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Effects of Channel Modulators on Cloned Large-Conductance Calcium-Activated Potassium Channels

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SUMMARY

Through expression of the cloned mouse (*mSlo*) or human (*hSlo*) large-conductance (BK) Ca^{2+}-activated K^+ channel in *Xenopus laevis* oocytes and HEK 293 cells, we characterized the effects of reported blockers and openers of BK channels to initiate the study of the molecular determinants of BK channel modulation. In oocytes, iberiotoxin and charybdotoxin, peptidyl scorpion toxins, were both equally effective blockers of BK current, although iberiotoxin was significantly more potent than charybdotoxin. The structurally related peptide kaliotoxin was not a potent blocker of BK current. Paxilline, a fungal tremorgenic alkaloid, was an effective but complex blocker of BK current. Tetrandrine, a putative blocker of type II BK channels, and ketamine were relatively ineffective. The putative BK openers NS004 and NS1619, phloretin, niflumic acid, flufenamic acid, and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) increased BK current in oocytes at μM concentrations; many of these produced biphasic concentration-response relationships. Coapplication of representative blockers and openers revealed several patterns of interaction, including competitive and noncompetitive antagonism. NS1619, niflumic acid, and phloretin were tested by using excised inside-out membrane patches from HEK 293 cells and were found to increase the activity of *hSlo* BK channels and produce a leftward shift in the G/G_{max}-versus-voltage relationship of these channels. These results represent the first comprehensive examination of the molecular pharmacology of BK channels.

BK channels have single-channel conductance values of ≫100 pS in symmetrical K^+ and are both Ca^{2+} and voltage dependent (1–5). They can be separated into at least two classes on the basis of channel kinetics and modulation by protein phosphorylation (6, 7) and may be capable of being further classified based on other criteria such as Ca^{2+} and toxin sensitivity (2, 3, 5, 6). Although found on many cell types, these channels play particularly important roles in the excitability and repolarization of both muscle cells and neurons and the regulation of neurotransmitter and hormone release (8–18). In general, their regulation by both voltage and Ca^{2+}, their large single-channel conductance, and their localization on neurons and other secretory and muscle cells suggest that they are a key element in the control of cellular excitability (3, 19). In this regard, they represent a major target for the development of pharmacological agents that can modulate muscle and neuronal excitability. In particular, agents that increase BK channel activity, during or after events that increase intracellular Ca^{2+}, would be expected to reduce cell excitability and could directly or indirectly reduce neurotransmitter and hormone release. Activation of BK channels could therefore have use in protecting cells such as neurons that are particularly sensitive to overstimulation and pathogenic Ca^{2+} entry resulting from conditions such as ischemia (20).

Until recently, activators of K^+ channels were limited to the class of K_{ATP} channels. However, blockers of several families of K^+ channels have been described and used as tools in studying K^+ channel structure and function. The recent discovery and characterization of the peptidyl scorpion toxin BK channel inhibitors ChTX and IbTX (21–25) have provided valuable tools in the study of BK channel function (e.g., see Refs. 26 and 27). Subsequently, a number of additional blockers and the first openers of these channels have been described (20, 28–30), but the diversity of BK channels, their complex regulation, and the absence of comprehensive pharmacological profiling in the same system leave many unanswered questions concerning the universal efficacy of various compounds on BK channels. An important example is the peptidyl toxin IbTX, which although quite specific for BK channels, apparently only blocks one of two major classes of BK channel in the central nervous system (6).

ABBREVIATIONS: BK, large-conductance calcium-activated potassium channel; ChTX, charybdotoxin; IbTX, iberiotoxin; KTX, kaliotoxin; K_{ATP}, ATP-dependent K^+; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney; 5-nitro-2-(3-phenylpropylamino)benzoic acid.

The recent cloning and expression of *Slowpoke*, a *Drosophila* BK channel (31, 32), and its mammalian (mouse, *mSlo*; Ref. 33) and, most recently, human (*hSlo*; Refs. 34 and 35) homologues offers an opportunity to more rigorously study the functional effects of putative modulators on relatively isolated and homogenous BK channel populations expressed in *Xenopus laevis* oocytes or in mammalian cells. In the current study, we evaluated the effects of a number of reported blockers and openers of BK channels on *mSlo*- and *hSlo*-mediated outward currents expressed in *X. laevis* oocytes. In addition, we tested representatives of each class of opener for their ability to increase the open probability of *hSlo* BK channels expressed in HEK 293 cells and recorded from excised inside-out membrane patches. The blockers tested include the peptides IbTX (21, 22), ChTX (24, 25), and KTX (36); the alkaloids paxilline (28) and tetrandrine (30); and the anesthetic (±)ketamine (37). The openers tested were the benzimidazolones NS004 and NS1619 (29, 38–41); phloretin (42); the Cl^- channel blockers niflumic acid, flufenamic acid, and NPPB (43–45); the K_{ATP} channel opener cromakalim (46); the tracheal relaxant SCA-40 (47, 48); and the dihydropyridine Ca^{2+} channel blocker niguldipine (49). The medicinal herb extract dehydrosoyasaponin I, reported to be a potent activator when applied to the inside of BK channels (50), was unavailable for testing.

This comprehensive study had three related goals: to further define the pharmacology of this very important class of ion channels, to determine the pharmacological similarity of the BK channel gene products of the highly homologous *mSlo* and *hSlo* genes, and to determine whether different classes of BK openers exerted their effects directly on isolated BK channels. The results allow for the comparison of the effects of most major classes of BK modulators in the same expression system.

Experimental Procedures

mSlo and *hSlo* mRNA Synthesis

The BK clone cDNAs (*mSlo* and *hSlo*; in pBluescript KS^+) were linearized with the restriction enzymes SalI (*mSlo*) or NotI (*hSlo*), sites that are downstream of the cDNA inserts, and *in vitro* transcribed with T3 RNA polymerase (Stratagene, La Jolla, CA) in the presence of the cap analogue $m^7G[5']ppp[5']G$ (Pharmacia, Piscataway, NJ). Template DNA was digested with RNase-free DNase I (Epicenter Technologies, Madison, WI); the cRNA was extracted with phenol/chloroform, precipitated with ammonium acetate and ethanol, rinsed with 70% ethanol, vacuum-dried and solubilized in RNase-free water, and stored at −70° at a concentration of 1.0 μg/μl. The *mSlo* and *hSlo* constructs used in the experiments did not contain any of the previously identified alternative splice exons (33–35).

