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SLO2 Channels Are Inhibited by All Divalent Cations That Activate SLO1 K^+ Channels*

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Two members of the family of high conductance K^+ channels SLO1 and SLO2 are both activated by intracellular cations. However, SLO1 is activated by Ca^{2+} and other divalent cations, while SLO2 (Slack or SLO2.2 from rat) is activated by Na^+. Curiously though, we found that SLO2.2 is inhibited by all divalent cations that activate SLO1, with Zn^{2+} being the most effective inhibitor with an IC_{50} of ~8 μM in contrast to Mg^{2+}, the least effective, with an IC_{50} of ~ 1.5 mM. Our results suggest that divalent cations are not SLO2 pore blockers, but rather inhibit channel activity by an allosteric modification of channel gating. By site-directed mutagenesis we show that a histidine residue (His-347) downstream of S6 reduces inhibition by divalent cations. An analogous His residue present in some CNG channels is an inhibitory cation binding site. To investigate whether inhibition by divalent cations is conserved in an invertebrate SLO2 channel we cloned the SLO2 channel from *Drosophila* (dSLO2) and compared its properties to those of rat SLO2.2. We found that, like rat SLO2.2, dSLO2 was also activated by Na^+ and inhibited by divalent cations. Inhibition of SLO2 channels in mammals and *Drosophila* by divalent cations that have second messenger functions may reflect the physiological regulation of these channels by one or more of these ions.

Potassium (K^+)[2] channels of the SLO family are the largest and most complex K^+ channels (1, 2). These high conductance K^+ channels are activated by voltage and modulated by intracellular ions as well as a host of different intracellular factors (3). Structurally they resemble voltage-gated K^+ channels but have additional conserved intracellular domains appended on the C terminus that allow them to be gated by different intracellular ions (reviewed in Refs. 1, 2). Two members of this family are SLO1, which is activated by intracellular Ca^{2+} (4) and SLO2.2 (also known as Slack), which is activated by intracellular Na^+ (5). As with Ca^{2+}-activated K^+ channels, Na^+-activated K^+ channels are widely expressed in the mammalian brain and other tissues, and are found in many species from *Caenorhabditis elegans* to humans (6, 7). Using immunohistochemistry (8) and inside-out patch recordings (9) it was shown that Na^+-activated K^+ channels are present in the soma and dendrites of many different classes of neurons. Although it has been suggested that Na^+-activated K^+ channels may serve a role in protecting cells from hypoxia (5, 7, 10), we (11, 12) and others (13, 14) have shown that Na^+-activated K^+ channels provide a major outward current component in MSN striatal neurons, tufted/mitral cells of the olfactory bulb, and a significant component in cortical pyramidal cells, and serve an important role in several aspects of normal physiology (7, 15).

The activating effect of divalent ions on the Maxi-K BK channel SLO1 is well documented, and serves as a negative feedback system for calcium entry in many cell types (16–18). On the other hand, although it is known that Na^+ activates SLO2.2 channels the effect of divalent cations on these channels is largely unstudied. In exploring this question we were surprised to observe that all divalent ions that were reported to activate SLO1 channels have the opposite effect on SLO2.2 channels: they inhibit SLO2.2 channels. As will be shown, this inhibition by divalent cations has none of the hallmarks of a pore-blocking effect, but appears to inhibit channel activity via an allosteric site. Interestingly, we show that Ba^{2+} is the single exception. We found that Ba^{2+} has two distinct inhibitory effects on SLO2.2 channels; it blocks in a manner similar to other divalent ions, but has an additional pore-blocking effect that increases at higher voltages. These results contrast with the effects of Ba^{2+} on SLO1 channels which has been reported to both activate and inhibit SLO1 channels, with the latter showing an obvious voltage-dependent pore-blocking effect (18). To investigate possible structural features in SLO2.2 channels involved with divalent cation inhibition we examined a comparative alignment of SLO2.2 with cyclic nucleotide-activated channels also known to be inhibited by divalent ions (19). The divalent cation inhibition of cyclic nucleotide gated channels involves a histidine residue located at a position immediately following the cytoplasmic end of the sixth transmembrane domain. We observed that a His residue is also found in a similar position in SLO2.2 channels from rat and *Drosophila*. By site-directed mutagenesis we showed that this His residue is also implicated in the divalent cation inhibition of SLO2.2 channels. Finally, we cloned and functionally expressed the *Drosophila* SLO2 channel (dSLO2) and observed that it is also activated by sodium ion, and like the rat SLO2.2 channel, is also inhibited by divalent cations.

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[2] The abbreviations used are: K^+, potassium; dSLO2, *Drosophila* SLO2 channel.

Experimental Procedures

Animals

All procedures described herein were reviewed and approved by the Animals Studies Committee of Washington University (St. Louis) and were performed in accord with the NIH Guiding Principles of the care and use of laboratory animals.

Molecular Biology

Channel Cloning Techniques—Channels of interest were cloned into our pOX *Xenopus* expression vector (28). The rat Slo2.2 (Slack, or rSlo2) wild type construct is previously described (5). The Slo2.2 H347Q mutant construct was made by the overlap extension PCR technique on a subcloned cDNA fragment using New England Biolab's Phusion polymerase and mutant oligos. The subcloned fragment was then inserted back into the original wild type pOX-Slack construct. The dSlo2 cDNA was synthesized by subcloning overlapping cDNAs obtained by rtPCR of adult stage *Drosophila* RNA, as well as subcloning overlapping PCR fragments from an adult stage cDNA library. The full-length cDNA was subcloned into pOX.

Electrophysiology

Defolliculated *Xenopus* oocytes were injected with 50 nl of cRNA (1 to 3 μg/μl) using a Drummond Scientific nanoinjector (Broomall, PA). Injected oocytes were incubated at 18 °C in ND96 medium (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES pH 7.5 with NaOH. Oocytes were electrophysiologically analyzed 3–5 days after injection.

Inside-out Patch Clamp Recordings from Oocytes and HEK Cells—(a) *Xenopus* oocytes: For patch-clamp experiments, the vitelline membranes were mechanically removed before recording. (b) Stably transfected HEK cells expressing Slo2.2 channels were incubated in DMEM complete medium containing 10% fetal bovine serum, Pen/Strep, and G418 (selection antibiotic). Cells were incubated at 37 °C. Inside-out patches from *Xenopus* oocytes and HEK cells were analyzed while perfusing the intracellular side of the membrane with a solution containing the following (in mM): 80 KCl, 80 NaCl (or 80 choline Cl for the 0 sodium condition), 5 HEPES and the divalent cation Cl salt needed to obtain the desired concentrations. In experiments with symmetrical potassium conditions, the pipette solution was (in mM) 80 KCl, 80 NaCl, 2 $MgCl_2$, and 5 HEPES. The pH of these solutions was adjusted to 7.4 using KOH. The pipettes were obtained by pulling borosilicate glass from Warner Instruments; macropatch pipette tip resistance ranged from 0.8 to 1.5 megohms, and single channel recording pipettes were 4 to 5 megohms. Traces were acquired using an Axopatch 200B (Molecular Devices, Palo Alto, CA), digitized at 10 kHz (macroscopic currents) or at 100 kHz (single-channel), and filtered at 2 kHz or 20 kHz, respectively. Data were analyzed using pClamp 9 (Molecular Devices), SigmaPlot (Jandel Scientific, Corte Madera, CA) or Origin 6.0 (Microcal Software, Northampton, MA). Drugs and pharmacological agents used in this study were purchased from Sigma. Control experiments were performed to show that the sodium dependence and divalent cation inhibition of rSlo2.2 channels showed no obvious differences when heterologously expressed in either *Xenopus* oocytes or HEK cells.

