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Pharmacological activation and inhibition of *Slack* (*Slo*2.2) channels

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Abstract

The *Slack* (*S*equence *l*ike *a c*alcium-activated *K* channel) (*Slo*2.2) gene is abundantly expressed in the mammalian brain and encodes a sodium-activated K^+ (K_{Na}) channel. Although the specific roles of *Slack* channel subunits in neurons remain to be identified, they may play a role in the adaptation of firing rate and in protection against ischemic injury. In the present study, we have generated a stable cell line expressing the *Slack* channel, and have analyzed the pharmacological properties of these channels in these cells and in *Xenopus* oocytes. Two known blockers of K_{Na} channels, bepridil and quinidine, inhibited *Slack* currents in a concentration-dependent manner and decreased channel activity in excised membrane patches. The inhibition by bepridil was potent, with an IC_{50} of 1.0 μM for inhibition of *Slack* currents in HEK cells. In contrast, bithionol was found to be a robust activator of *Slack* currents. When applied to the extracellular face of excised patches, bithionol rapidly induced a reversible increase in channel opening, suggesting that it acts on *Slack* channels relatively directly. These data establish an important early characterization of agents that modulate *Slack* channels, a process essential for the experimental manipulation of *Slack* currents in neurons.

Keywords: Potassium channels; Slack; Sodium-activated channels; Electrophysiology

1. Introduction

The significance of potassium (K^+) conductances in the regulation of neuronal excitability and their involvement in neuronal pathologies has been well documented (Hille, 2001; Levitan and Kaczmarek, 2002). K^+ channels encoded by the *Slack* (*S*equence *l*ike *a c*alcium-activated *K* channel) (*Slo*2.2) gene are gated by intracellular Na^+, and *Slack* channels are located in the brain regions (Joiner et al., 1998; Bhattacharjee et al., 2002; Yuan et al., 2003) reported to possess sodium-activated K^+ (K_{Na}) channels (Egan et al., 1992; Dryer, 1994; Bhattacharjee and Kaczmarek, 2005). Nevertheless, the physiological significance of these K_{Na} conductances in the central nervous system (CNS) is not yet fully understood. The discovery of potent blockers and activators of *Slack* channels may therefore lead to a better understanding of the roles these channels play under physiological and/or pathophysiological circumstances.

Abbreviations: BK_{Ca}, large conductance calcium-activated K^+; CHO, Chinese hamster ovary (cells); CNS, central nervous system; HEK, human embryo kidney (cells); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; K_{Na}, sodium-activated K^+; $[Na^+]_{in}$, intracellular concentration of Na^+; *Slack*, Sequence like a calcium-activated K; TEA, tetraethylammonium.

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Other than the closely related K_{Na} channel subunit *Slick* (Bhattacharjee et al., 2003), the channel with the highest similarity to *Slack* is the Ca^{2+}-activated potassium channel *Slo*, with ~7% identity (Joiner et al., 1998). *Slack* has several domains in its long carboxy terminal region that show clear homology to *Slo*. Nevertheless, the pharmacology of the *Slack* current is quite distinct from that of *Slo* channels. *Slack* channels are insensitive to the large conductance calcium-activated K^+ (BK_{Ca} or maxi-K) channel blocking agent iberiotoxin and the BK_{Ca} channel opener NS-1619, but are inhibited by millimolar (10 mM) concentrations of tetraethylammonium ions (TEA) (Joiner et al., 1998; Bhattacharjee et al., 2003).

In this study we have characterized the actions of quinidine and bepridil, two compounds that have been shown to inhibit K_{Na} channels in ventricular myocytes (Mori et al., 1998; Li et al., 1999), on the properties of *Slack* channels expressed in oocytes and mammalian cells. Quinidine and bepridil are mixed ion channel blockers and have been used as antiarrhythmic drugs. They are known to block several types of K^+ channels, as well as other types of voltage-dependent ion channels, in cardiac myocytes, neurons and other cell types (Kehl, 1991; Zilberter et al., 1994; Lesage et al., 1996; Sato et al., 1996; Chouabe et al., 1998; Leonoudakis et al., 1998; Reyes et al., 1998; Kobayashi et al., 2001; Yumoto et al., 2004). In contrast to these blocking agents, there have been no previous descriptions of activators or openers of either native K_{Na} channels or of the *Slack* channel. We now report that bithionol, a commercially available bis-phenol anti-parasitic compound (Enzie and Colglazier, 1960; Barr et al., 1965), is an effective opener of *Slack* channels. The pharmacological characterization of *Slack* currents in transfected cells will allow a more rigorous comparison of these currents to K_{Na} currents in native neurons, and may also represent an important first step in the development of more potent and specific K_{Na} modulators.

2. Methods

2.1. Stable cell line expressing the Slack channel

The SlackHA.pCDNA3 construct, containing the full-length wild type *Slack* sequence, was used to transfect HEK-293 cells. Transfection was performed using the SuperFect Transfection Reagent (QIAGEN Inc., Valencia, CA). The stable *Slack*-expressing HEK cell line was confirmed by patch-clamp recordings and Western blot. These HEK cells were cultured in a modified low sodium DMEM medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (Invitrogen Inc, Carlsbad, CA).

2.2. Electrophysiological recordings from Slack-expressing Xenopus oocytes

Two-electrode voltage clamp recordings were carried out on *Xenopus laevis* oocytes using standard techniques (Gribkoff et al., 1996). Each oocyte was injected with approximately 50 nL of *Slack* mRNA and maintained at 17 °C in ND96 medium consisting of (in mM): 90 NaCl, 1.0 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 5.0 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.5; horse serum (5%) and penicillin/streptomycin (5%) were added to the incubation medium. After 3–7 days, oocytes were placed in a recording chamber and incubated in Modified Barth's Solution (MBS) consisting of (in mM): 88 NaCl, 2.4 $NaHCO_3$, 1.0 KCl, 10 HEPES, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, pH 7.5. Oocytes were impaled with electrodes (1–2 MΩ, borosilicate glass, filled with 3 M K-acetate pulled on a Brown-Flaming P-87 pipette puller (Sutter Instrument Co., Novato, CA), and allowed to stabilize before recording.

Voltage-clamp protocols typically consisted of a series of voltage steps of 500 ms in duration, in +10 mV steps from a holding potential of −80 mV to a maximal potential of +140 mV. Records were obtained using a two-electrode voltage clamp amplifier (TEV-200A, Dagan Instruments, Minneapolis, MN), digitized at 5 kHz and stored on a computer using pClamp data acquisition and analysis software (Axon Instruments, a Division of Molecular Devices, Sunnyvale, CA). Compounds were dissolved in incubation medium to the desired concentration from 10 mM stock solution in dimethylsulfoxide (DMSO), and solutions were introduced into the recording chamber via gravity (1–2 mL/min). Solution switching occurred via electronic valves. Oocytes were exposed to compounds for 5–10 min, and washes, when attempted, lasted for 10–15 min. Compounds were tested in at least 5 different oocytes to evaluate the effect of each single drug concentration and data were expressed either as percent inhibition or as the average percentage change in *Slack* current relative to drug-free control (100%). Since these values may change as a function of the voltage used for a determination, values were sometimes obtained for more than a single membrane voltage value.