Oocyte Preparations

Frog oocytes were surgically harvested from mature *X. laevis* (Nasco and *Xenopus* 1) that had been anesthetized with 0.15% 3-aminobenzoic acid ethyl ester (Tricaine; Sigma Chemical Co., St. Louis, MO). The overlying follicle cell layers were removed manually. Only late-stage V and VI oocytes were selected for cRNA injection. Each oocyte was injected with ~50 nl of the appropriate cRNA. After injection, oocytes were maintained at 17° in ND96 medium consisting of 90 mM NaCl, 1.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 5.0 mM HEPES, pH 7.5. Horse serum (5%) and penicillin/streptomycin (5%) were added to the incubation medium.

Electrophysiology

Two-electrode voltage-clamp recording from oocytes. Standard two-electrode voltage-clamp techniques were used throughout the experiments (51, 52). In general, recording began 2–6 days after mRNA injection. Before the start of an experiment, oocytes were placed in a recording chamber and incubated in modified Barth's solution consisting of 88 mM NaCl, 2.4 mM $NaHCO_3$, 1.0 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, and 0.41 mM$CaCl_2$, pH 7.5. Oocytes were impaled with electrodes (1–2 MΩ), and whole-cell membrane currents were recorded under two-electrode voltage-clamp conditions [GeneClamp 500 (Axon Instruments, Foster City, CA) or Turbo TEC01C (Adams and List,). Voltage-clamp protocols typically consisted of a series of 500–750-msec voltage steps in +20-mV steps from a holding potential of −60 mV to a maximal potential of +140 mV (Fig. 1). In most cases, a P/4 leak subtraction protocol was used. A family of outward currents was generated under control conditions for comparison with currents elicited in the presence of an experimental compound. The currents evoked in control medium were not typically observed at depolarized potentials at <+40 mV. Further descriptions of individual experimental protocols and data concerning the possible contributions of Ca^{2+}-activated Cl^- current to drug effects are presented in Results. Control and drug solutions were introduced into the recording chamber continually through the use of a gravity-flow system; solutions were switched with the use of a rotary valve, which completely exchanged solutions in <10 sec. In initial experiments, the time required for maximal drug effect was determined for each compound; all drugs were applied for 5–10 min, which was adequate for maximal activation or inhibition of BK current in every case. A minimum of five oocytes was used to generate each data point for each compound. All experiments characterizing the effects of putative BK channel openers and blockers, except where noted, involved application of the opener until steady state current values were obtained, followed by application of 50 nM IbTX (alone) to estimate the percentage of total current that was attributable to BK channel expression in the oocyte under voltage-clamp conditions. In this manner, BK modulator effects could be expressed as the percentage change in IbTX-sensitive current, controlling for the variable levels of channel expression from oocyte to oocyte. Data acquisition and analyses were performed using pClamp or AxoData-AxoGraph software (Axon Instruments). Graphs illustrating concentration-response relationships, best-fit estimations using one-site or two-site logistic models, and estimations of EC_{50}, values (where possible) were accomplished with Kaleidograph graphics software (Abelbeck/Synergy, Reading, PA) and Prism software (Graphpad Software, San Diego, CA).

Patch-clamp recordings. HEK 293 cells were plated onto poly-D-lysine-coated coverslips at 10–20% confluency and transiently transfected 3 days later with *hSlo* (in pcDNA3, Invitrogen; 1 μg DNA/35-mm dish) using the lipofectamine method according to the manufacturer's instructions (GIBCO-BRL, Gaithersburg, MD). Stably transfected HEK 293 cells were also prepared and used for some of the experiments. To prepare clonal cell lines stably expressing *hSlo*, cells were split 2 days after transfection and selected for resistance to G-418 (GIBCO-BRL). After 10–12 days, surviving cells were clonally isolated, grown to confluence, plated onto coverslips, and tested for *hSlo* expression through three methods: reverse transcription-polymerase chain reaction to test for the level of *hSlo* mRNA, Western blot analysis to test for protein levels with an *hSlo* antibody, and electrophysiologically with whole-cell patch-clamp to determine *hSlo*-mediated outward current levels. In the stably transfected clone used for these experiments, as well as the transiently transfected HEK 293 cells selected for patch recordings, very high levels of expression were encountered. In the case of transiently transfected HEK 293 cells, excised patch voltage-clamp recordings began 24–72 hr after transfection. All patch-clamp recordings (inside-out configuration only) were made according to standard techniques (53, 54). An Axopatch 200A amplifier and pClamp 6.0 soft-