Results

The Inhibitory Effect of Divalent Cations—Inside-out patch recordings obtained from a HEK cell line constitutively expressing SLO2.2 channels were used to study the effect of intracellular divalent cations on SLO2.2 channels. It had been reported that calcium ions inhibit native Na^+-dependent K^+ channels, likely to be SLO2.2, in thick ascending limb of mouse kidney (20) but the effect of calcium has not been confirmed in heterologously expressed SLO2.2 channels. To investigate this question, we pulled inside-out patches from the HEK cell line constitutively expressing SLO2.2 channels and exposed the cytoplasmic surface to different concentrations of Ca^{2+}. In the presence of 80 mM sodium we observed that calcium inhibits SLO2.2 channels in a concentration-dependent manner with an EC_{50} of ~260 μM (Figs. 1 and 2). Furthermore we observed that the blocking effect of calcium ion is not voltage dependent in the range of voltages studied as might be expected for a pore blocking ion. In the absence of intracellular divalent cations, a voltage-dependent blocking effect of SLO2.2 channels is noticeable at voltages greater than +60 mV (*red symbols* in Fig. 1 and Ref. 5). This blocking effect has been attributed to the presence of intracellular Na^+ (6). Experiments with inside-out patches expressing single SLO2.2 channels showed that the block seen in macroscopic currents at high voltages was due to a diminishing amplitude of single channel currents (5). This appears to be almost entirely due to the action of sodium, because increasing concentrations of Ca^{2+} in the presence of sodium ion did not result in a leftward shift in the blocking effect, as seen in Fig. 1. Taking into consideration a small time-dependent run-down of SLO2.2 channels observed in inside-out patches, we found that the inhibitory effect of calcium ion, and all other divalent ions studied, was completely reversible.

Different Divalent Cations Inhibit Slack Channels in a Broad Range of EC_{50} Values—The inhibitory effect of seven different divalent cations applied to the cytoplasmic surface of inside-out patches expressing SLO2.2 channels was tested in the presence of 80 mM sodium. The range of concentrations used was adjusted in accordance with the observed blocking effect of the individual cation. Remarkably, all divalent cations tested inhibited SLO2.2 currents (Fig. 2). In all cases, the effect was reversible and all except for Ba^{2+} (as discussed below) showed similar blocking effects at positive and negative potentials. Zn^{2+} was the strongest inhibitor of the SLO2.2 channel. The relative strength of inhibition was: $Zn^{2+} > Cd^{2+} > Ni^{2+} > Ba^{2+} > Ca^{2+} > Mn^{2+} > Mg^{2+}$.

Only Barium Ion Shows a Voltage-dependent Blocking Effect—Ramps from −90 mV to +90 mV were applied to inside-out patches. The ramp protocol reveals currents over a wide voltage range similar to the voltage range traversed using discrete voltage steps; however, currents are revealed in response to a single voltage step. In this way, we can reduce the likelihood of rundown or losing the patch during the recordings. Among all the divalent cations tested only Ba^{2+} showed a greater blocking effect at more positive potentials (Fig. 3). This observed effect of barium ion is consistent with what is

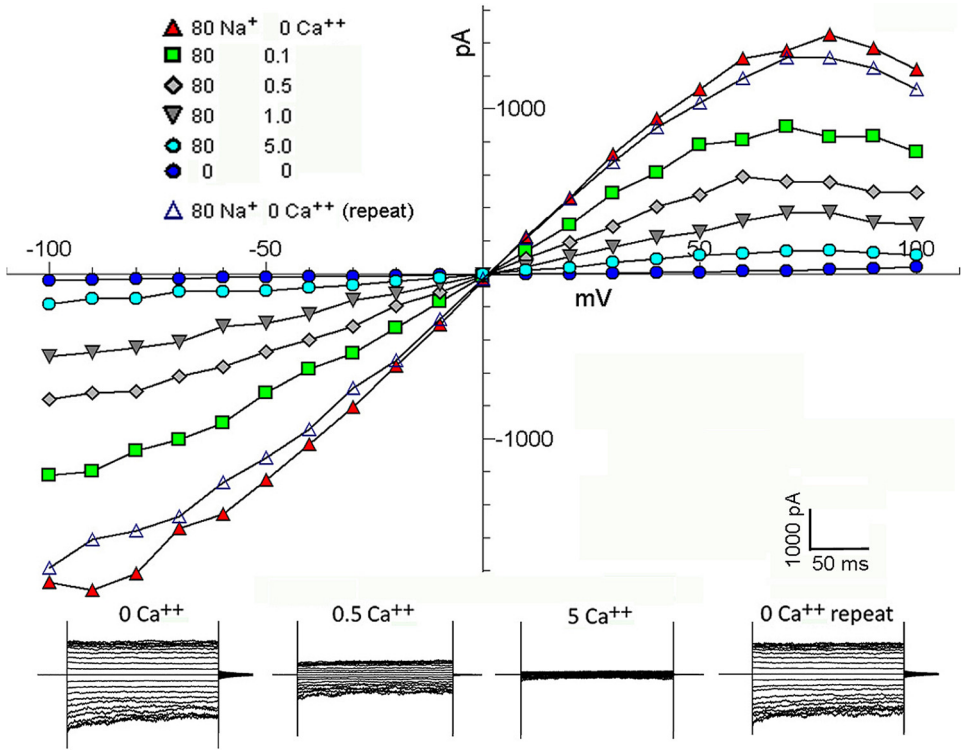

FIGURE 1. **Family of I-V curves from an inside-out macropatch at different internal calcium concentrations.** *Top*: representative I-V curves obtained from inside-out macropatches from HEK cells stably expressing rSLO2.2 in the presence of 80 mM Na^+ and exposed to different calcium concentrations. The experiments were performed in symmetrical K^+; the patch was held at 0 mV and 200 ms steps applied from −100 to +100 mV in 10 mV increments. The currents in the plot are averages of the last 50 ms of each trace where the currents are relatively stable. All concentration units are mM. Note that 5 mM calcium reduces the current to levels close to the residual leak current obtained in 0 mM Na^+. Reversibility was observed by applying 80 Na^+ 0Ca^{2+} solution at the end of the experiment. *Bottom*: representative Inside-out patch current traces at indicated [Ca^{2+}].

