SLO-2 isoforms with unique Ca\(^{2+}\)- and voltage-dependence characteristics confer sensitivity to hypoxia in *C. elegans*

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Introduction

The BK (Maxi K, Slo) potassium channel family includes Slo1, Slo2 and Slo3 as its members. Among them, the Slo1 channel is gated by Ca\(^{2+}\),1,2 while the Slo3 channel is activated by alcalinization.3,4 Slo2 channel gating exhibits species differences; the rat Slo2.2 (rSlo2.2 or Slack) and human Slo2.1 (hSlo2.1 or Slick) channels are each activated by both Na\(^{+}\) and Cl\(^{-}\),5,6 whereas the *C. elegans* Slo2a (SLO-2a) has been reported to be sensitive to Ca\(^{2+}\) and Cl\(^{-}\).7 Based on structure and sequence alignment, the C-termini of Slo1 channels are organized into two functional RCK (regulation of conductance for K) domains, RCK1 and RCK2. Three distinct Ca\(^{2+}\) binding sites, two high-affinity and one low-affinity sites, have been reported in Slo1 C-termini based on electrophysiological experiments.8 One of the high-affinity Ca\(^{2+}\) binding sites, located in the RCK2 domain, is composed of five consecutive Asp amino acid residues and referred to as the “Ca\(^{2+}\) bowl.”8 The second high-affinity Ca\(^{2+}\) binding site located predominantly in the RCK1 domain is composed of five consecutive Asp amino acid residues and referred to as the “Ca\(^{2+}\) bowl.”8 The second high-affinity Ca\(^{2+}\) binding site located predominantly in the RCK1 domain is composed of five consecutive Asp amino acid residues and referred to as the “Ca\(^{2+}\) bowl.”8

Slo channels are large conductance K\(^{+}\) channels that display marked differences in their gating by intracellular ions. Among them, the Slo1 and *C. elegans* SLO-2 channels are gated by calcium (Ca\(^{2+}\)), while mammalian Slo2 channels are activated by both sodium (Na\(^{+}\)) and chloride (Cl\(^{-}\)). Here, we report that SLO-2 channels, SLO-2a and a novel N-terminal variant isoform, SLO-2b, are activated by Ca\(^{2+}\) and voltage, but in contrast to previous reports they do not exhibit Cl\(^{-}\) sensitivity. Most importantly, SLO-2 provides a unique case in the Slo family for sensing Ca\(^{2+}\) with the high-affinity Ca\(^{2+}\) regulatory site in the RCK1 but not the RCK2 domain, formed through interactions with residues E319 and E487 (that correspond to D362 and E535 of Slo1, respectively). The SLO-2 RCK2 domain lacks the Ca\(^{2+}\) bowl structure and shows minimal Ca\(^{2+}\) dependence. In addition, in contrast to SLO-1, SLO-2 loss-of-function mutants confer resistance to hypoxia in *C. elegans*. Thus, the *C. elegans* SLO-2 channels possess unique biophysical and functional properties.
Cloning and expression of the slo-2b gene in Xenopus oocytes.

The slo-2 gene in C. elegans encodes two protein isoforms. The slo-2a isoform has been previously described, and here we describe the slo-2b isoform. slo-2b is identical to slo-2a starting from residue R40 of slo-2a, but its N-terminal end is shorter by 22 and variant in 17 amino acids from slo-2a. The full-length slo-2b gene encodes a 1086 amino acid protein, which is named F08B12.3c, as an alternative splice form of the slo-2 in the Worm database. All amino acids of the slo-2b from R18 (corresponds to R40 in slo-2a) are identical to the slo-2a in contrast to a previous report. Moreover, we provide experimental evidence that the RCK1 domain residue E487 (corresponding to D362 of Slo1, respectively) control Ca²⁺ sensitivity in slo-2b, independently of the RCK2 domain. Furthermore, we show that SLO-2 loss-of-function mutations confer resistance to hypoxia in C. elegans (contrary to the results by Yuan et al., 2003), while a slo-1 loss-of-function mutant does not share this phenotype. These results establish the C. elegans SLO-2 channels to be functionally more similar to the Slo1 than Slo2 mammalian channels but with important differences from Slo1 channels in the Ca²⁺ sensing mechanism.