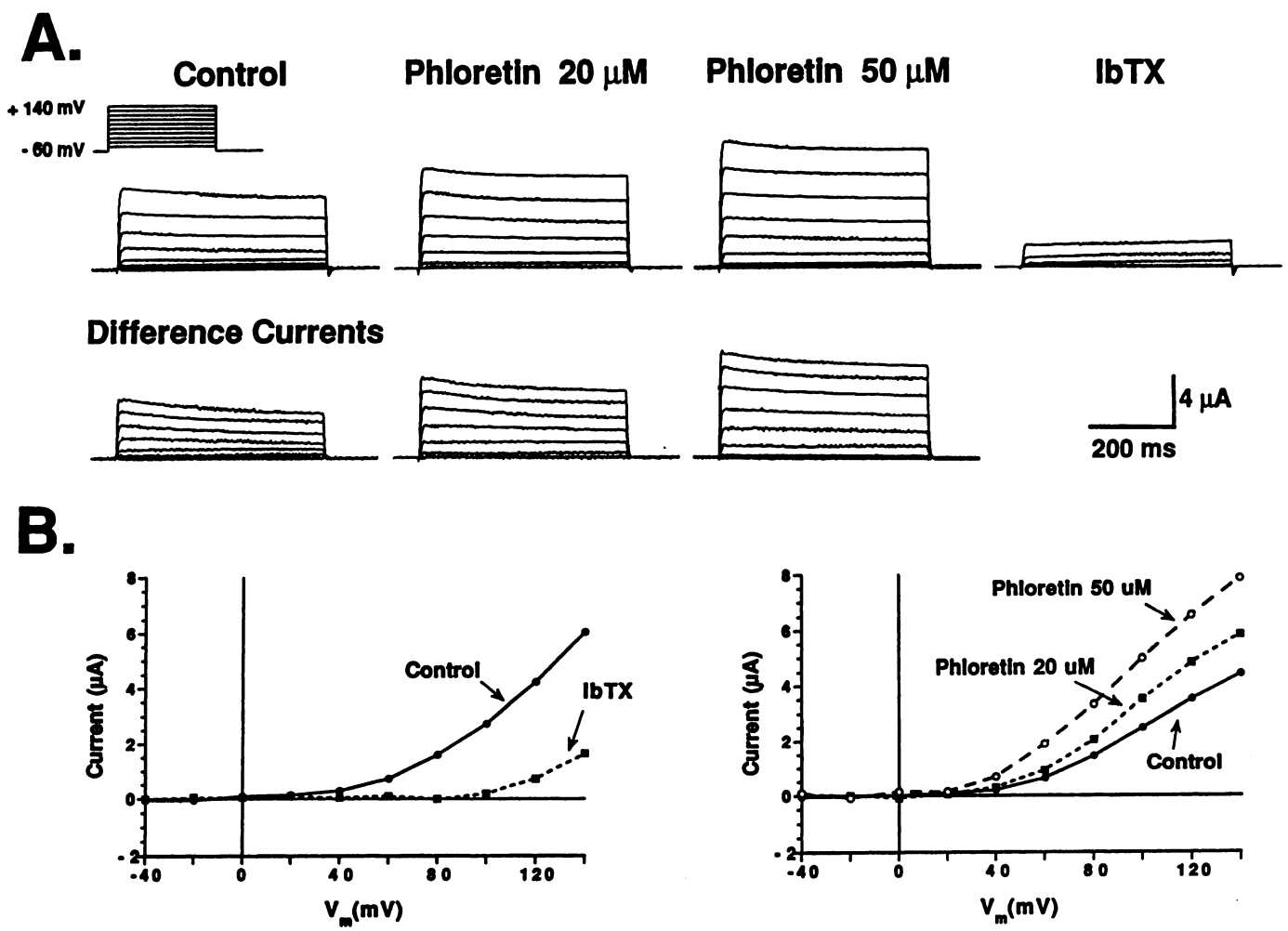

Fig. 1. Effects of an opener and a blocker of BK channels on *hSlo*-mediated outward current expressed in a *Xenopus* oocyte. A, *Top traces*, membrane currents generated in response to depolarizing voltage steps applied to an *hSlo*-injected oocyte incubated in control medium or the BK channel opener phloretin (20 and 50 μM, 5 min) recorded under two-electrode voltage-clamp. Subsequent incubation of the same oocyte in the peptide BK channel blocker IbTX (50 nM, 10 min) resulted in suppression of a significant proportion of the outward current, consisting of the rapidly activating *hSlo* current component (*top traces, far right*). The remainder of the current is more slowly activating and has an inward tail current on return to the −60 mV holding potential that is consistent with the native Ca^{2+}-activated Cl^- conductance. *Bottom traces*, difference currents from which the IbTX-insensitive current has been digitally subtracted to isolate BK current. B, *Bottom*, I-V relationships for the control currents and currents remaining in IbTX (*left*) and for the difference currents in control medium and phloretin.

ware (Axon Instruments) were used for all recordings, and no differences were observed in the response of channels from transiently transfected or stably transfected HEK 293 cells. The pipette filling solution and the bath solution used for inside-out excised patch recordings contained 140 mM KCl, 20 mM 3-(*N*-morpholino)-propanesulfonic acid, and 0.8 mM K_2EGTA, pH 7.2. $CaCl_2$ was added (0.8 mM) to adjust free [Ca^{2+}] to a calculated value of 10 μM. Pipettes (2.5–5.0 M½ in bath solution) were pulled from borosilicate glass, coated with Sylgard, and fire-polished. Actual free [Ca^{2+}] was determined with a Fura-2 fluorometric assay in accord with the manufacturers' instructions (Molecular Probes, Inc., Eugene, OR). In these experiments, the free intracellular Ca^{2+} concentration was empirically determined to be 4.52 μM by this method.

Excised inside-out membrane patches were voltage-clamped at V_m = −80 mV (where $V_m = -V_{pipette}$) and stepped to potentials from −100 mV to +100 mV in 10-mV, 300-msec steps. The protocol was repeated 10 times in each case, and the currents elicited were averaged before further analysis. Background levels of native current were very low in HEK 293 cells, generally <5% of the *hSlo* value. Due to the high levels of *hSlo* expression, patches generally contained many channels, and drug effects on single channels, although obvious, were difficult to quantify. However, a small number of patches contained only a small number of channels, which allowed us to confirm that the expressed channels had the single-channel conductance (>280 pS) and Ca^{2+} sensitivity of *hSlo* BK channels (34, 35). To compare results obtained from different patches, current/voltage relationships were constructed with the last 50 msec of each current trace at each step voltage. These values were averaged, and the averages were plotted against the corresponding voltage steps. Conductance (G) values were calculated as $G = I/V_m$. Each experiment was normalized by dividing G values by G_{max}, where G_{max} was defined as the largest G value obtained in each experiment. The values for the group data were calculated by averaging the G/G_{max} values for each potential within each experimental group. Data are presented as mean ± standard error and represent a minimum of five patches for each drug. Drugs (NS1619, niflumic acid, and phloretin; all at 25 μM) were applied to patches after a control sequence of voltage steps. Recording began after ~1–2 min, and each patch was used for a single drug. G/G_{max} voltage curves for control and drug experiments were fitted with a Boltzmann relationship of the form $G/G_{max} = (1 + \exp[(V_{½} - V_m)/k])^{-1}$ with the use of Kaleidograph software.