expected for a charged molecule blocking the pore of the channel; more block is seen when more electrostatic force is applied pushing the molecule further into the pore. Thus, a blocking effect of Ba^{2+} is noted at all positive voltages, in contrast to all other divalent ions tested (as well as experiments without a divalent ion on the intracellular surface) where a sodium blocking effect is noted only at intracellular voltages greater than +60 mV. This well characterized pore blocking effect of Ba^{2+} on K^+ channels and its voltage-dependence has been especially well studied on SLO1 BK channels (21, 22). More recently, a dual effect of Ba^{2+} has been reported; it blocks the pore of SLO1 channels at positive voltages, but also activates the channel by binding to the calcium bowl (18). Similarly, we show in Fig. 3 that Ba^{2+} also has a dual effect on SLO2.2 channels. However, both effects are inhibitory; Ba^{2+} blocks SLO2.2 channels with a voltage-dependent effect at positive potentials, but also inhibits the channel in a non-voltage-dependent manner, which is most obvious at negative potentials. The obvious voltage-dependent block of Ba^{2+} was unique and not observed with any of the other divalent cations tested, the absence of which suggests that the effect of all other divalent cations is achieved by interacting with a site away from the pore.

Single Channel Conductance Is Not Decreased by Divalent Cation Application—We studied the effect of divalent cations on SLO2.2 using single channel recordings in inside-out patches from *Xenopus* oocytes expressing rSLO2.2 channels. Our results show that the application of divalent cations produces an obvious decrease in open channel probability (Fig. 4*A*) without producing a significant reduction in single channel conductance (Fig. 4*B*) or flickering of the channel. These results also suggest that the effect of divalent cations on SLO2.2 channels is by allosteric modification of the channel and not by blocking of the pore.

Divalent Cation Inhibition Is Less at High Sodium Concentrations—Experiments were performed in inside-out macropatches to test the inhibiting effect of Zn^{2+} at different Na^+ concentrations. Our results showed that Zn^{2+} is a more effective inhibitor of the channel at low sodium concentrations. When the same patch is exposed to different sodium concentrations where the concentration of Zn^{2+} is held constant we observed that, as the sodium ion concentration is increased, the inhibitory effect of Zn^{2+} is measurably smaller (Fig. 5). Conversely, when the sodium concentration is lower, the inhibitory effect of divalent cations is much greater. To give a specific example, at 40 mM Na^+, a Zn^{2+} concentration of .05 mM resulted in an ~87% inhibition of the reference (no Zn^{2+}) current, while at 240 mM Na^+, the same Zn^{2+} concentration of 0.05 mM resulted in only an ~18% inhibition of the reference current. This may have important consequences under physiological conditions. The IC_{50} values reported in Fig. 2 were derived in the presence of 80 mM Na^+; however, the actual physiological intracellular Na^+ concentration is much lower and thus the inhibitory effect of these divalent cations *in vivo* is likely to be much greater. Hence, free Mg^{2+} which may have an intracellular concentration of 1 mM or greater (23), may exert a considerable inhibitory effect under physiological conditions.

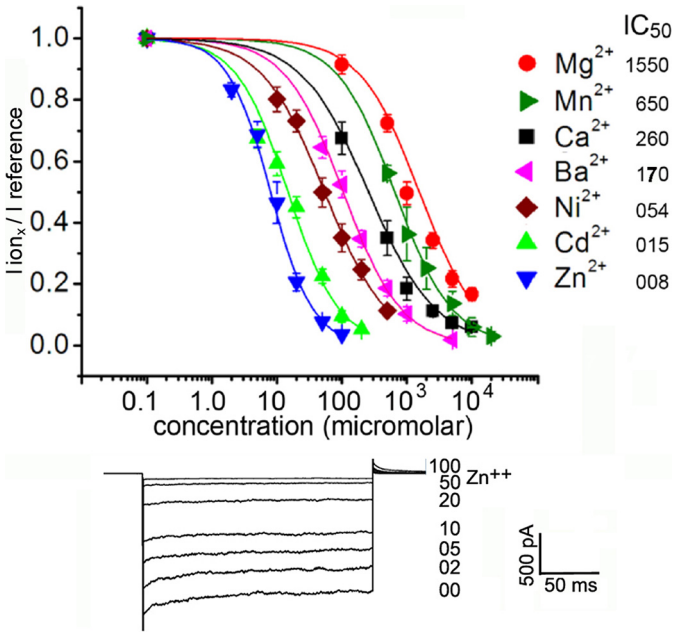

FIGURE 2. **Different divalent cations inhibit SLO2.2 channels with differing effectiveness.** All divalent cations tested showed an inhibitory effect on SLO2.2 currents when applied to the intracellular side of the membrane. The effect of internal divalent cations was measured at −50 mV in the presence of 80 mM sodium at different divalent concentrations. Inside-out patches were exposed to the same solution except for increasing divalent cation concentrations. The current obtained in the presence of zero intracellular sodium was subtracted from all the recordings, and currents were normalized to the current obtained with 80 mM Na^+ in the absence of divalent cation (Reference). *Top*: plot shows the averages of the normalized currents at different divalent concentrations fitted with the logistic function for all divalent cations tested. The number of patches for each point was between 5 and 8. IC_{50s} were derived from the fits. *Bottom*: representative inside-out patch current traces in the presence of 80 mM Na^+ at different Zn^{2+} concentrations.