Figure 1. Amino acid sequence of the C. elegans SLO-2b channel and Ca²⁺ dependence of its macroscopic conductance. (A) The full amino acid sequences of SLO-2b (in red, F08B12.3c) and SLO-2a (in black, F08B12.3b). (B) Current traces of inside-out macropatches expressing SLO-2b, bathed in the indicated Ca²⁺ concentrations. Pipette Solution: 140 mM KMES (potassium methanesulphonate), 20 mM KOH, 10 mM HEPES, 2 mM MgCl₂ (pH 7.0). The bath solution used in inside-out patch recordings contained 140 mM KMES, 20 mM KOH, 10 mM HEPES and one of the following: 5 mM EGTA (for nominally 0 Ca²⁺), 100 nM Ca²⁺ buffer for 600 nM and higher Ca²⁺. (C) The normalized conductance is plotted as a function of [Ca²⁺] for a range of command potentials. Solid lines show the best fits of the conductance at each voltage by the Hill equation. The x-axis is shown in a Log scale.
amino acids (Fig. 1A). The slo-2b cDNA was subcloned into the vector pGEMsh and expressed in Xenopus oocytes. Recording from patches in the inside-out patch configuration, using a step protocol from –200 to 200 mV, we found that SLO-2b is a Ca\(^{2+}\)- and voltage-dependent potassium channel (Fig. 1B). The macroscopic current reached the range of 2–10 nA at 60 \(\mu\)M Ca\(^{2+}\) with 200 mV depolarization in the absence of internal Cl\(^{-}\), a finding that differed from previously reported SLO-2a data. To provide estimates of the Ca\(^{2+}\) dependence of the SLO-2b current regulation, conductance estimates were re-plotted showing the effect of Ca\(^{2+}\) on conductance at different depolarized potentials (Fig. 1C). Such plots could be fit with the Hill equation with a half maximal activation of the conductance at a voltage range of over 80 mV occurring at [Ca\(^{2+}\)] concentrations of 10.4 – 39.5 \(\mu\)M. The Hill coefficients for such relationships ranged from 1.8 to 2.4, suggesting that at least two Ca\(^{2+}\) ions cooperate to activate SLO-2b.