Compounds

Small-molecule BK modulators, alkaloids, and peptide toxins used in these experiments were either obtained commercially or, when necessary, synthesized by our group using modifications of standard procedures. ChTX was obtained from Peptides International (Louisville, KY), KTX was obtained from Peptides International or Research Biochemicals (Natick, MA), paxilline was obtained from Sigma/Aldrich/Fluka Bulk Chemicals, tetrandrine was obtained from Aldrich (Milwaukee, WI), and ketamine was obtained from Sigma. Due to the large amount of IbTX used in this study, we used synthetic IbTX produced in our laboratories. Synthetic IbTX was prepared using standard solid phase 9-fluorenylmethoxycarbonyl chemistry protocols similar to those described previously for the synthesis of ChTX (25); the principal difference in our protocol was that each amino acid was coupled through the use of hydroxybenzotriazole/diisopropylcarbodiimide activation on an Advanced Chemtec 200 (Louisville, KY) batch peptide synthesizer. The purified peptide was tested for potency and was found to be identical to that obtained commercially.[1] The benzimidazolones NS004 and NS1619 and SCA-40 were prepared in-house. Phloretin, cromakalim, niflumic acid, and flufenamic acid were obtained from Sigma. NPPB and niguldipine were obtained from Research Biochemicals.

All peptides were prepared as 100 μM aqueous stock solutions, and individual aliquots were brought to the final desired concentrations in modified Barth's solution just before use. Other compounds were initially prepared as 20 mM stock solutions in DMSO, and final solutions were prepared immediately before use. An exception was tetrandrine, which was prepared as a 5 mM stock solution in DMSO. Paxilline and niguldipine are light sensitive, and care was taken to protect them from light.

Results

Oocyte Experiments with Blockers

IbTX and ChTX. Outward currents expressed in oocytes previously injected with the *hSlo* construct were qualitatively and quantitatively different from currents in control cells. In particular, control oocytes had currents that typically were slowly activating, and at −60 mV holding potentials had tail currents that were inward. In *mSlo* and *hSlo* cRNA-injected oocytes, current amplitudes were generally two to four times that of control oocytes and had very rapid activation and some degree of inactivation or current reduction over the course of voltage clamp episodes of 500–750-msec duration. Unfortunately, the native current, primarily Ca^{2+}-activated Cl^- current, had much the same voltage activation range as *hSlo*-mediated currents, although it was more slowly activating than BK currents [Figs. 1 and 2 (see also Fig. 5)]. To facilitate this differentiation, all of the currents used for drug evaluation were elicited at step voltages of +120 to +140 mV and were measured at the earliest possible time point during the step depolarization. This corresponded to the initial peak of the control current, usually at ~30 msec into the voltage step, and maximized the difference in the activation kinetics of the two currents. This was necessary because the use of Cl^- channel blockers to attenuate the native oocyte current was precluded, due to the influence of many of these blockers on BK currents (43, 44). To isolate and quantify BK current and its response to drug solutions, we used the peptide BK channel-blocking toxin IbTX. This was previously shown to be effective at blocking most *hSlo*-mediated current at concentrations >25 nM (34); therefore, at 50 nM (used throughout these experiments), we could be certain of nearly complete BK blockade (see Fig. 2A), allowing for an estimate of the percentage of the total current mediated by *hSlo* expression. This was used to normalize expression levels in most of the following experiments (e.g., Figs. 1 and 5).

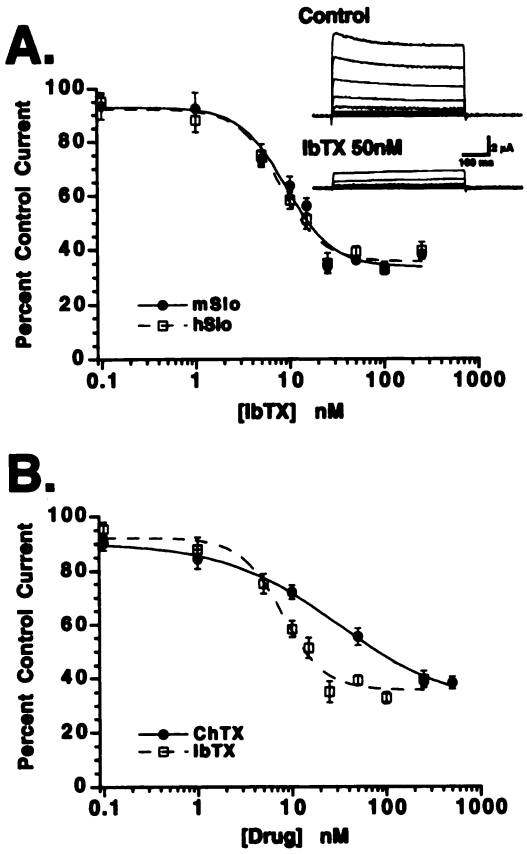

Fig. 2. A, Depression of *hSlo* and *mSlo* BK currents by the specific peptide BK blocker IbTX. In this and all subsequent graphs, each data point was obtained by measurements on a minimum of 5 and a maximum of 10 oocytes. All error bars represent ± standard error. Exposure to IbTX over a wide concentration range (0.1–250 nM) produced very similar current responses and EC_{50} values for both *hSlo* and *mSlo* (values in text). These data were best fit assuming a single site of IbTX interaction. The traces represent typical current families in control medium and in IbTX. B, Amplitudes of current responses from *hSlo*-injected oocytes were reduced in the presence of another potent peptidyl inhibitor of BK channels, ChTX. Compared with IbTX, ChTX (0.1–500 nM) was not as potent or sensitive in blocking *hSlo* currents, as revealed by a shallower concentration response and a significantly greater EC_{50} (values in text). It was not clear whether the shallower curve of ChTX inhibition represents two sites of interaction because the curve was not clearly biphasic.

To demonstrate the utility of IbTX, we first characterized the concentration-response relationship for the blockade of outward current; as with other compounds used in this study, we were also interested in determining the degree of pharmacological identity between *hSlo* and its close homologue *mSlo* (identity ~ 98%). IbTX was applied to oocytes expressing either *mSlo* or *hSlo* at concentrations of 0.1–250 nM for 10 min (maximal effects were always observed by 10 min). IbTX produced a consistent concentration-dependent blockade of the *hSlo*- and *mSlo*-mediated component of outward current, at higher concentra-

[1] V. K. Gribkoff, J. T. Lum-Ragan, C. G. Boissard, D. J. Post-Munson, N. A. Meanwell, J. T. Starrett, Jr., E. S. Kozlowski, J. L. Romine, J. T. Trojnacki, M. C. McKay, J. Zhong, and S. I. Dworetzky, unpublished observations.

tions leaving a more slowly activating residual current that was essentially identical to native current (Fig. 2A). The IbTX concentration-response relationship for both *hSlo* and *mSlo* was best fit assuming a single site of interaction; the estimated EC_{50} for *hSlo* was 7.9 nM, and the EC_{50} for *mSlo* was 9.1 nM. IbTX was equally effective on both BK channel homologues and was maximally effective at 25–50 nM. The reduction of *hSlo* current by IbTX was reversible; although washout began quickly, complete recovery required wash times >2 times the duration of exposure to the peptide. IbTX produced no significant effect on native current when applied at high concentrations to uninjected control oocytes (250 nM for 10 min; currents 104.1 ± 7.5% of control, five oocytes). As mentioned above, for the subsequent examination of BK openers, IbTX was used at a supramaximal concentration of 50 nM.