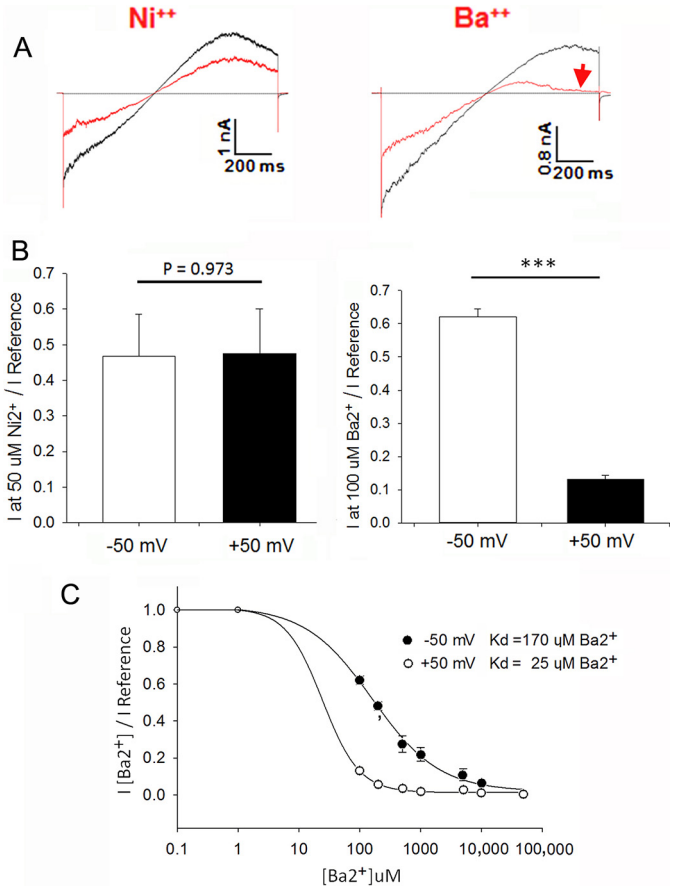

FIGURE 3. **Among divalent cations tested, (Zn^{2+} Cd^{2+}, Ni^{2+}, Ba^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Ba^{2+}) only Ba^{2+} showed a voltage-dependent block.** Ramps from −90 mV to +90 mV were applied to inside-out patches expressing SLO2.2. Note that block shown at higher voltages in the control traces (*black*) is due to the high intracellular Na^+ (80 mM), which is necessary to activate these sodium-activated channels (5). *A*, *left*: when nickel is added to the intracellular solution the decrease in the current observed is similar for positive and negative potentials. *Right*: voltage-dependent block of Ba^2 is seen at all positive voltages. Barium was the only cation tested that had a blocking effect at voltages less than +60 mV. Inside-out macropatches were obtained from HEK cells stably expressing rSLO2.2. *B*, bar diagrams showing the relative current at −50 mV and +50 mV when Ni^{2+} (*left*) or Ba^{2+} (*right*) is applied. The fraction of current remaining after divalent cation application at positive and negative potentials is not different for Ni^{2+} ($p = 0.769$) but significantly differs for Ba^{2+} at positive and negative voltages ($p < 0.0001$). *C*, plot of the average currents at different barium concentrations relative to Imax in the absence of barium (reference). Data are fitted with a logistic function. *Open circles* are normalized currents at +50 mV; *filled circles* are at −50 mV; $n = 4$.

SLO2.2 Channels Are pH-dependent—Since Zn^{2+} was the most effective inhibitor of the divalent cations tested we investigated amino acid residues usually present in its coordination. We focused our attention on histidine or cysteine residues which are often present in Zn^{2+} binding sites (24). Our first approach was to use histidine and cysteine modifiers to detect whether the modification of one or more residues of either class might alter channel gating when modified. When the cysteine modifier MTSEA was applied to inside-out patches no change in currents or in divalent cation inhibition was observed. In contrast the histidine modifier diethyl pyrocarbonate decreased the macroscopic currents when applied to the inside of the membrane. Also, as previously reported (25), we confirmed that low pH inhibits SLO2.2 in inside-out macropatches (Fig. 6). Thus, both of these results suggest the importance of a histidine residue in channel gating.

A His Residue Downstream of S6 Affects Divalent Cation Inhibition—Since our results indicated that a histidine could be present in divalent cation binding we undertook mutagenesis of histidine residues in the C-terminal domain of the SLO2.2 channel. We found that substitution of two histidines by arginine residues (His-823 and His-824) in a putative SLO2 sodium binding site (26) had no effect on divalent cation inhibition of the mutant channel. Subsequently a comparative analysis of the sequence of SLO2.2 and SLO1 channels revealed a histidine present in SLO2.2 channels on the cytoplasmic side of S6 that is not present in SLO1. Interestingly, the relative position of this histidine residue is conserved in a cyclic nucleotide-gated channel (Fig. 7), and was shown to be involved in the inhibition of those channels by Ni^{2+} (19). As was found for cyclic nucleotide-gated channels, the substitution of glutamine for this histidine (H347Q) in SLO2.2 channels significantly decreased the inhibitory effect of Zn^{2+} on SLO2.2 currents recorded in inside-out macropatches (Fig. 7, lower current traces and bar graph). We also tested the effect of Ba^{2+} on the SLO2.2H347Q mutant (Fig. 8). Remarkably, at the concentration of Ba^{2+} tested (0.1 mM), there was no blocking effect whatsoever at negative potentials suggesting that the allosteric blocking by Ba^{2+} seen in the WT channel had been removed by the H347Q mutation. At positive potentials, however, the current was reduced in a potential-dependent manner suggesting a voltage-dependent block, just as

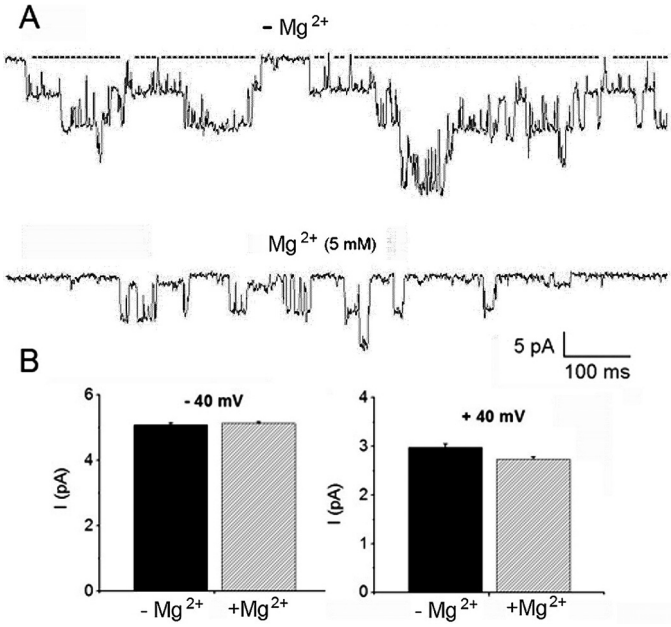

FIGURE 4. **Divalent cations decrease open probability but not single channel conductance.** *A*, single channel currents of an inside-out patch containing at least four channels. The open probability of SLO2.2 channels decreases in the presence of divalent cations. The recordings shown here were performed at −80 mV using symmetrical potassium and high internal sodium in the absence and presence of 5 mM magnesium. *B*, statistical analysis of single channel current amplitude before and after the addition of Mg^{2+} to the cytoplasmic surface of inside-out patches. At −40 mv (*left*) there is no decrease in single channel current after Mg^{2+} is added. At a positive voltage (+40) a very slight but significant decrease in single channel amplitude is present. (mean amplitudes (pA) At −40 mV: 5.09 -Mg, 5.14 + Mg; p = 0.5. At +40 mV: 2.98 -Mg, 2.74 +Mg; p = .01. n = 20 for each population carefully measured by eye)