The SLO-2b channel has a similar conductance with the SLO-2a channel but is not Cl\(^{-}\) dependent. In order to further investigate the effect of Cl\(^{-}\) on the activation of SLO-2b channels, we performed inside-out patch recordings in symmetrical potassium solutions, either in 160 mM KMES or 160 mM KCl. Plotted normalized G-V curves in different Ca\(^{2+}\) concentrations established the Ca\(^{2+}\)-dependent activation of SLO-2b under both conditions (Fig. 2A and B). However, at high Ca\(^{2+}\) (ranging from 10 – 300 \(\mu\)M) concentrations and at negative potentials the conductance values were much higher in 160 mM KCl than in 160 mM KMES. Thus, superficially, Cl\(^{-}\) seemed to increase the activity of the SLO-2b channel. We tested the possibility that Cl\(^{-}\) activated a conductance independent of SLO-2b in Xenopus oocytes by running a ramp protocol on the same patch perfused in its inner surface with either 160 mM KCl or 160 mM KMES (with 160 KMES in its outer surface) in 60 \(\mu\)M Ca\(^{2+}\). The recorded currents obtained by perfusion with 160 mM KCl (Fig. 2C, black line), following offsetting the holding current at 0 mV, showed relatively symmetrical currents (Fig. 2C, solid blue line). This was in contrast to the currents obtained in 160 mM KMES that showed outwardly rectifying currents (Fig. 2C, red line). The difference current between the two conditions (the current recorded in 160 mM KMES subtracted from that recorded in 160 mM KCl) revealed a weak inwardly rectifying current (Fig. 2C, dashed blue line). Uninjected oocytes tested in symmetrical 160 mM NaCl in 60 \(\mu\)M Ca\(^{2+}\), yielded a weak inwardly rectifying current similar to the difference current obtained in Figure 2C (Fig. 2D, blue line). Almost identical G-V relationships of the two Cl\(^{-}\)-induced conductances (corresponding to ramp currents shown in the dashed blue trace in Fig. 2C and the solid blue trace in Fig. 2D) indicated that the greater conductance seen in KCl solutions resulted from an oocyte endogenous Ca\(^{2+}\) activated Cl\(^{-}\) current that is independent of SLO-2b expression (Fig. 2E and F). Furthermore, we performed experiments to measure and compare the reversal potentials achieved by different concentrations of internal Cl\(^{-}\) in uninjected versus SLO-2b expressing oocytes. In each Cl\(^{-}\) concentration we tested, the measured reversal potential values were comparable in the two groups of oocytes and consistent with the predicted value based on the Nernst Equation for a Cl\(^{-}\) selective conductance, rather than a K\(^{-}\) activated one (Fig. S1). Finally, we performed single channel recordings at +100 mV in 0 Ca\(^{2+}\) with different concentrations of Cl\(^{-}\). The data showed no obvious increase in P\(_o\) with elevated Cl\(^{-}\) concentrations (Fig. 2G and H). This result was in sharp contrast to similar recordings using the Slack channel as a positive control. At –100 mV the Slack P\(_o\) was increased with the elevated Cl\(^{-}\) concentration (to 30 or 100 mM Cl\(^{-}\) in 50 mM Na\(^{+}\)) (Fig. S2). At 1 \(\mu\)M Ca\(^{2+}\) the P\(_o\) of SLO-2b in different Cl\(^{-}\) concentrations could not be measured because large Cl\(^{-}\) currents were evoked even in uninjected oocytes. Thus, we obtained inside-out recordings in HEK cells transfected with the cSlo2b cDNA. With 10 \(\mu\)M Ca\(^{2+}\) at 60 mV, P\(_o\) was measured in different Cl\(^{-}\) concentrations, clearly failing to show P\(_o\) increases with increasing Cl\(^{-}\) concentrations (Fig. S3A–C). However, the increased Ca\(^{2+}\) concentration clearly led to a higher P\(_o\) of cSlo2b channels (Fig. S3A–C). These results combined suggest that the SLO-2b channel isoform is not Cl\(^{-}\) sensitive, in marked contrast to previous results reported for SLO-2a.7

In order to further compare SLO-2b to SLO-2a channels, the unitary conductance of SLO-2b was determined (Fig. S4A–B). Although the activation of SLO-2b is voltage dependent, openings can be observed even at strongly hyperpolarized potentials, provided that there is high enough channel expression. The unitary conductance in symmetrical 160 mM KMeS was 107 ± 3 pS. This value is consistent with the unitary conductance reported for SLO-2a.7 The single-channel open probability at 100 mV in 60 \(\mu\)M Ca\(^{2+}\) was calculated to be 0.39 ± 0.02 by measuring and fitting single-channel open and closed events with a Gaussian function (Fig. S4C and D). Thus, the open probability (P\(_o\)) at 100 mV in 60 \(\mu\)M Ca\(^{2+}\) was about 67% of the maximal Po (Fig. 3B, blue dashed line), the maximal Po was calculated at around 58%.