The close structural homologue of IbTX, ChTX (0.1–500 nM, 10 min), was as effective as IbTX at high concentrations in blocking BK current (Fig. 2B). In these experiments, the effects on *mSlo*-mediated current were not examined. Although equally effective, the potency of ChTX was significantly lower than the potency of IbTX, and the resulting curve was shallower (EC_{50} values: ChTX, 30.8 nM; IbTX, 7.9 nM). Similar to IbTX, the effects of ChTX were reversible on return to control medium. ChTX had no effect on native current in uninjected control oocytes when applied at 500 nM for 10 min (current amplitudes, 98.4 ± 6.3% of control, five oocytes).

Paxilline. The alkaloid paxilline was applied at 11 different concentrations between 1.0 nM and 7.5 μM to oocytes expressing *hSlo*. As with the peptide blockers IbTX and ChTX, maximal effects of the compound were observed by 10-min incubation. Unlike the peptide blockers, the concentration-dependent inhibition of *hSlo* current by paxilline was characterized by at least two components (Fig. 3); the resultant concentration-response relationship was best fit assuming two sites of interaction: a high affinity site (9.1 nM) and a low affinity site (0.53 μM). The high affinity interaction accounted for approximately one half of the total inhibition of current produced by paxilline. The effects were reversible with washing, but currents returned to baseline values more slowly than was observed with the peptide blockers, particularly after high concentrations of paxilline. Paxilline had only a small effect on native current in uninjected oocytes; at 7.5 μM (10 min), a small increase in native current was observed (to 110.4 ± 6.9% of control, five oocytes).

Kaliotoxin, tetrandrine, and ketamine. The effect of a third putative peptide blocker of BK channels, KTX, was examined at 0.1–1.0 μM (Fig. 4). Only a small reduction in *hSlo* current was observed relative to the effects of IbTX; however, this reduction was concentration dependent and reversible. Higher concentrations were not tested due to limited availability of the peptide. This peptide was much less potent than either IbTX or ChTX, although its efficacy could not be estimated from these limited data.

Two additional compounds were examined for their ability to inhibit *hSlo* current: the alkaloid tetrandrine and the anesthetic (±)ketamine. Even at very high concentrations (>100 μM), neither compound produced a significant degree of reduction of *hSlo* current (Fig. 4). The small decrease observed in response to tetrandrine may represent a vehicle effect rather than an effect of the compound. Due to the limited solubility of tetrandrine in DMSO, the final concentration of the vehicle at 100 μM tetrandrine was 2%, which depressed BK currents to almost the same degree as the solutions containing both tetrandrine and DMSO. This was the only instance in this study when vehicle effects were demonstrated; in all other cases, vehicle concentrations were kept well below levels that directly influenced outward currents.

Oocyte Experiments with Openers

Two of the putative openers examined in this study, the benzimidazolones NS004 and NS1619, were found to in-

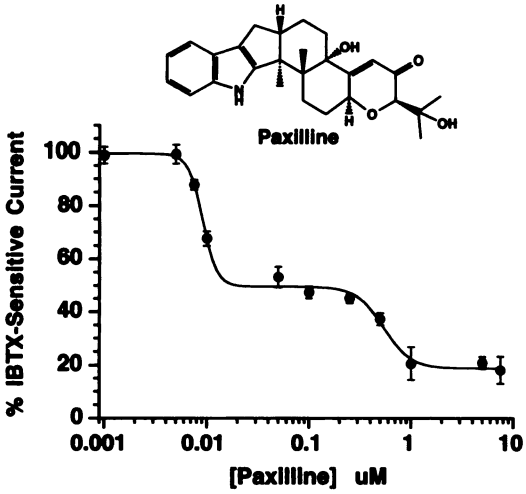

Fig. 3. Potent inhibition of *hSlo* currents by the nonpeptidyl BK blocker paxilline. Oocytes were exposed to this diterpene alkaloid over a wide concentration range (1 nM to 7.5 μM), and paxilline seemed to interact at two sites, with high and low affinities. Note that some IbTX-sensitive current was present even at high paxilline concentrations.

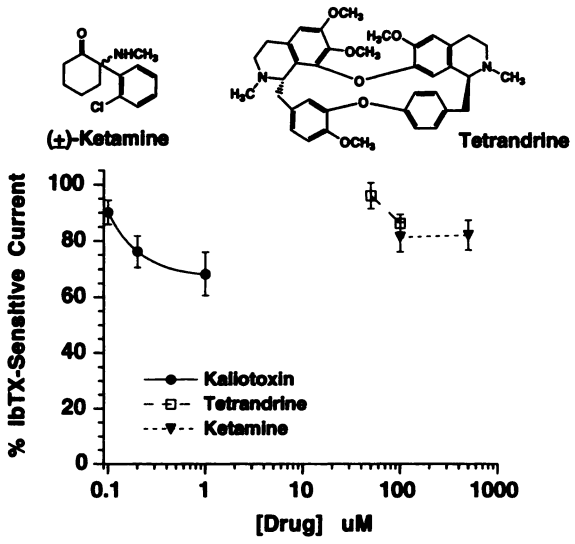

Fig. 4. The effects of several putative BK channel blockers on *hSlo*-injected oocytes. The peptide KTX produced a relatively small but concentration-dependent depression of current. The medicinal alkaloid tetrandrine had no significant effect on *hSlo* currents at these concentrations, which were very high relative to effective concentrations of IbTX or ChTX. Likewise, the anesthetic agent (±)ketamine, also reported to be a BK channel blocker, produced only a marginal depression at a high concentration.