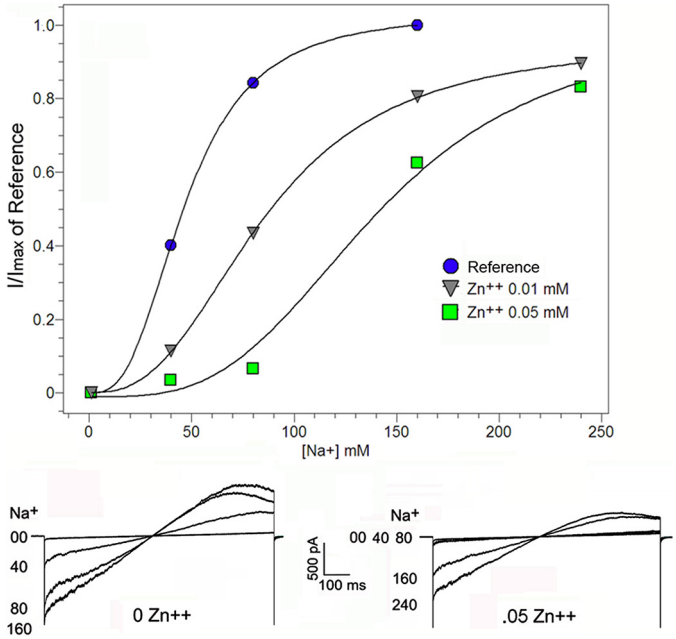

FIGURE 5. **Plots of normalized currents from inside-out macropatches with increasing sodium concentrations in the absence and presence of zinc.** Averaged normalized currents were recorded at −50 mV under symmetrical potassium conditions. Results were fitted with the Hill equation. Hill coefficients indicate cooperativity for sodium binding which is little changed by the presence of Zn^{2+} (h (Reference) = 2.8; h (0.01 Zn^{2+}) = 2.7; h (0.05 Zn^{2+}) = 3.3). In the presence of zinc, EC_{50} values for sodium ion are significantly increased but estimated V_{max} values were similar (V_{max} (Reference) = 1.03; V_{max} (0.01 Zn^{2+}) = 0.95; V_{max} (0.05 Zn^{2+}) = 1.01). This may indicate competitive inhibition. However, this does not mean that the site of sodium and divalent ion binding are necessarily the same. The inhibitor can bind to the channel at a different site and produce a conformational change that reduces the affinity of the activator for the binding site. Zinc concentrations used are .01 and .05 mM as indicated. n = 4 patches for each zinc concentration. *Bottom*: representative **c**urrent traces of a macropatch at different sodium concentrations in the absence and presence of zinc (0.05 mM).

had occurred in the WT SLO2.2 channel (Fig. 8). This result suggests that barium inhibits the channel by an allosteric effect only in the wild-type channel and not in the mutant channel, while it blocks the pore in a voltage-sensitive manner in both wild-type and mutant channels. This experiment is especially illustrative in distinguishing the two distinct blocking effects of Ba^{2+}.

The Divalent Cation Inhibition of SLO2 Channels Is Conserved in Drosophila—Prior results had shown that a Na^+-activated potassium current was present in *Drosophila* neurons (27), but nothing was known about the genetic identity of the channel underlying the current. To investigate the presence of a SLO2 channel in *Drosophila* we Blasted the amino acid sequence of the rat SLO2.2 channel against the translated whole genome sequence of *Drosophila,* which revealed the nucleotide sequence of several homologous exons in the *Drosophila* genome; all were clustered on chromosome 2R at CG42732. This nucleotide sequence was then used to isolate several cDNAs by rtPCR of *Drosophila* adult stage RNA, or PCR of an adult stage cDNA library. A full-length dSLO2 cDNA was then constructed from these overlapping cDNA fragments and cloned into our pOX *Xenopus* expression vector (28) for heterologous functional expression. The translated sequence of this functional cDNA clone does not correspond to any of the gene products predicted by *Drosophila* Genome Consortium. This sequence has now been deposited with GenBank™ (accession number KT852572). Fig. 9 shows a comparison of the amino acid alignment of the dSLO2 channel with the rat Slack (SLO2.2) channel. The two sequences have an overall identity of ~61% through the conserved portions (membrane spanning domains 1 through 6), although sequence conservation drops off radically toward the end of RCK2. A histidine residue downstream of S6 is also conserved in the dSLO2 sequence so we hypothesized that dSLO2 may also be subject to inhibition by divalent cations. Notably, this histidine residue is not conserved in the *C. elegans* SLO2 channel, which is activated by the divalent cation, Ca^{2+}(29), rather than inhibited as in the rat SLO2.2 channel. Functional expression studies showed that, like the rat SLO2.2 channel, the dSLO2 channel is also activated by internal Na^+ (Fig. 10, *A* and *B*) with sensitivity similar to that of mammalian SLO2 channels. We also showed that the dSLO2 channel is similarly inhibited by divalent cations (Fig. 10, *C* and *D*).

Discussion

SLO2 channels are high conductance K^+ channels of the SLO channel family that encode Na-dependent K^+ currents. K_{Na} channels were initially identified in cardiac cells where their suggested role was that of providing a reserve conductance that would protect cells during hypoxia; during hypoxia an elevation in $[Na^+]_i$ may occur as a result of blocking the Na^+/K^+ ATPase (10). This idea arose as a result of observations

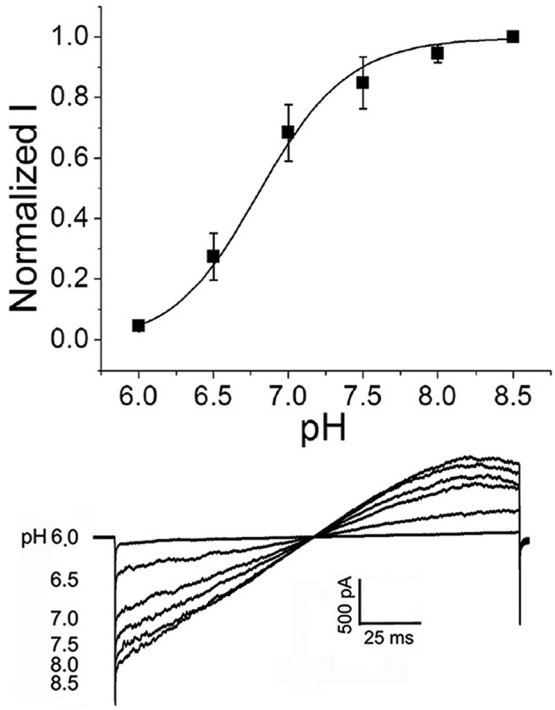

FIGURE 6. **Effect of intracellular pH on Slack (SLO2. 2) currents.** Inside-out macropatch currents of SLO2.2 channels were recorded in the presence of 80 mM sodium at indicated pH. The 50% inhibition of current occurs at pH 6.8 suggesting an IC_{50} for protons of ~160 nM. *Top*: plot shows normalized macropatch currents in the presence of 80 mM sodium at different pH values. The currents were normalized to the current at pH 8.5 recorded at −50 mV. *Bottom*: representative current traces at different pH as indicated.