2.3. Electrophysiological recordings from a stable cell line expressing the Slack channel

Whole-cell and excised patch recordings from the stable *Slack*-expressing HEK cells were obtained at room temperature (21–23 °C) using the gigaseal patch-clamp technique (Hamill et al., 1981). Electrodes of 3–5 MΩ resistance for whole-cell recordings and 7–9 MΩ resistance for excised patch recordings were pulled from TW150F-6 micropipettes (World Precision Instruments Inc., Sarasota, FL) on a horizontal Flaming/Brown micropipette puller (Model P-87, Sutter Instrument Co., Novato, CA), fire-polished, coated with dental wax, and filled with appropriate filling solutions.

For whole-cell recordings, cells were bathed in a solution containing (in mM): 140 NaCl, 1 $CaCl_2$, 5 KCl, 29 glucose, and 25 HEPES, pH 7.4. The pipette solution contained (in mM): 100 K-gluconate, 30 KCl, 5 Na-gluconate, 29 glucose, 5 EGTA, 2 Na_2ATP, 0.2 GTP, and 10 HEPES, pH 7.3. For excised patch recordings, the bath and pipette solution contained (in mM): 130 KCl, 10 Na-gluconate, 10 HEPES, 5 EGTA, and 19 glucose, pH 7.3. All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO).

Following the establishment of a >2 GΩ seal, the whole-cell configuration was achieved. In some experiments outside-out membrane patches were excised for single channel recordings (Hamill et al., 1981). Signals were processed using an EPC-7 amplifier (HEKA Electronic, Lambrecht, Germany) or a MultiClamp 700B amplifier (Axon Instruments, Sunnyvale, CA). Data were collected and analyzed with PClamp 9.2 software (Axon Instruments, Sunnyvale, CA). Series resistance was <12 MΩ for whole-cell configuration or <20 MΩ for single channel recordings, and compensated at 70–80%. An Ag-AgCl electrode connected to the bath solution via a KCl-agar bridge served as reference. All signals were digitized at 5 kHz for whole-cell recordings or 20 kHz for single channel recordings, filtered at 2 kHz, and stored on computer for off-line analysis.

To determine the I–V relationships, the membrane potential was held at −70 mV, and stepped to levels between −120 mV and +120 mV in 20-mV increments. For single-channel recordings from excised patches in the outside-out configuration, the sign of the applied voltage refers to the patch pipette with respect to the bath. Outward currents refer to the flow of cations from the pipette (cytosolic side) into the bath (extracellular side). In every outside-out patch experiment, patches were excised from cells, and the membrane potential was stepped to +60 mV from a holding potential of 0 mV. For single-channel analysis, NPo (N is the number of active channels, Po is the open probability) was calculated using the pClamp 9.2 software.

2.4. Drugs

The actions of bepridil, quinidine and bithionol on the amplitude and kinetics of macroscopic *Slack* currents, as well as on the behavior of these

currents detected in single-channel recordings, were assessed in these experiments. All experimental compounds were prepared on the day of experiment by diluting stock the solutions (10 mM in DMSO) stored at appropriate temperatures into perfusate. The concentration ranges tested in this study were initially in accordance with those shown to be effective in the literature cited above, and adjusted as necessary when effective ranges were determined for each compound. All of the chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, MO).

2.5. Statistical analysis

Data were compared, where applicable, using Student's t-test. All values are plotted as means ± SEM. In the oocyte experiments determinations of EC_{50} values were made using logistic fits of data and Kaleidograph software (Synergy Software, Reading, PA). In the patch clamp experiments, the concentration-response curves were constructed from a sigmoidal function of nonlinear regression (either Origin, OriginLab Corp., Northampton MA, or Prism; GraphPad Software Inc., San Diego, CA), from which the IC_{50} and EC_{50} values were obtained.

3. Results

3.1. Bepridil inhibits macroscopic Slack currents

We first examined the actions of bepridil on *Slack* currents expressed in *Xenopus* oocytes. As reported previously (Joiner et al., 1998), oocytes injected with *Slack* mRNA responded to depolarizing voltage pulses with large (2–5 μA) outwardly rectifying currents. In all cases, uninjected oocytes displayed relatively low-amplitude currents only at very high depolarizing potentials (reflective of endogenous Ca^{2+}-activated chloride current), and were not significantly affected by any of the compounds used in these studies. In *Slack* mRNA-injected oocytes, bepridil produced a concentration-dependent decrease in outward current at the concentrations used (1–50 μM; Fig. 1). The effects of bepridil appeared to be largely voltage-independent (Figs. 1A, B). The maximal inhibition of current that was attained at the three membrane voltages examined (+40, +100 and +140 mV) was approximately 80%. It is likely, however, that the residual current reflects the native Ca^{2+}-activated Cl^- current, because the absolute values for these residual currents were close to those recorded in uninjected oocytes at the same voltages, and bepridil did not reduce these native currents in uninjected oocytes. The calculated EC_{50} values were 9.2, 7.0 and 6.5 μM for at +40, +100 and +140 mV, respectively (Fig. 1B). The effects of bepridil were largely reversible upon washout into control medium (Fig. 1C).

Application of depolarizing voltage steps under whole-cell voltage clamp in HEK cells expressing *Slack* subunits resulted in large outward currents with an activation voltage near −60 mV (Fig. 2). Untransfected cells had only very low levels of endogenous outward current, even with large depolarizing voltage steps. Application of bepridil (10 nM–20 μM) produced a concentration-dependent inhibition of *Slack* currents with an estimated IC_{50} of ~1.0 μM, lower than that measured in oocytes (Figs. 2A, C, D). The inhibition was observed through almost the entire voltage range of channel activation, although at −40 mV no observable effect was seen. The

