application.