of K_{Na} channels in inside-out patches which showed that K_{Na} channel activation required a much higher $[Na^+]_i$ than that normally found in bulk cytoplasm. However, these channels are widely distributed, being present in a wide variety of neurons (8). We recently showed that Na^+ entering a neuron via a small but persistent sodium current is surprisingly effective in activating the sodium-activated K^+ current (IK_{Na}), even in the total absence of bulk internal Na^+ (11). These observations suggest a mechanism by which IK_{Na} can commonly participate in providing a large outward conductance in many neuronal types. K_{Na} channels are expressed throughout the brain where they might contribute to resting conductance as well as action potential repolarization (7, 8, 11, 12). In some neurons such as tufted/mitral cells of the olfactory bulb, sodium-activated K^+ channels are densely packed into the soma (9, 11, 12). If active, such channels will hyperpolarize the soma of a neuron or shunt synaptic potentials, making it more difficult for summed postsynaptic potentials to initiate an action potential. Thus, because of their properties and strategic location, SLO2 channels could play a "gate keeper" role in synaptic integration, and its modulation may contribute to use-dependent mechanisms of plasticity in the mammalian brain. The somatodendritic localization of SLO2 channels and their modulation by neurotransmitter receptors (30) make SLO2 channels good candidates for regulating intrinsic neuronal excitability. Because of the important role that SLO2 channels may play both in physiological and pathophysiological conditions, it is important to understand how they are regulated. Both Ca^{2+} and Mg^{2+} which are important physiological second messengers have been shown to be activators of the SLO1 channel (1, 2), but their effect on SLO2 channels had not yet been studied in any detail.

In this study we found that the mammalian Slack channel (SLO2.2) is inhibited by all divalent cations that activate SLO1. The relative strength of inhibition was: $Zn^{2+} > Cd^{2+} > Ni^{2+} > Ba^{2+} > Ca^{2+} > Mn^{2+} > Mg^{2+}$. None of these ions showed any voltage-sensitivity of block except for Ba^{2+}, suggesting an allosteric mode of inhibition rather than a pore blocking effect. An allosteric mode of action is also supported by the fact that the addition of Mg^{2+} to the inside surface of patches expressing single channel currents did not show any reduction in single channel current amplitudes or any obvious increase in single channel flickering (Fig. 4). Other evidence to discount a pore blocking effect is the fact that the block is smaller when a divalent ion is added in the presence of higher concentrations of sodium suggesting a possible competition between sodium and divalent ions in the mechanism of inhibition.

We also showed that SLO2 activation by sodium ion and inhibition by divalent cations is conserved in *Drosophila*. It had been reported that a Na^+-activated potassium current was present in *Drosophila* neurons (27), but the genetic identity of the channel underlying the current was unknown. Our cloning and functional expression of the *Drosophila* dSLO2 gene product, showing the conservation of its properties with the mammalian SLO2.2 channel, now opens the door for the use of *Drosophila* as a model system to study the physiological role of SLO2 channels in this genetically versatile system.

Site-directed mutagenesis of the SLO2.2 channel implicated a His residue downstream S6 as a possible divalent cation binding site at a position analogous to an inhibitory cation binding site in some CNG channels. Notably, this His residue is conserved in the *Drosophila* SLO2 channel which we also show to be inhibited by divalent cations. Prior studies showed that, in contrast to rat SLO2.2 channels, SLO2 channels in *C. elegans* are activated rather than inhibited by Ca^{2+} (29). This may reflect the fact that, unlike most metazoans, *C. elegans* lacks a true voltage-sensitive sodium channel, and relies on voltage-dependent Ca^{2+} channels for the propagation of electrical signaling. Significantly, the amino acid sequence of the *C. elegans* SLO2 orthologue does not include the His residue implicated in divalent ion inhibition and conserved in rat and *Drosophila* orthologues.

The fact that the inhibition by divalent cations in SLO2 channels is conserved across species (rodent/fruit fly) intriguingly suggests that this property may have an important physiological role in modulating SLO2 channels; however, we cannot say for certain which (if any) of the divalent cations we have tried is actually involved. Although calcium and magnesium ion are two candidates which conceivably may have a physiological role in modulating SLO2 channels, any of the other ions could plausibly be factors under unknown conditions where ion concentrations in limited submembrane regions rise to a sufficient level to influence channel activity. In addition, it is important to note that we measured the IC_{50} values in the presence of high (80 mM) sodium ion. We also showed, however that the relative blocking effect of these divalent ions is much greater at lower

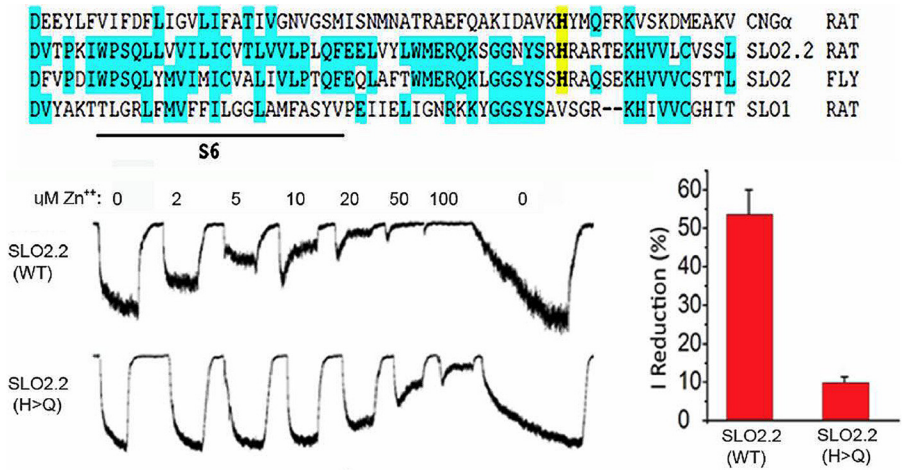

FIGURE 7. **A histidine residue downstream of S6 affects zinc inhibition of SLO2.** *Top*: sequence alignment for the CNG, SLO2, and SLO1 channels of the region extending from the transmembrane segment S6 into the S6-RCK1 linker. Conserved residues are *shaded*. The conserved His residue is shaded *yellow*. *Bottom*: gap-free recordings of SLO2 WT and the SLO2 H347Q mutant in the presence of different intracellular zinc concentrations. Membrane voltage was maintained at −40 mV with an intracellular sodium concentration of 80 mM. Increasing zinc concentrations were added as noted. The bar graph on the *right* shows the % current reduction recorded at −50 mV, after addition of 10 μM zinc for WT and the H347Q mutant ($n > 4$, $p < 0.01$). Data are from heterologous expression in inside-out patches from *Xenopus* oocytes.