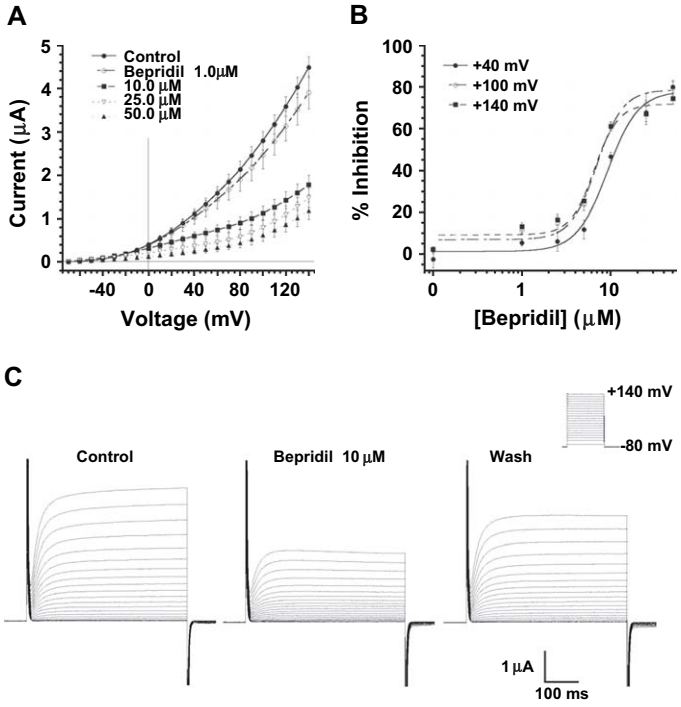

Fig. 1. Bepridil inhibition of *Slack* currents in *Xenopus* oocytes. (A) Current-voltage (I–V) relationships (−70 mV–+140 mV) for Bepridil (1.0 μM–50 μM) versus control medium from a holding potential of −80 mV. Experiments were conducted in two groups of five oocytes with one group receiving 1.0 and 25 μM and the second group receiving 10 and 50 μM Bepridil. Control data were combined for this figure. Drug exposures were 5min/concentration. Following the final drug concentration, a 5 min wash was performed. The effects were reversible. In all oocyte experiments, a minimum of 5 oocytes contributed to group data. (B) Concentration-response curves for Bepridil (1.0–50 μM) taken at +40, +100 and +140 mV voltages. Percent inhibition data was derived from data in Panel A. (C) Family of currents generated in a single oocyte in response to voltage commands (−70–+140 mV) from a −80 mV holding potential. Bepridil (10 μM) was applied for 5 min followed by a 5 min wash.

degree of inhibition was slightly greater at higher voltages. The bepridil-induced inhibition was reversible upon return to control medium (not shown).

3.2. Bepridil inhibits Slack currents in outside-out and inside-out excised patches

Because *Slack* currents are dependent on intracellular Na^+, we could not rule out the possibility that the inhibition of *Slack* currents by bepridil in oocytes resulted from an inhibition of a small-amplitude endogenous Na^+ current by this compound (Yatani et al., 1986; Sperelakis, 1987). To determine if the effects of the compound are independent of an effect on voltage-dependent Na^+ current, we carried out experiments to measure the effect of bepridil on *Slack* channel activity under conditions of controlled intracellular Na^+ concentration ($[Na^+]_{in}$). For these experiments we recorded *Slack* channels in patches excised from HEK cells stably expressing the *Slack* gene. These channels could clearly be attributed to the *Slack* subunit because they were absent in recordings from untransfected HEK cells. Moreover, in whole-cell recordings, untransfected

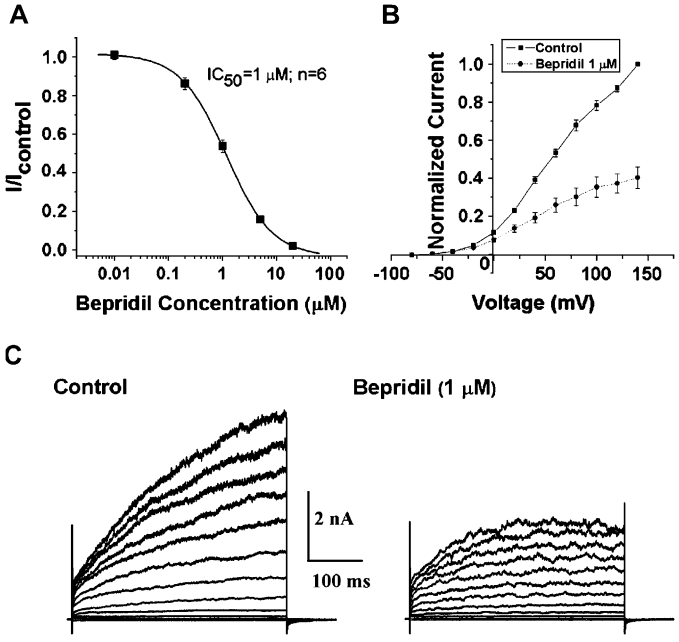

Fig. 2. Bepridil inhibited whole-cell *Slack* currents in HEK cells. (A) Concentration-response relationship. Bepridil was applied to 6 cells sequentially and the degree of inhibition noted. The inhibition was essentially complete at the highest concentration tested (20 μM). (B) Current-voltage relationships for the cells exposed to 1 μM bepridil. (C) Examples of currents generated by voltage steps between −80 and +140 mV from a holding potential of −60 mV, in the presence of control medium (left panel) and 1 μM bepridil (right panel).

HEK cells displayed outward currents of very low amplitude (tens of pA) only at very positive potentials and these currents were not significantly affected by any of the compounds used in these studies.

Application of bepridil to the extracellular face of outside-out patches excised from stable *Slack*-expressing HEK cells induced a marked and reversible decrease in channel activity (Fig. 3A). Activity in multi-channel patches was quantified by measuring the product of the number of channels (N) multiplied by probability of opening (Po). The ratio of activity of *Slack* channels in the presence and the absence of 10 μM bepridil ($NPo_{(Bepridil)}/NPo_{(Control)}$) was 0.471 ± 0.026 ($n = 11$). After reperfusion with control bath solution the ratio $NPo_{(Washout)}/NPo_{(Control)}$ returned to 0.885 ± 0.026 ($n = 11$) (Fig. 3B). Although we did not determine the number of channels in these patch experiments, we rarely detected simultaneous openings of two or more channels. We therefore also calculated the mean open time of channel openings in these experiments. Bepredil (10 μM) decreased the mean open time of the *Slack* channels from 111 ± 5 to 21 ± 5 ms ($n = 11$). These results indicate that bepridil reversibly blocks the *Slack* channel directly rather than though effects on intracellular sodium levels. In addition to these experiments with outside-out patches, we also tested the effects of application of bepridil to the intracellular face of inside-out patches excised from stable *Slack*-expressing HEK cells. As with the outside-out patches, bepredil produced a marked and reversible decrease in channel activity (Figs. 3C, D).