crease native current when applied at high concentrations to uninjected or water-injected control oocytes. However, the activation kinetics of the native Cl^- current was very slow, and the effects of these compounds were principally observed late in long depolarizing voltage steps. Thus, effects on these currents did not contribute significantly to evaluations of opener efficacy (performed very early in the voltage step; see Fig. 5C). For example, application of 100 μM NS1619 for 5 min, which caused large increases in native current late with higher voltage steps (>100 mV), produced only a small increase in the native current, to 117.1 ± 9.0% of control (seven oocytes), when the effect was measured early in the voltage step (at times comparable to those used for BK current measurement). In oocytes expressing BK channels, the effect of NS1619 on BK current was more than 1 order of magnitude greater, and the contribution of increases in native current was negligible. Likewise, the effects of NS004 and phloretin on peak BK currents could not be attributed to their effects on native background current. NS004 at 100 μM, the highest concentration used in these experiments, produced a small decrease in the native current (to 83.3 ± 5.3% of control, five oocytes), and phloretin also produced a small decrease in native current (to 84.7 ± 2.2% of control at 225 μM, five oocytes). Similarly, compounds that were putative blockers of the native current, but openers of BK channels (e.g., niflumic acid) had only small effects on the quantification of the results (reducing the apparent effect of the compounds), due to the methods of current measurement and to our observation that at the concentrations used in these experiments, the Cl^- channel blockers were modestly effective at reducing the small contribution of native current (values given below).

Benzimidazolones: NS004 and NS1619. The benzimidazolones NS004 and NS1619 were both highly effective at increasing BK current in oocytes (Fig. 5, A–C). Both of these compounds, which differ structurally only by the substitution of a trifluoromethyl substituent at the para position on the phenol ring for NS1619, produced a maximum increase of BK current in excess of 200% of control values. Calculation of absolute maximum effects and accurate estimates of EC_{50} values were prevented by the limited solubility of the compounds at >100 μM. NS1619 was soluble to 200 μM, but it was used at this concentration only in some of the competition studies (see below) because its effects were difficult to reverse. No significant difference in the effect of NS004 was observed on currents produced by expression of *mSlo* or *hSlo*.

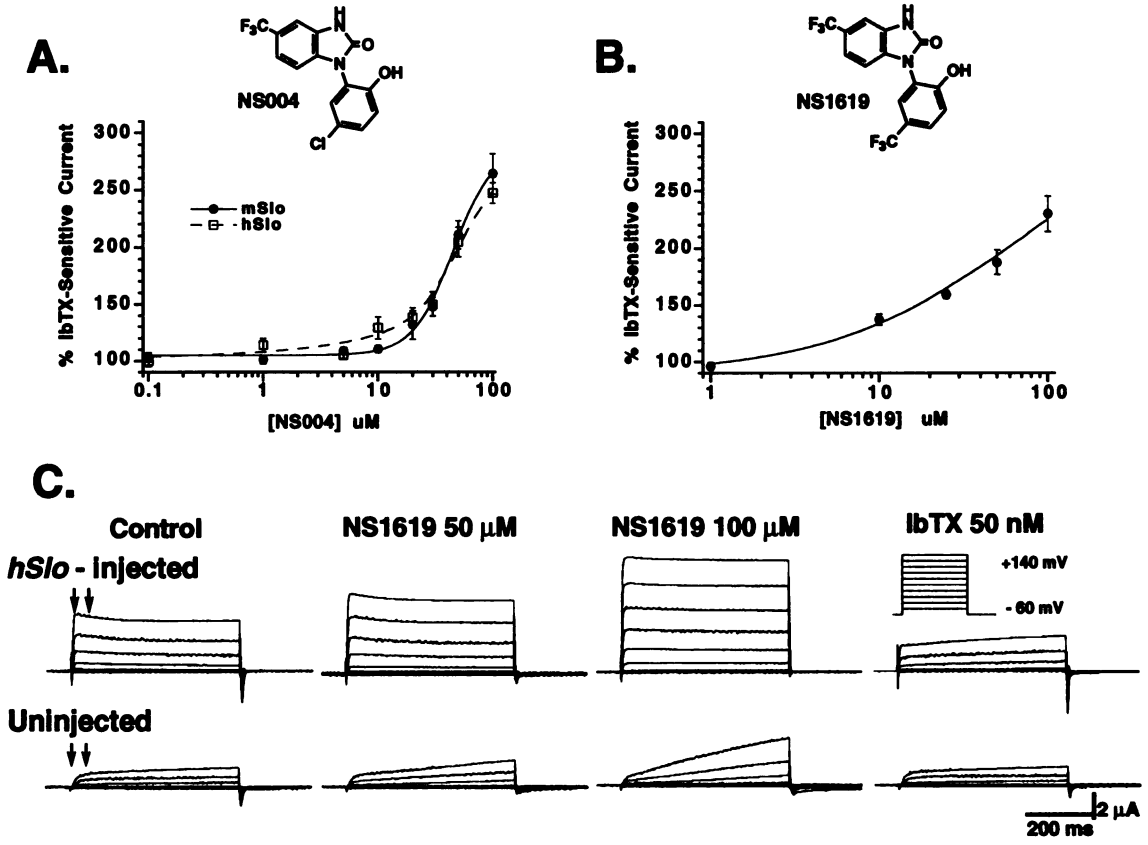

Fig. 5. A, Application of the benzimidazolone BK channel opener NS004 to oocytes expressing *mSlo* or *hSlo* BK channels produced similar concentration-dependent increases in BK outward current. Although not very potent, NS004 and the other effective openers characterized in this study have great potential utility as pharmacological tools because extracellular application under whole-cell recording conditions resulted in significant increases in BK current. B, The closely related compound NS1619 also effectively increased BK (*hSlo*) current. The shallow concentration-response relationship may suggest a multiphasic interaction of this compound not observed with NS004 (see text and Fig. 10A). C, Current families generated by identical voltage-clamp protocols in *hSlo*-injected (*top*) and control (*bottom*) oocytes. The application of NS1619 to the *hSlo*-injected oocyte produced a concentration-dependent increase in the outward current measured at an early point in the voltage pulse. *Arrows*, the region of the current responses used for measuring the effects of compounds on *hSlo*. Note that although NS1619 produced a significant increase in a slowly activating component of native current visible late in these long current pulses, there is no significant effect on native current at the early points typically used for assessing drug effects on *hSlo* (see text).

Both compounds achieved maximal effect within 5 min of application and were generally easily reversible to 100 μM.