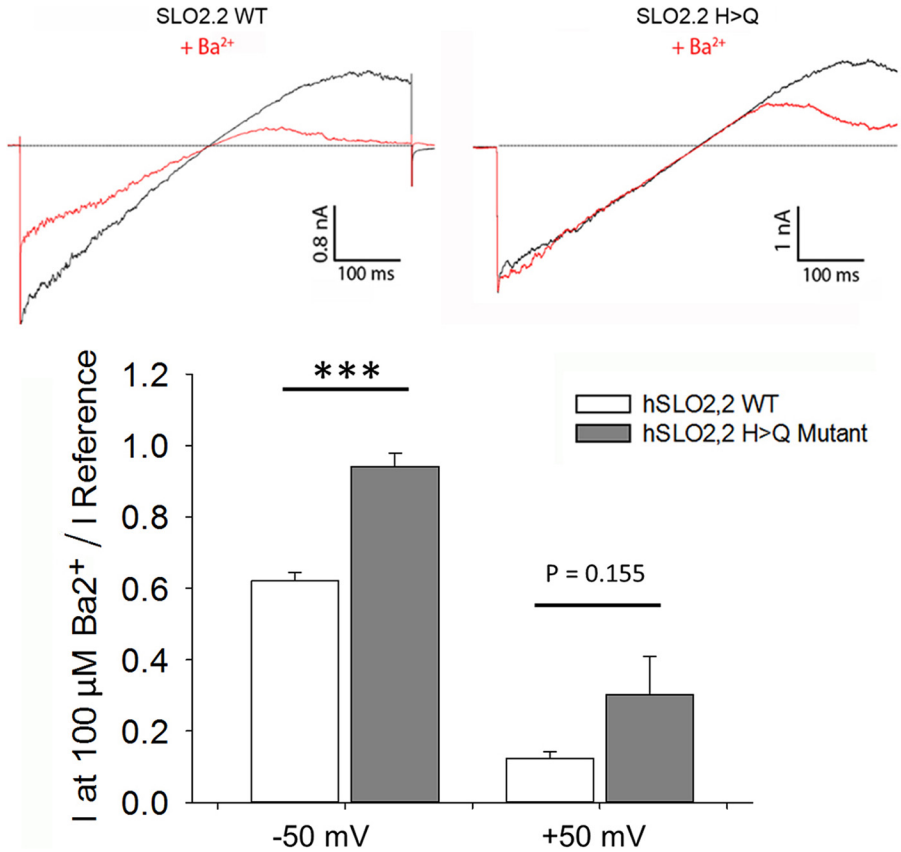

FIGURE 8. **Effect of barium in SLO2.2 WT compared with the SLO2.2H347Q mutant.** *Top*: *left*, Slo2.2 WT currents evoked by a voltage ramp from −90 to 90 mV in the presence of 80 mM sodium before (*black*) and after (*red*) the application of 0.1 mM barium. On the *right*, the same experiment was performed in macropatches expressing the (H347Q) mutant. Note that the application of 0.1 mM barium produces no effect at negative potentials in the H347Q mutant but blocks the current in a voltage-dependent manner at positive potentials. Data are from heterologous expression in inside-out patches from *Xenopus* oocytes. *Bottom*: bar diagram illustrating the lack of current block in the H347Q mutant at −50 mV by 0.1 mM barium. This differs with WT, which is blocked at both negative and positive voltages.

sodium ion concentrations (Fig. 5), and thus the IC_{50} values reported in Fig. 2 may be much lower under physiological conditions where the actual intracellular Na^+ concentration is likely to be much lower.

The possibility that Mg^{2+} may have a role is interesting to consider because free [Mg^{2+}] may have an intracellular concentration of 1 mM or greater (31) which may exert a considerable inhibitory effect at physiological conditions. Magnesium is the least effective

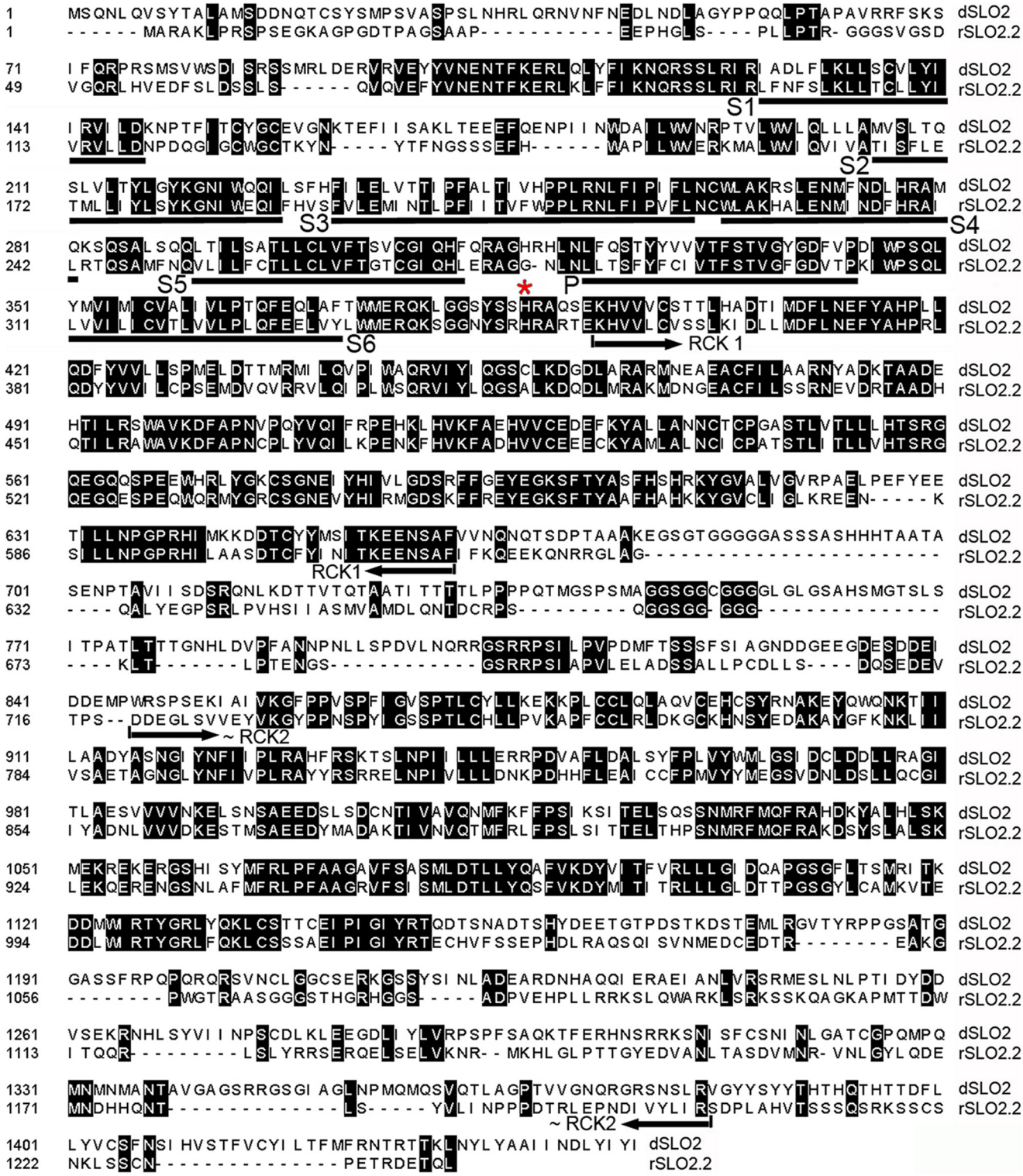

FIGURE 9. **Comparative alignment of the amino acid sequence of the *Drosophila* SLO2 channel (dSLO2) with the rat Slack (rSLO2) channel.** Putative membrane spanning domains, S1-S6, are noted with *black bar* underlining. RCK1 and RCK2 gating ring structure is delimited by *arrows*. A conserved histidine residue following S6, which is important in inhibition by divalent cations is marked by a *red asterisk*. Notably, this residue is not conserved in the *C. elegans* SLO2 channel, which is activated by the divalent cation, Ca^{2+}, rather than inhibited by it (29).

divalent cation tested in inhibiting SLO2.2 channels. Nevertheless, it is conceivable that it modulates SLO2.2 channels within its physiological range of concentrations.