The biophysical properties of SLO-2 channels are closer related to Slo1 than Slo2 channels. Since SLO-2a and SLO-2b are regulated by Ca\(^{2+}\) and voltage, we used the Horrigan-Aldrich model aiming to describe the gating behavior of the SLO-2b channel (Fig. S5A). Due to the low open probability of SLO-2b in low Ca\(^{2+}\) concentrations, we depolarized the membrane to 300 mV. First, at very negative potentials, the SLO-2b voltage sensor is likely to be located in the resting state because the voltage dependence of the SLO-2b channels is weaker than that of the mouse Slo1 (mSlo1) channel.7 Based on the equation for P\(_o\) (see Materials and Methods, Eqn. 3), Z\(_{g}\) was given a value of 0.003e, suggesting lack of a voltage dependence. We thus estimated the D value based on the Log(G) vs. V relationship and P(Vmax)/P(Vmin). With some constraints (see Materials and Methods) we could fit all parameters based on Equation 2 (Table 1). The G-V relationship of the wild-type SLO-2b could be fitted well by Equation 2, indicating that the Horrigan-Aldrich model is suitable for describing the properties of SLO-2b (Fig. S5B). Furthermore, the lower value of the SLO-2b Z\(_{g}\), compared to that of mSlo1, is consistent with the fact that the voltage sensor of SLO-2b lacks a number of positively charged residues located on its S4 segment. The higher value of the Ca\(^{2+}\) dissociation constant C reflects the fact that SLO-2b is also less sensitive to Ca\(^{2+}\).
than mSlo1. The G-V of the SLO-2a channel could also be fitted well by the Horrigan-Aldrich model although the parameters used were somewhat different from those for the SLO-2b channels (see Table 1). The activation time course was well fitted by a single exponential function over the full range of Ca\(^{2+}\) concentrations (Fig. S5C).
is much slower than that of the mSlo1 channel. For SLO-2b, increases in Ca\textsuperscript{2+} from 10 to 60 μM produced a 10-fold change in the activation time constant at a given potential (Fig. S5D).

In order to directly compare SLO-2b with SLO-2a, we also performed similar recordings with the SLO-2a isoform. SLO-2a also revealed a large Ca\textsuperscript{2+}-dependent current that was insensitive to internal Cl\textsuperscript{−} (Fig. 3A). The conductance versus voltage relationship (G-V) showed that SLO-2a exhibits greater activation at any given Ca\textsuperscript{2+} concentration than SLO-2b (dashed lines for SLO-2b are shown in the same color for a given Ca\textsuperscript{2+} concentration). The G-V curve of SLO-2a was left-shifted about 60 mV in each Ca\textsuperscript{2+} concentration (Fig. 3B and C). In high Ca\textsuperscript{2+} (60–300 μM), the channel remained in the open state even under highly hyperpolarized conditions (Fig. 3B). These results indicate that the N-terminus of SLO-2a can allosterically regulate channel activity. We further tested whether SLO-2a was sensitive to Cl\textsuperscript{−} by...
measuring the single-channel activity in different Cl− concentrations. Our data clearly demonstrate that the P₀ of SLO-2a was insensitive to increasing Cl− concentrations (Fig. 3D–F).

The current properties of SLO-2a and SLO-2b seemed to resemble those of mSlo1 but with significant differences. Thus, we investigated whether the Ca²⁺ sensing mechanism of SLO-2b was similar to that of mSlo1 channels. Since there is no Ca²⁺ bowl in the RCK2 domain of SLO-2b, we focused on the RCK1 high-affinity Ca²⁺ binding site. We aligned the SLO-2b (or cslo2) amino acid sequence with that of the Ca²⁺-sensitive mSlo1 and the related but Ca²⁺-insensitive rSlo2 (or slack) (Fig. 4A). The E325 and E487 residues of SLO-2b corresponded to the D362 and E535 of mSlo1, respectively, which are shown to greatly affect Ca²⁺ sensitivity (e.g., 12). We performed alanine scanning mutagenesis on and around these negatively charged residues (Fig. 4E). The E325A mutant did not substantially change the Ca²⁺ sensitivity of the SLO-2b channel (Fig. 4C and E). In contrast, the E319A mutant (corresponding to the D362 residue in mSlo1) largely abolished the Ca²⁺ sensitivity of the SLO-2b channel (Fig. 4B). The E319D, E319N and E319R mutants also decreased the Ca²⁺ sensitivity of SLO-2b (Fig. S6A and B). Similarly, the Ala mutant at position E487, which corresponds to the critical Ca²⁺-sensitive Slo1 residue E535, dramatically decreased Ca²⁺ sensitivity of SLO-2b channel activity (Fig. 4D). All other mutants we tested did not substantially change the Ca²⁺ sensitivity of the SLO-2b channel, exhibiting Vₘ values similar to the wild-type SLO-2b (Fig. 4E). We also tested mutants of the two positive SLO-2b residues (i.e., E319 and E487) at the corresponding SLO-2a channel positions (i.e., E340A and E508A). The results show that these mutations also largely abolished Ca²⁺ sensitivity in SLO-2a channels (Fig. S6C and D).