Phloretin. Application of phloretin to oocytes expressing either *mSlo* or *hSlo* resulted in significant increases in outward current, but the profile of the concentration-response relationship was different from that obtained with the benzimidazolones (Fig. 6). In particular, the data for both constructs were best fit assuming two sites of interaction, as was the case with the inhibitor paxilline. The estimated EC_{50} values for the higher affinity site were 31.4 and 34.6 μM for *mSlo* and *hSlo*, respectively. The values for the lower affinity site could not be estimated due to the limited solubility at >200 μM. The effects of phloretin were maximal at 5 min and were reversible.

The Cl^- channel blockers niflumic acid, flufenamic acid, and NPPB. Niflumic acid and its closely related analogue flufenamic acid were both effective at increasing BK-mediated currents (Fig. 7, A and B), and niflumic acid (1–500 μM) had very similar effects on both *mSlo*- and *hSlo*-mediated currents. The concentration-response relationships of these openers could also be best described by assuming two sites of interaction, although the degree of interaction with the higher affinity site was less with these compounds than with phloretin. NPPB increased BK-mediated outward current (Fig. 7B), but no effect was observed at 20 μM. Therefore, NPPB was the least potent of all of the effective BK openers that we tested. The maximum effect of NPPB could not be estimated because it was insoluble at >100 μM. As expected, application of these Cl^- channel blockers produced a reduction in the native current in uninjected oocytes at the highest concentrations used in these experiments (niflumic acid at 500 μM reduced currents to 30.7 ± 2.5% of control, five oocytes; flufenamic acid at 500 μM reduced currents to 27.8 ± 2.8% of control, five oocytes; NPPB at 100 μM reduced currents to 40.4 ± 3.0% of control, five oocytes).

Effects of other putative BK openers: cromakalim, SCA-40, and niguldipine. Three additional compounds, the K_{ATP} opener cromakalim, the tracheal relaxant SCA-40, and the dihydropyridine niguldipine, previously reported to open natively expressed BK channels, were tested on oocytes expressing BK channels (Fig. 8). Even at high concentrations, cromakalim and SCA-40 did not produce any significant effect on BK-mediated outward current. Due to limited solubility, niguldipine could not be evaluated at >5 μM; there was no effect of the compound at the tested concentrations.

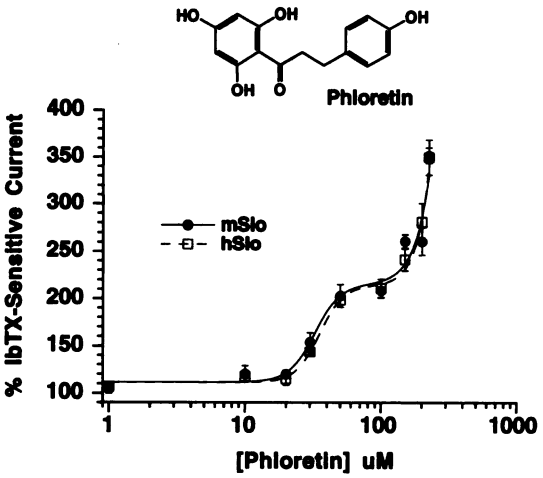

Fig. 6. The putative BK channel opener phloretin produced similar biphasic concentration-response relationships when applied to oocytes expressing either *mSlo* or *hSlo* BK channels.

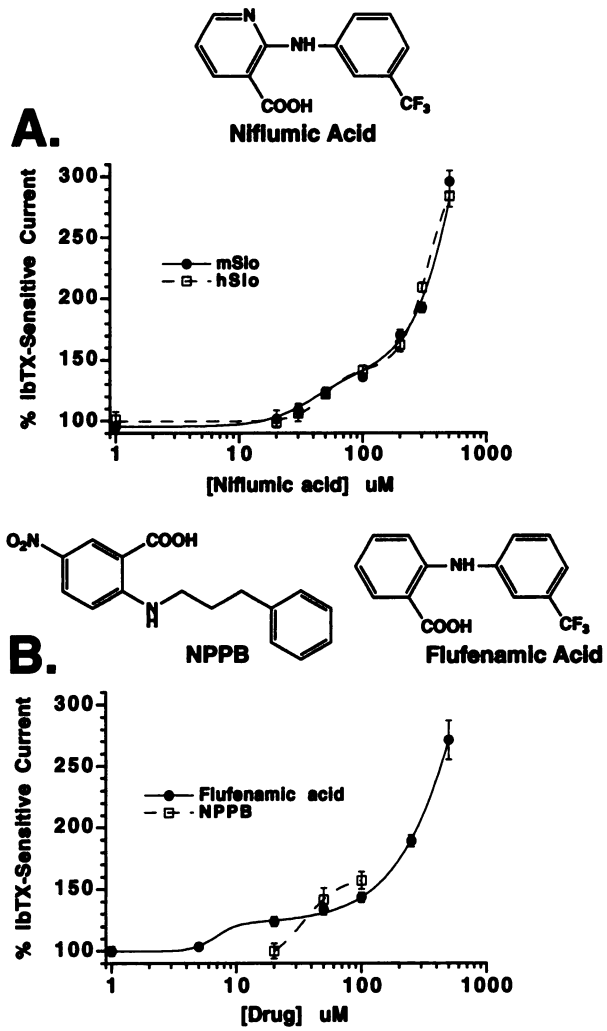

Fig. 7. Increases in BK-mediated outward current produced by the Cl^- channel-blocking compounds niflumic acid (A, *mSlo* and hSlo) and flufenamic acid and NPPB (B, *hSlo* only). Note biphasic relationships for both niflumic acid and flufenamic acid. NPPB could not be evaluated at concentrations of >100 μM because of marginal solubility in the recording medium. NPPB has not previously been reported as an opener of BK channels.