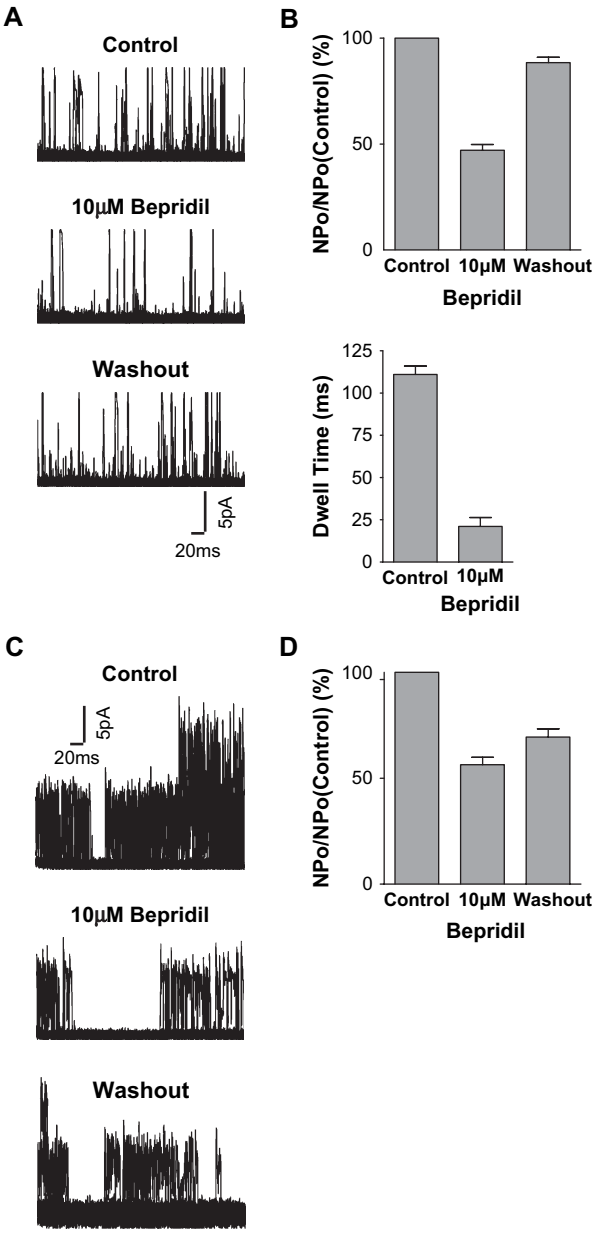

Fig. 3. Bepridil decreased the open probability of *Slack* channels in HEK cells. (A) The membrane potential of outside-out patches was stepped to +60 mV from a holding potential of 0 mV. *Slack* channel activity decreased rapidly and reversibly in response to 10 μM bepridil. Scale bars: 20 ms, 5 pA. (B) Summary bar graph showing the ratios $NPo_{(Bepridil)}/NPo_{(Control)} = 0.471 \pm 0.026$ ($n = 11$) with 10 μM bepridil and $NPo_{(Washout)}/NPo_{(Control)}$ ($n = 11$) expressed as percentages. Also shown is the mean open time in control ($n = 11$) and 10 μM bepridil ($n = 11$). (C) In inside-out patches, *Slack* channel activity decreased rapidly and reversibly in response to 10 μM bepridil. Scale bars: 20 ms, 5 pA. (D) Summary bar graph showing the ratios $NPo_{(Bepridil)}/NPo_{(Control)}$ ($n = 6$) with 10 μM bepridil and $NPo_{(Washout)}/NPo_{(Control)}$ ($n = 6$) expressed as percentages.

3.3. Block of the Slack-mediated macroscopic currents by Quinidine

We have previously reported that 1 mM quinidine inhibits *Slack* currents in CHO cells transiently transfected with the *Slack* gene (Bhattacharjee et al., 2003). We have now determined the full dose-response relationship for the inhibition

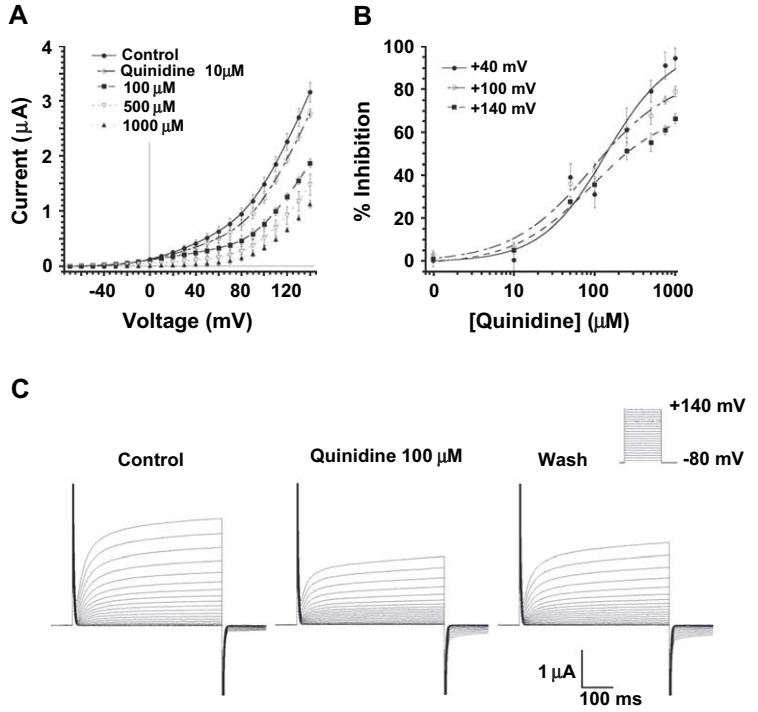

Fig. 4. Quinidine blocked *Slack* currents in a concentration-dependent manner in *Xenopus* oocytes. (A) I–V curves in control medium and in several concentrations of quinidine. (B) Concentration-response relationships for quinidine on *Slack* currents at +40, +100 and +140 mV. (C) Examples of membrane currents generated in oocytes in response to depolarizing steps in control medium and in quinidine (100 μM).

of *Slack* currents by quinidine. Application of quinidine to oocytes expressing *Slack* produced levels of inhibition similar to those observed with bepridil, with a maximal inhibition of 80–90% of the total outward current (Fig. 4). The effects again appeared to be voltage-independent (Fig. 4A), but were significantly less potent than those of bepridil, with calculated EC_{50} values of 138.2, 94.4 and 91.6 μM at +40, +100 and +140 mV, respectively (Fig. 4B). The effects of quinidine in *Slack*-injected oocytes were more difficult to reverse on washout of this compound, although in most cases some degree of recovery was observed (Fig. 4C).

As in oocytes, bath application of quinidine (1 μM–10 mM) to voltage-clamped stably *Slack*-transfected HEK cells, produced a rapid (2–3 min to peak) decrease in current ($n = 35$) (Fig. 5A). In contrast to oocytes, however, the inhibitory effects of quinidine on the transfected HEK cells were readily reversible, and a second bath application of this agent resulted in similar blocking effects on the *Slack* currents. Analysis of the group data in response to quinidine concentrations ranging from 10^{-6} to 10^{-2} M demonstrated that the reduction in current was concentration-dependent with an EC_{50} of 89.6 μM (Fig. 5B), in relatively close agreement with the values obtained in oocytes.