Although the E319A and E487A mutants dramatically decreased the Ca²⁺ sensitivity of SLO-2b channels, neither of them alone completely abolished Ca²⁺ sensitivity of SLO-2b channels, nor did the double mutant (it showed a similar effect as the single mutants). We thus investigated whether the RCK2 domain of the SLO-2b channels contributed to Ca²⁺ sensitivity. Neutralization mutants of the non-conserved negative residues around the residue that corresponds to the Na⁺ regulatory site D818 in Slo2.2 were tested for potential effects on Ca²⁺ sensitivity. None of the D759N, D763N and the triple mutant E754N/E756N/E757N showed obvious Ca²⁺ sensitivity changes (Fig. S6B). We constructed a chimeric channel, in which the entire RCK2 domain of the SLO-2b channel was replaced by the RCK2 domain of the rSlo2.2 (slack) channel, to test the role of the RCK2 domain in Ca²⁺ sensing (Fig. 5A). This chimeric channel showed no obvious decrease in Ca²⁺ sensitivity. Although the Vₘ in 60 μM Ca²⁺ was right-shifted by 200 mV, the Vₘ in 0 Ca was also right-shifted because the G/Gmax in 0 Ca at 300 mV was only 1/3 value of the wild-type SLO-2b (Fig. 5B). Since replacement of the SLO-2b RCK2 domain right-shifted the voltage-dependent activation even in the absence of Ca²⁺, we concluded that it reflected a Ca²⁺-independent effect on channel gating. Also, the RCK2 of Slack did not confer Na⁺ sensitivity to the chimeric channel, even though it contained the Na⁺ coordination site of Slack suggesting further differences from the Slack channel. Thus, the RCK2 of SLO-2 is likely to contribute less to the Ca²⁺-sensitivity of the channel than the RCK1 domain. Based on the recent crystal structure of the C-terminal domain of hSlo1, we constructed a homology model of SLO-2b. This model shows the E487 and E319 residues within reasonable proximity to be able to coordinate Ca²⁺ (Fig. 5C).

We tested if Cd²⁺ or Mg²⁺ ions could also activate the SLO-2b channel. Cd²⁺ has been previously shown to activate mSlo1 channels by binding to the D362 residue, which corresponds to E319A in the SLO-2b channel. However, Cd²⁺ could not activate the SLO-2b channel at concentrations up to 200 μM (Fig. S7), whereas it does activate the mSlo1 channel at concentrations in the range of tens of micromolar. Mg²⁺ activated the SLO-2b channel but less potently than mSlo1 (Fig. 6A). Using a ramp protocol from −100 mV to +100 mV, we obtained the Mg²⁺ dose-dependent activation curve. At 100 mV, the current level with 100 mM Mg²⁺ in the bath was only 10% of the current level at 300 μM Ca²⁺ (Fig. 6A). Similarly, Mg²⁺ dose-dependent activity could also be observed with increasing voltage steps in the range from 60–200 mV. This activity was higher in bath solutions including 10 mM Mg²⁺ compared to 1 mM Mg²⁺ but was much lower than at 300 μM Ca²⁺ (Fig. 6C–3). The G-V relationship of the Mg²⁺ activation for SLO-2b was plotted and fitted with the Boltzmann equation (Fig. 6B), showing an obvious left shift with increasing Mg²⁺ concentrations. Next, we determined how the Ca²⁺ binding site influences Mg²⁺ sensitivity by measuring the activity of the E487A mutant. Since the G-V relationship was largely out of the measurable range, it appears that the E487A only right-shifted the G-V relationship but did not abolish Mg²⁺ sensitivity (compare Fig. 6B and D). These results indicate that G-V relationships could be right-shifted by the E487A mutant but that the Mg²⁺ sensitivity was maintained. Considering that the E487A also right-shifted the G-V curve at 0 μM Ca²⁺, the possibility that an independent Mg²⁺ binding site exists cannot be excluded.