Interestingly, it has been suggested that native sodium-activated K^+ channels represent a reserve conductance that can be activated during times of stress resulting from ischemia or hypoxia to prevent depolarization when Na^+ accumulates in cells (6, 10). However, it is well established that the concentration of many divalent cations increases during hypoxia or ischemia. In particular, the free concentration of magnesium is ele-

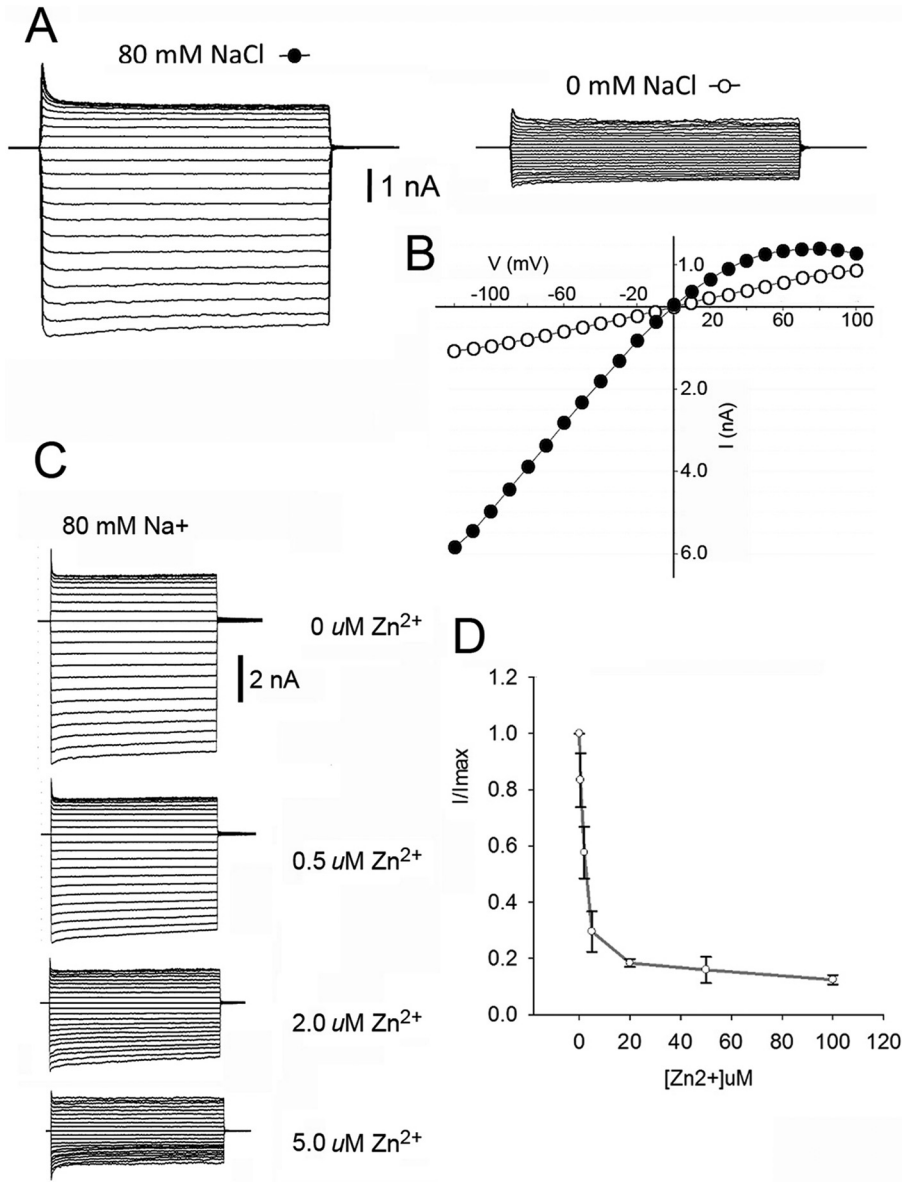

FIGURE 10. **Na$^+$ activation of *Drosophila* SLO2 and inhibition by Zn^{2+}.** *A*, dSLO2 currents expressed in inside-out patches from *Xenopus* oocytes in the presence and absence of 80 mM Na$^+$. *B*, plot of current-voltage data from *A*. *C*, exposure of dSLO2 currents to intracellular Zn^{2+} at indicated concentrations. *D*, fit of the plotted data from *C* indicated an IC_{50} of ~1.5 μM suggesting a greater sensitivity to Zn^{2+} than the rat SLO2.2 (Slack) channel. Data are from heterologous expression in inside-out patches from *Xenopus* oocytes.

vated due to the decrease in ATP which buffers Mg^{2+} (32). Thus, this work may indicate that SLO2.2 channels are inhibited rather than activated during hypoxia or ischemia (Fig. 5).

Among the divalent cations tested, the most effective in inhibiting SLO2.2 channels was Zn^{2+} with an IC_{50} close to 8 μM. Such intracellular Zn^{2+} concentrations are probably never reached in the cytoplasm. However, as has been observed in calcium measurements (33), it is possible that intracellular Zn^{2+} concentrations increase in microdomains, close to channels permeable to Zn^{2+} or other Zn^{2+} sources, and that increase is high enough to modulate SLO2.2 activity. Thus, it is conceivable that some channels or receptors permeable to Zn^{2+} co-localize with SLO2.2 channels in neurons. In SLO1, which is activated by similar concentrations of Zn^{2+}, co-expression with TRP channels that are permeable to Zn^{2+} increased SLO1 channel activity when extracellular Zn^{2+} was present (3) indicating a rise in a local intracellular submembrane domain where SLO1 channels are present and possibly co-localized with TRP channels.

SLO2 channels are high conductance and only weakly voltage dependent and may be active in a negative voltage range where they contribute to the resting conductance of neurons. In addition to a possible "gatekeeper" role in synaptic integration as previously discussed, SLO2 channels are also possibly involved in shaping the characteristics of a back-propagating action potential that could be involved in dendro-dendritic synaptic activity. Thus, the somatodendritic localization of SLO2 channels and their modulation by neurotransmitter receptors (30) make SLO2 channels good candidates for regulating intrinsic neuronal excitability that potentially plays several roles in the electrical life of neurons. Our findings presented in this manuscript suggest yet

another layer of control over these interesting channels in their diverse roles.

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