3.4. Inhibition of Slack channels by quinidine in outside-out and inside-out membrane patches

In order to confirm that the inhibitory effects of quinidine on *Slack* current are direct, we carried out experiments to examine its effects on *Slack* channel activity under conditions of controlled $[Na^+]_{in}$. Application of quinidine to the extracellular face of outside-out active patches excised from stably *Slack*-transfected HEK cells produced a marked and reversible decrease in channel activity (Fig. 6A). The decrease in activity produced by 1 mM quinidine was quantified by measuring the ratio $NPo_{(Quinidine)}/NPo_{(Control)}$, which was 0.410 ± 0.050 ($n = 6$) (Fig. 6B). We also measured the mean open time of unitary events that were uncontaminated by simultaneous openings. Quinidine decreased the mean open time of the *Slack* channels from 107.02 ± 5.91 to 36.60 ± 6.58 ms ($n = 6$). These results strongly indicate that quinidine directly inhibited *Slack* channel activity when applied to the extracellular side of the plasma membrane. As with outside-out patches, application of quinidine to the intracellular face of excised inside-out patches also produced a marked and reversible decrease in *Slack* channel activity in HEK cells (Figs. 6C, D).

3.5. Activation of Slack channels by Bithionol

In oocytes expressing *Slack* channels, bithionol was an impressively effective activator of these currents (Fig. 7). The compound was tested to the limits of its solubility (25 μM), and produced very marked increases in the amplitudes of the outward currents (Figs. 7A–C). The estimated EC_{50} values at +40, +100 and +140 mV were 12.1, 9.5 and 9.6 μM, respectively (Fig. 7B). These values may, however, be less accurate than those obtained in previous experiments, due to the limited solubility of this compound. The effects of bithionol

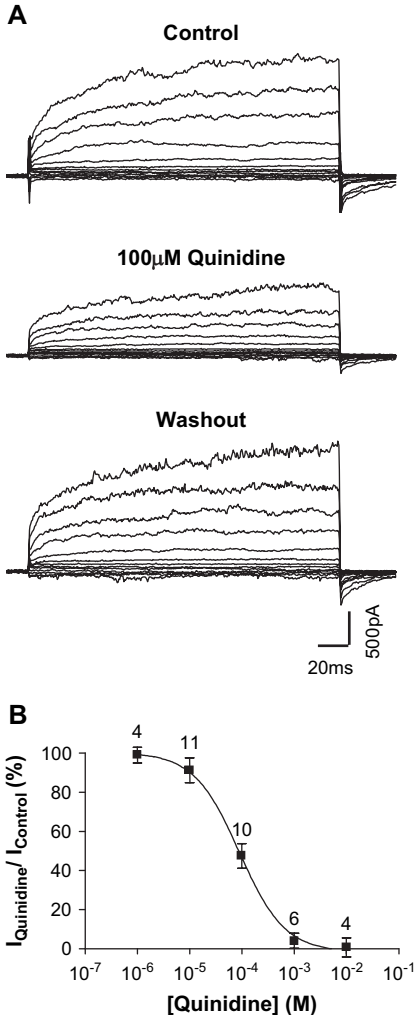

Fig. 5. Quinidine inhibited the *Slack* currents in a concentration-dependent manner in HEK cells. (A) Whole-cell K^+ conductances evoked by voltage steps between −120 and +120 mV in 20-mV increments from a holding potential of −70 mV. Bath application of quinidine (100 μM) produced a rapid (2–3 min) and reversible decrease in *Slack* currents. Scale bars: 20 ms, 500 pA. (B) Inhibition of *Slack* currents by quinidine is concentration dependent. Changes in percentage current ($I_{Quinidine}/I_{Control}$) measured during responses to 10^{-6} ($n = 4$), 10^{-5} ($n = 11$), 10^{-4} ($n = 10$), 10^{-3} ($n = 6$), and 10^{-2} ($n = 4$) M extracellular quinidine were plotted against bath quinidine concentrations. Data are shown as mean ± SEM and are fitted to a sigmoid concentration-response function; $IC_{50} = 8.96 \times 10^{-5}$ M.

were readily and nearly completely reversible during brief washes (Fig. 7C).

The increase in *Slack* current produced by bithionol was associated with a very marked alteration in the voltage-dependence of currents. In contrast to other voltage-dependent channels such as *Slo* or *Shaker*-family channels, the S4 region of the *Slack* protein does not have basic residues at every third position, a motif that is thought to contribute to voltage sensing by these other channels. Nevertheless, the probability of opening of *Slack* channels increases at positive potentials (Joiner et al., 1998; Bhattacharjee et al., 2003). We constructed conductance-voltage relations for *Slack* currents in the presence or absence of bithionol by normalizing currents to maximal current measured +140 mV, and assuming that current