SLO-2 confers sensitivity to hypoxia and functions differently from SLO-1 in C. elegans. SLO-2 channels have previously been reported to have a role in the C. elegans response to hypoxia. In this report, worms lacking a functional SLO-2

| Table 1. Allosteric constants comparing Slo1, Slo3 and SLO-2b activation
| Slo1 Horrigan/ Aldrich | Slo3 Zhang/ Lingle | SLO-2b | SLO-2a |
|-------------------------|-------------------|--------|--------|
| L | 9.8E-07 | 1.59E-03 | 5.29E-07 | 1.38E-03 |
| Zₓa | 0.3 | 0.04 | ~0 | ~0 |
| Jₓ | 0.3 | 0.34 | 0.05 | 0.09 |
| Zᵧ | 0.58 | 0.34 | 0.27 | 0.48 |
| D | 25 | 4.03 | 30.48 | 3.83 |
| Kₒ(μM) | 11 | 50.16 (H⁺) | 48.4 | 57.8 |
| C | 8 | 0.125 | 6.57 | 3.38 |
| E | 2.4 | 0.7 | 1.37 | 5.05 |
| Vₒ | 152 | 80.1 | 371 | 267 |
channel were more sensitive to hypoxia-induced lethality than wild type. Given the similarities of SLO-2 channels to mSlo1, we tested the effects of loss of function in both slo-2 and slo-1 on sensitivity to hypoxia in C. elegans. To our surprise, when we tested slo-2 mutant animals in three different paradigms of hypoxia, we found that they were significantly resistant to hypoxia, rather than sensitive. slo-1; slo-2 double mutant animals were also resistant to hypoxia. In contrast, slo-1 mutants were not different from wild-type strains.

There are several methods for inducing hypoxic responses in the worm, and we used three different approaches to induce hypoxia. First, we incubated the animals with cobalt chloride, which induces expression of the hypoxia inducible transcription factor, HIF-1α. second, we incubated worms in a bio-bag environmental chamber that uses an oxygen scavenger to remove oxygen from the enclosed environment, which generates O2 levels well less than 1%, as tested by a chemical indicator enclosed in the bag. Third, we used a hypoxia chamber similar to the one used previously (Fig. 7C). In this chamber, oxygen in the atmosphere is replaced by a gas that is pumped in, and oxygen levels are monitored by a sensor. In our experiments, we maintained the oxygen at 0.1%. Unlike the previous study that used a gas mixture of 5% CO2, 10% H2, and 85% N2, we used 100% N2 gas, as 10% H2 is flammable. Additionally our experiments were performed at 25°C rather than the 27°C used in the previous study. Regardless of the method used to cause or mimic hypoxia, we found that slo-2 mutant animals were significantly resistant to hypoxia, whereas slo-1 mutant animals were not. Loss of function of the SLO-2 channel improved survival under hypoxic conditions, strongly suggesting that slo-2 confers sensitivity to hypoxia.

Discussion

We have shown that the C. elegans SLO-2b and SLO-2a isoforms are both voltage and Ca2+-dependent potassium channels. Neither of these SLO-2 channels exhibits Cl− sensitivity, as previously reported for the SLO-2a channel. Thus, the N-terminal amino acid differences could not account for this difference, although the N-terminus of SLO-2 allosterically led to a shift in the G-V relationship. Our results suggest that contamination by an endogenous Cl− current present in Xenopus oocytes is likely to have misled the SLO-2a work. Endogenous Cl− currents in oocytes have been reported to arise from several Cl− channels that can be activated by Ca2+.