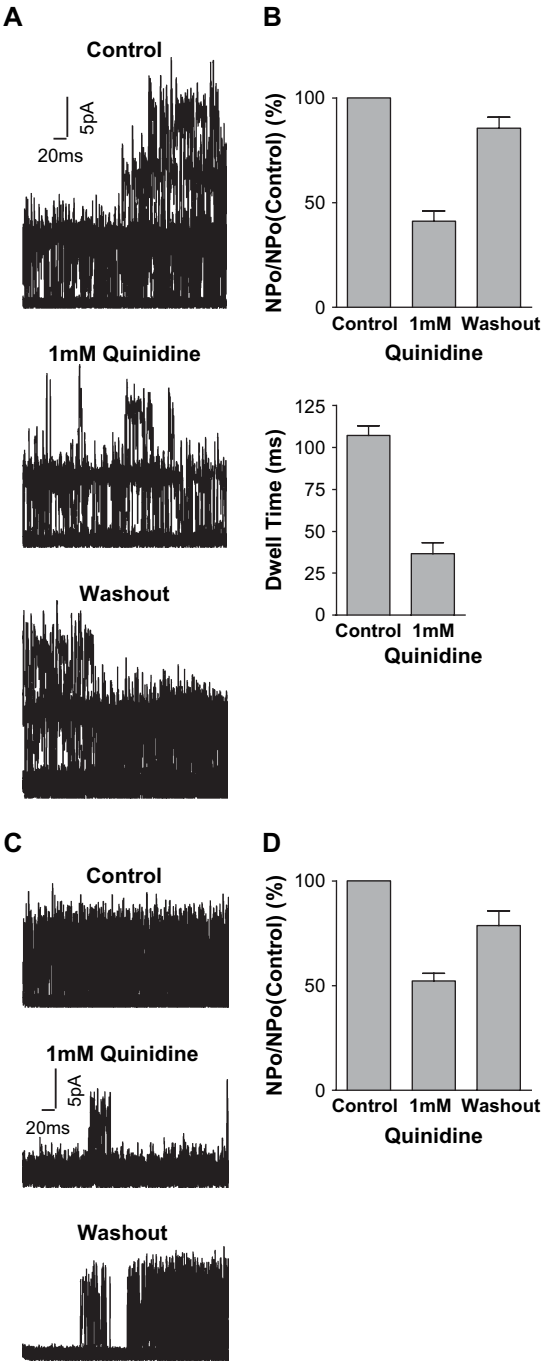

Fig. 6. Quinidine inhibition of *Slack* channels in membrane patches from HEK cells. (A) The membrane potential of outside-out patches was stepped to +60 mV from a holding potential of 0 mV. *Slack* channel activity decreased rapidly and reversibly in response to 1 mM quinidine. Scale bars: 20 ms, 5 pA. (B) Summary bar graph showing the ratios $NPo_{(Quinidinel)}/NPo_{(Control)}$ ($n = 6$) with 1 mM quinidine and $NPo_{(Washout)}/NPo_{(Control)}$ ($n = 6$), expressed as percentages. Also shown is the mean open time in control (control, $n = 6$) and in 1 mM quinidine ($n = 6$). (C) In inside-out patches, *Slack* channel activity decreased rapidly and reversibly in response to 1 mM quinidine. Scale bars: 20 ms, 5 pA. (D) Summary bar graph showing the ratios $NPo_{(Quinidine)}/NPo_{(Control)}$ ($n = 5$) with 1 mM quinidine and $NPo_{(Washout)}/NPo_{(Control)}$ ($n = 5$) expressed as percentages.

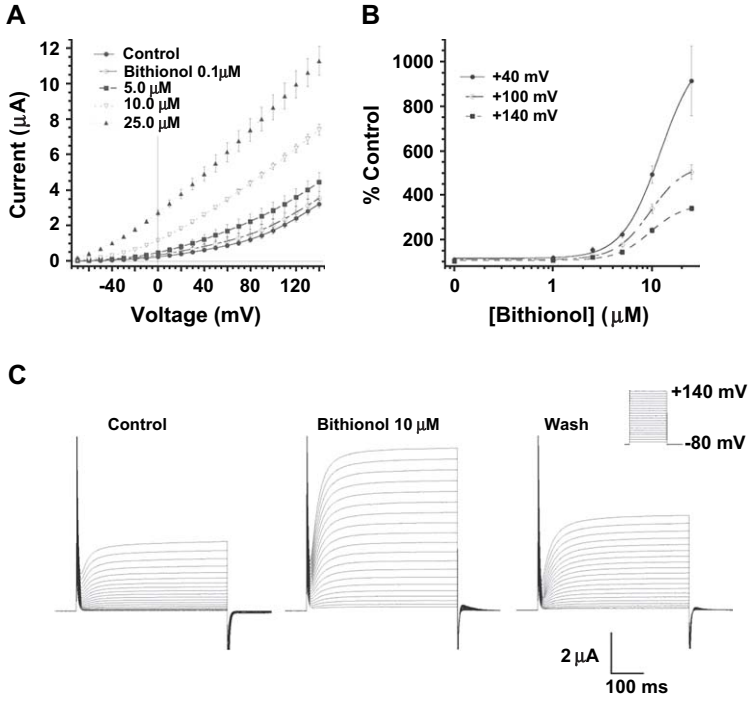

Fig. 7. Bithionol activation of *Slack* currents in *Xenopus* oocytes. (A) I–V curves generated in control medium and in bithionol demonstrating a leftward drug-induced shift in the activation voltage, and a significant increase in outward current amplitude produced by bithionol. (B) Concentration-response relationships for bithionol at +40, +100 and +140 mV. Note that at +40 mV the currents have increased over 9-fold at 25 μM. (C) Examples of membrane currents produced in *Xenopus* oocytes in response to depolarizing steps in control solution and bithionol, showing the very large increase in outward current and the reversibility of the effect.

though open channels in physiological solutions obeys the Goldman-Hodgkin-Katz equation, as suggested by single channel recordings (Joiner et al., 1998). Fig. 8 shows that, in the absence of bithionol, the calculated conductance-voltage relationship for *Slack* currents in oocytes does not saturate even at potentials up to +140 mV. In contrast, in the presence of bithionol the activation voltage of currents is shifted to negative potentials. Full activation in the presence of 10 and 25 μM bithionol is apparently complete at potentials of 0 and −40 mV, respectively.

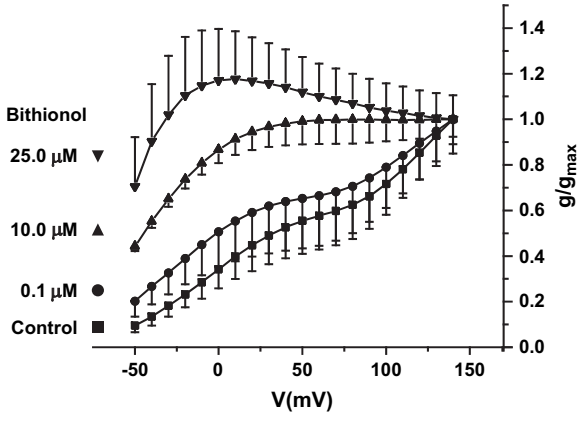

Fig. 8. Bithionol shifts activation of *Slack* currents in *Xenopus* oocytes to negative potentials. Relative conductance voltage-relations were calculated for *Slack* currents in the absence or presence of 0.1, 10 and 25 μM bithionol. Currents were normalized to those recorded at +140 mV and the relative conductances (g/g_{max}) were calculated using predictions of the Goldman-Hodgkin-Katz current equation for current flow though open channels (intracellular $[K^+] = 100$ mM; extracellular $[K^+] = 1.0$ mM).

Bithionol also produced a marked increase in current and a shift in the activation voltage to more negative potentials in whole-cell patch clamp experiments in HEK cells expressing *Slack* channels (Figs. 9A–C). The augmentation of *Slack* current was concentration-dependent with an apparent EC_{50} of 0.77 μM (Fig. 9A), a value lower than that recorded in Xenopus oocytes. Untransfected cells did not respond to the application of bithionol (not shown). As observed with Slack mRNA-injected oocytes, activation of Slack currents in HEK cells could be detected at more negative potentials after treatment with bithionol (Fig. 9B).