The G-V relationship of the SLO-2b channel could be described well by the Horrigan-Aldrich model, which was developed for describing gating properties of the Slo1 channel. Recently, one crystal structure of the gating ring from the human BK channel in the presence of 50 mM [Ca2+] (PDB ID code: 3MTS) revealed that one Ca2+ ion was coordinated by residues in the Ca2+ bowl of the RCK2 domain of the Ca2+-bound crystal.
Figure 5. The RCK2 domain contributes less to the Ca\(^{2+}\) sensitivity of SLO-2b than the RCK1 domain. (A) The cartoon of a chimera (CS736) between Slack (that is not Ca\(^{2+}\)-sensitive) and SLO-2b. The RCK2 domain of SLO-2b was replaced by the RCK2 domain of Slack (blue). (B) The normalized G-V curve of the CS736 chimera shows a rightward Vh shift of about 200 mV in both 0 and 60 \(\mu\)M intracellular Ca\(^{2+}\). (C) An approximation of the Ca\(^{2+}\) coordination site obtained from the C-terminal homology model of SLO-2b based on the crystal structure of the Slo1 C-terminus (3NAF).
slo-2b was obtained by PCR from EST clone 1522e1. The primers were flanked with BamHI and XmaI restriction endonuclease sites, at 5' and 3' respectively. This 5' fragment was inserted into the *Xenopus* oocyte expression vector pGEM-sh, using the BamHI and XmaI sites. The nucleotides 1350-1356 of the slo-2b cDNA represent an endogenous XmaI site. A 3' cDNA fragment was isolated by restriction endonuclease digestion from the EST clone 1193 in the pME18S-FL3 vector, using XmaI and XhoI. This fragment was inserted into the 5' fragment-containing pGEM-sh construct that had been digested with XmaI and XhoI, so that the 5' and 3' cDNA fragments were joined together at the XmaI site. The N-terminal 450bp cDNA encoding sequence of slo-2a was synthesized by the GenScripts Company and flanked by BamHI and NgoMIV endonuclease sites. A NgoMIV endonuclease site mutation was made in the corresponding sequence of slo-2b. Then the corresponding N-terminus of slo-2b constructs was cut from the BamHI/NgoMIV site and replaced by a synthesized N-terminus of slo-2a. Finally, the inserted NgoMIV endonuclease site was deleted by mutagenesis.

The CS736 chimeras were made by replacing the RCK2 domain of slo-2b from amino acid 736 with the Slack RCK2 domain from amino acid 803 to the end of the protein. Mutants were made following previously published procedures.35

**Oocyte isolation and culture.** Stage VI oocytes from female *Xenopus laevis* frogs were harvested and used for cRNA injection,
previously described procedures. Each series of $G/V$ curves were obtained from the same patch, and conductances were normalized to estimates of maximal fitted conductance obtained with 60 μM Ca²⁺. Individual $G/V$ curves were fitted with a Boltzmann function of the following form:

$$G(V) = \frac{G_{\text{max}}}{1 + \exp \left( \frac{V - V_h}{z} \right)}$$  

Eqn. 1

where $G_{\text{max}}$ is the fitted value for maximal conductance at a given Ca²⁺ concentration. $V_h$ is the voltage of half maximal activation of conductance, and $z$ reflects the net charge moved across the membrane during the transition from the closed to the open state. $\Delta V_h = V_h$ at 0 – 100 μM [Ca²⁺].

Allosteric models of activation of BK channels. The Ca²⁺ and voltage dependence of activation of the SLO-2b channels and SLO-2a could be reasonably well described by the Horrigan-Aldrich model that was developed for Slo1 channels. For the scheme used here (Fig. S5A), we assumed that the effects of Ca²⁺ involved a single Ca²⁺-dependent equilibrium occurring with each of the four subunits. The system was described by three relatively independent equilibrium constants: $L$ indicates the intrinsic equilibrium constant for the close-open transition; $J$ defines the equilibrium constant for the voltage sensor moving between resting and activation states; $K$ defines the equilibrium constant for interaction with Ca²⁺. In turn, each process was allosterically coupled to the other two processes by coupling constants. $D$ defines the coupling between voltage sensor movement and channel opening; $C$ defines the coupling between Ca²⁺ binding and channel opening; $E$ defines the coupling between Ca²⁺ and voltage sensor movement. The open probability was determined by the equation:

$$P(V,[Ca]) = \frac{1}{1 + L(1 + K + J + K_E + C + E + C_D + D + E + C_D)}$$

Eqn. 2

where $L = L(0) \exp (Z, V/kT)$, $J = J(0) \exp (Z, V/kT)$, $K = [Ca^{2+}]/K_d$, where $K_d$ is the dissociation constant of free Ca²⁺. Hence, at very negative potentials lower than –100 mV and at all Ca²⁺ concentrations, the normalized conductance is < 0.01, (1 + K) > L(1 + KC). The equation that is used to define $Z_L$ can be simplified as:
Detailed information regarding the Horrigan-Aldrich model has been previously described.\textsuperscript{4,38} Some parameters were constrained ($0 < L < 1$, $0 < Z_1 < 10$, $0 < D < 100$, $0 < C < 100$) when the G-V was fitted with Eqn. 2.

**C. elegans** methods. Nematode Culture and Strains. *C. elegans* were maintained using standard methods.\textsuperscript{39} Strains used were N2 (var. Bristol), slo-1(js379), slo-2(nf100), slo-2(nf101), slo-1(js379);slo-2(nf100) and slo-1(js379);slo-2(nf101).

Assays of severe hypoxia. Cobalt chloride treatment. Healthy age matched first day adult animals were used. Five hundred microliters of filter sterilized 100 mM cobalt chloride (Acros Organics) was added to 3-d-old Nematode Growth Media (NGM) plates. Plates were seeded with OP50 bacteria 24 h before addition of cobalt chloride. Fifty animals were incubated on plates for 16 h at 20°C (for each 50 worm experiment $n = 1$). Worms were moved to new NGM plates and scored for survival after 24 h. A 1-way analysis of variance (ANOVA) was performed with a significance value of $p < 0.05$ with Bonferroni’s post hoc test comparing each mutant strain to N2.

Environmental chamber. Healthy age matched first day adult animals were used. Fifty animals on NGM plates seeded with OP50 were placed in Bio-Bag Environmental Chamber Type A (Becton, Dickinson and Company). Animals were exposed to oxygen levels of less than 1% for 8 h at 27°C, then removed from the bag and moved to new NGM plates and incubated in room atmosphere at 20°C. Survival was scored 24 h after incubation. A 1-way analysis of variance (ANOVA) was performed with a significance value of $p < 0.05$ with Bonferroni’s post hoc test comparing each mutant strain to N2.

Hypoxic Cabinet: Healthy age matched second day adult animals were used. Animals were washed with 1 mL of M9 buffer into 1.5 mL polypropylene tubes. The M9 was replaced three times with 1 mL of M9 that had been bubbled for 30 min with 100% nitrogen gas. Worms were incubated in a Hypoxic Cabinet with an oxygen controller (Coy Laboratory Products) in a final volume of 100 $\mu$L of M9 at an oxygen level of 0.1% for 16 h at 25°C. Animals were removed from the chamber and moved to new NGM plates and incubated in room atmosphere at 20°C. Survival was scored 24 h after incubation. A 1-way analysis of variance (ANOVA) was performed with a significance value of $p < 0.05$ with Bonferroni’s post hoc test comparing each mutant strain to N2.

Reagents. KMES, potassium metanesulphonate; KOH, potassium hydroxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MgCl$_2$, magnesium chloride; EGTA, ethylene glycol tetraacetic acid; HEDTA, N-(hydroxyethyl)-ethylendiaminetriacetic acid were all purchased from Sigma-Aldrich.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Material**

Supplemental materials may be found here: http://www.landesbioscience.com/journals/channels/article/24492

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**Channels**

Volume 7 Issue 3
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