3.6. Effects of bithionol on Slack channels in outside-out and inside-out membrane patches

In order to confirm that the activating effects of bithionol on *Slack* current are direct, we carried out experiments to examine its effects on *Slack* channel activity under conditions of controlled $[Na^+]_{in}$. Application of bithionol to the extracellular face of outside-out active patches excised from stably *Slack*-transfected HEK cells produced a marked and reversible increase in channel activity (Fig. 10A). The increase in activity produced by 10 μM bithionol was quantified by measuring the ratio $NPo_{(Bithionol)}/NPo_{(Control)}$, which was 2.610 ± 0.256 ($n = 7$). The ratio returned to 1.017 ± 0.093 ($n = 7$) after

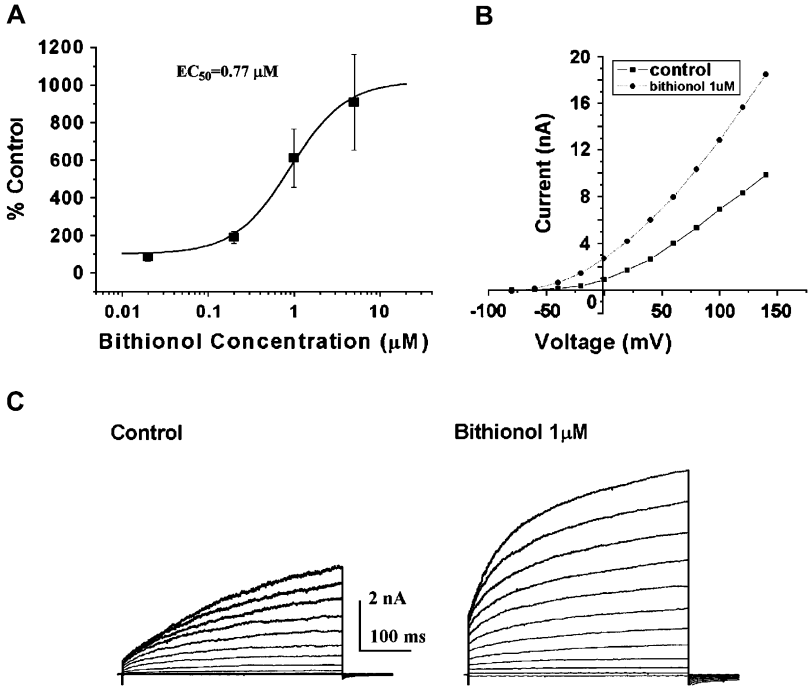

Fig. 9. Bithionol increased whole-cell currents in HEK cells expressing *Slack* subunits. (A) Concentration-response relationship generated by administering 4 concentrations of buithionol to 6 cells. The effects were potent, but the limited solubility of bithionol suggests that this is estimate of potency may not be highly accurate. (B) Current-voltage-relationship for a cell exposed to 1 μM bithionol. (C) Current families generated by voltage steps from −80 to +140 mV from a holding potential of −60 mV. The instantaneous component of the current appeared to be generally increased to an even greater degree than the later, slowly activating component.

washout of bithionol with control bath solution (Fig. 9B). In contrast to the actions of bepredil and quinidine, 10 μM bithionol did not affect the mean open time of *Slack* channels ($n = 7$). This suggests that bithionol may decrease closed times, but this was not measured directly because of the absence of true single channel patches. As with the outside-out patches, application of bithionol to the intracellular face of excised inside-out patches also produced a marked and reversible increase in *Slack* channel activity (Figs. 10C, D).

4. Discussion

The significance of voltage-gated potassium conductances in the regulation of neuronal excitability and their involvement in neuronal pathologies has been well documented (Shieh et al., 2000; Levitan and Kaczmarek, 2002). *Slack* is a ligand-gated K^+ channel that is activated by intracellular Na^+ ions and is abundantly expressed in those mammalian CNS regions reported to possess K_{Na} channels (Joiner et al., 1998; Bhattacharjee et al., 2002; Bhattacharjee and Kaczmarek, 2005).

Since the first description of K_{Na} channels in cardiac cells by Kameyama et al. (1984), there has been much debate about what roles these channels may play during physiological and patho-physiological conditions (Dryer, 1994). This is largely because the activation of these K_{Na} channels usually requires $[Na^+]_{in}$ to rise above 20 mM, while $[Na^+]_{in}$ measured by ion-selective electrodes in control and stimulated cardiac preparations is generally lower than 10 mM (Lee and Fozzard, 1975; Ellis, 1977; Nakaya et al., 1990). Nevertheless, substantially higher local levels of $[Na^+]_{in}$ may arise if Na^+ channels or non-selective cation channels are closely physically associated with individual K_{Na} channels, and/or if there are significant diffusion barriers that allow for the accumulation Na^+ in submembranous regions. In fact, such barriers are present in neurons in small elongated structures such as distal dendrites or nodes of Ranvier (Koh et al., 1994). Moreover, recent imaging studies of have determined that localized intracellular $[Na^+]_{in}$ may rise by as much as 40–80 mM in restricted neuronal compartments during repetitive stimulation (Rose, 2002; Rose and Konnerth, 2001; Zhong et al., 2001). Furthermore, even in non-neuronal cells there is micro-heterogeneity of $[Na^+]_{in}$. At the inner side of the cell membrane of cardiac cells (Carmeliet, 1992), for example, $[Na^+]_{in}$ accumulates up to about 40 mM within 20 nm of the inner side of the sarcolemma during repetitive electrical activity. This is a result of Na^+ influx via Na^+ channels and Na^+-Ca^{2+} exchangers (Wendt-Gallitelli et al., 1993). Consistent with these findings, K_{Na} channel activity can be measured experimentally during paired-pulse stimulation of guinea-pig ventricular cells (Wendt-Gallitelli et al., 1993).

The recent cloning, expression and identification of *Slack* and *Slick* subunits as molecular substrates of K_{Na} (Joiner et al., 1998; Bhattacharjee et al., 2002, 2003; Yuan et al., 2003) will allow a more detailed analysis of K_{Na} at a molecular level. This should permit detailed study of the contributions of these channels to the properties of cells in which they form physiologically relevant currents, a task that will be made

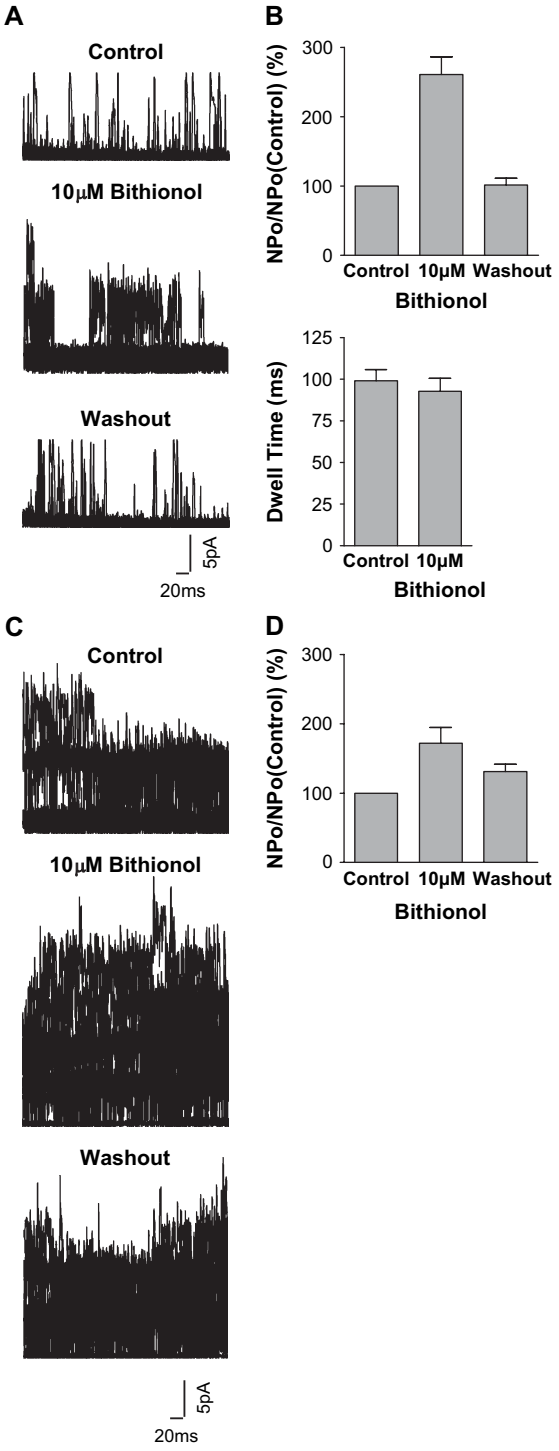

Fig. 10. Bithionol increases the open probability of *Slack* channels in HEK cells. (A) The membrane potential of outside-out patches was stepped to +60 mV from a holding potential of 0 mV. The *Slack* channel activity increased rapidly and reversibly in response to 10 μM bithionol. Scale bars: 20 ms, 5 pA. (B) Summary bar graph showing the ratios $NPo_{(Bithionol)}/NPo_{(Control)}$ ($n = 7$) with 10 μM bithionol and $NPo_{(Washout)}/NPo_{(Control)}$ ($n = 7$), expressed as percentages. Also shown are the mean open times for control ($n = 7$) and 10 μM bithionol ($n = 7$). (C) In inside-out patches, *Slack* channel activity increased rapidly and reversibly in response to 10 μM bithionol. Scale bars: 20 ms, 5 pA. (D) Summary bar graph showing the ratios $NPo_{(Bithionol)}/NPo_{(Control)}$ ($n = 6$) with 10 μM bithionol and $NPo_{(Washout)}/NPo_{(Control)}$ ($n = 6$) expressed as percentages.

practical when sufficient pharmacological tools are available for the study of these channels *in situ*. Specifically, the development and characterization of potent and selective blockers and openers of these channels may help us to better understand the roles these channels play under both physiological and/or patho-physiological circumstances. An important first step in the development of specific ligands for these channels is to identify known agents with significant activity against one or both K_{Na} subunits. In this study, we generated the first stable cell line expressing the *Slack* channel, and demonstrated that both bepridil and quinidine block the *Slack* channel in a concentration-dependent manner when expressed in this mammalian cell line, as well as in *Xenopus* oocytes. We have analyzed the actions of bepridil and bithionol on both the amplitude and kinetics of the macroscopic currents, as well as on the behavior of the channels detected in single-channel recordings. Our data strongly suggest that both bepridil and quinidine directly block the *Slack* channel, and the effects of bepridil are quite potent. In particular, the finding that both these agents effectively decreased single channel activity in excised outside-out patches renders it unlikely that the inhibition of *Slack* conductances by these compounds is secondary to an effect on Na^+ channels limiting available Na^+ for K_{Na} activation by voltage. Our findings are consistent with data demonstrating that class I, III and IV antiarrhythmic drugs, including bepridil, inhibit the K_{Na} channel current in guinea-pig ventricular cells (Mori et al., 1996, 1998), and indicate that at least some of this effect may result from direct K_{Na} inhibition.

Bithionol, a bis-phenol compound previously known for its anti-parasitic actions (Enzie and Colglazier, 1960; Barr et al., 1965), was shown in this study to be a relatively potent and very effective activator of *Slack* currents. Single-channel recordings revealed a significant increase in mean channel open probability in the presence of bithionol. These data represent, to the best of our knowledge, the first demonstration of activation of *Slack* currents (or K_{Na}) by any compound. We have found that bithionol also activates maxi-K channels (Gribkoff, Boissard and Starrett, unpublished observations), but not all maxi-K channel openers/activators result in an increase in Slack currents; for example, NS-1619 does not (Joiner et al., 1998). The use of bithionol may therefore prove particularly useful in cell types that express little or no maxi-K current, such as fast-spiking neurons including those in the auditory brainstem, where *Slack* is expressed at high levels (Kaczmarek et al., 2005).

We have compared the responses of Slack channels to bepredil, quinidine and bithionol in two different expression systems, *Xenopus* oocytes and mammalian HEK cells. While the IC50 for quinidine is similar in these two expression systems, the IC50 for bepredil and the EC50 for bithionol are approximately ten-fold higher in oocytes. This finding is not unusual and likely reflects both the complex structure of *Xenopus* oocytes, which requires that a pharmacological agent cross several membranes before interacting with a channel, and the fact that lipophyllic compounds may accumulate in the large volume of yolk. The more complex structure of oocytes may, however, provide a more accurate predictor of potency in

intact tissues. Nevertheless, disparities between *Xenopus* oocytes and monolayers of mammalian cells could also reflect differences in posttranslational modification of channels in the two expression systems.

Pharmacological tools that target ion channels, either through a direct interaction or through their effects on channel modulation, have a variety of uses. For example, they permit a rigorous comparison of the properties of channels such as those produced by the S*lack* gene expressed in transfected cells with corresponding currents in native neurons. Both inhibitors and activators may, however, also ultimately have value as therapeutics. The *Slack* K_{Na} channel is expressed in both neurons and cardiac myocytes and it is possible that the effects of bepridil that we have characterized may contribute to its known antiarrhythmic action. The *Slack* channel is also likely to play a role in the adaptation of firing rate to maintained stimulation, and may also contribute to the protection of neurons against ischemic injury (Yuan et al., 2003). Thus the present initial characterization of agents that influence these channels is a starting point for the discovery of more potent and specific modulators of K_{Na} channels. While none of these compounds is highly selective, both bepridil and bithionol are highly potent against *Slack* currents, and carefully designed experiments may be able to use these initial agents to examine the role of K_{Na} in native tissues. Moreover, they serve as a starting point for the synthesis of new and novel modulators of these channels.

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