Effects of Coapplication of BK Openers and Blockers: Iberiotoxin and Paxilline Antagonism of the Effects of the BK Openers NS1619 and Phloretin

Limited competition studies were performed with representatives of two classes of BK opener, NS1619 and phloretin, and two blockers, the peptide IbTX and the alkaloid paxilline. Coapplication of NS1619 (1–100 μM) and a supramaximal concentration of IbTX (50 nM) resulted in some reduction of the effects of NS1619 but not the expected complete blockade (Fig. 9A). Likewise, phloretin (1–225 μM) was coapplied with IbTX (50 nM), and there was a reduction but not a complete block of the actions of the opener. Specifically, in the presence of IbTX, phloretin seemed to interact with the BK channels in a monophasic manner, with an apparent elimination of the higher affinity interaction (Fig. 9B). Overall, the potencies of NS1619 and phloretin were reduced in

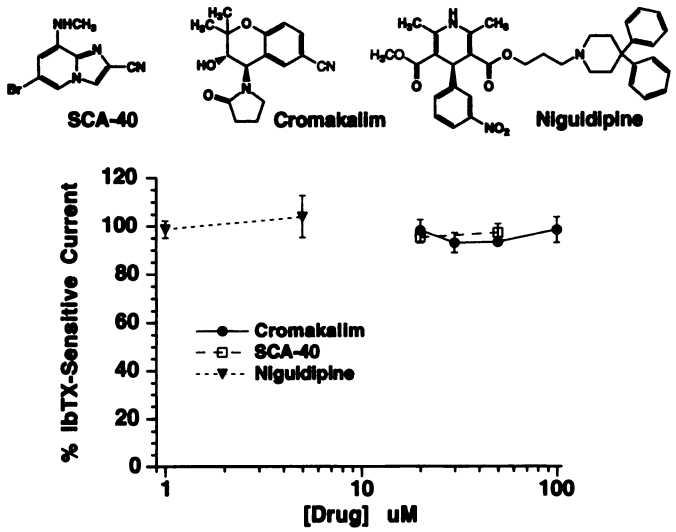

Fig. 8. The putative BK channel openers cromakalim SCA-40 and niguldipine had no significant effect on BK current. All of these compounds have been reported to open native BK channels, but SCA-40 and cromakalim did not produce any detectable increase in current, even at very high concentrations. Niguldipine could not be evaluated at >5 μM because of limited solubility.

IbTX, but it was not possible to estimate the effects on efficacy due to limited solubilities and because of the unique requirements of this group of experiments. In particular, the control values for the curves generated in the presence of the openers alone in Fig. 9, A and B, are included for visual qualitative comparison, but caution must be exercised in using these for direct quantitative comparisons with the curves generated in the presence of IbTX. The control current values in the presence of supramaximal IbTX, against which the effects of openers were compared, normally defined for activators as the percent increase in IbTX-sensitive current, were defined for these experiments as the percentage increase in remaining outward current. This was necessary because at the outset of the experiments, the IbTX-sensitive current had been antagonized. This was not an issue in the subsequent series of experiments with paxilline, as the IbTX-sensitive component could still be used for control purposes.

Coapplication of NS1619 (1–200 μM) or phloretin (1–225 μM) and paxilline (100 nM and 1.0 μM) produced complex profiles of interaction (Fig. 10, A and B). In both groups of experiments, paxilline concentrations were chosen based on the data presented in Fig. 3. In these earlier experiments, 100 nM paxilline was found to produce maximal antagonism of a higher affinity site, although 1.0 μM produced maximal antagonism of both higher and lower affinity sites. In the present experiments, paxilline at 100 nM competitively antagonized the effects of NS1619, and the concentration-response relationship for NS1619 was now clearly biphasic (Fig. 10A). In 1.0 μM paxilline, the effects of NS1619 were completely blocked (Fig. 10A). The coapplication of paxilline and phloretin, both of which had biphasic interactions with BK channels when applied alone (Figs. 3 and 6), produced a concentration-dependent competitive antagonism of the higher affinity component of the effects of phloretin. The lower affinity component may have been completely blocked at both concentrations of paxilline, but this could not be tested further due to the limited solubility of phloretin.

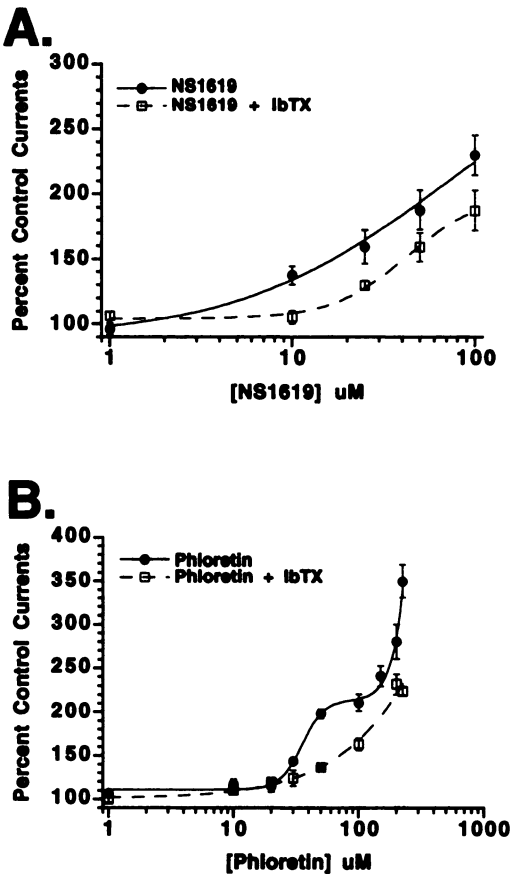

Fig. 9. Coapplication of a supramaximal concentration of IbTX (50 nM) and NS1619 (1–100 μM, A) or phloretin (1–225 μM, B) resulted in a reduction but not total suppression of the increases in BK current produced by these openers. The effect of IbTX on the biphasic relationship generated by phloretin suggests that the greatest effect of the peptide is to block the higher affinity interaction. See text for details of normalization procedures.

HEK 293 Cell Excised Patch Experiments

Application of 25 μM NS1619 (six patches), 25 μM niflumic acid (five patches), or 25 μM phloretin (five patches) to excised inside-out membrane patches produced increases in channel activity, as indicated by increases in the amplitudes of averaged currents, throughout the tested voltage range. When normalized relative to control values (11 patches), G/G_{max} relationships were shifted to the left by each of these compounds (Fig. 11). The $V_{½}$ values were 25.18 ± 0.76 mV for control patches, −9.22 ± 1.03 mV for NS1619, 8.39 ± 1.11 mV for niflumic acid, and 7.66 ± 0.87 mV for phloretin. The differences in $V_{½}$ values between the groups were highly significant (analysis of variance; $F = 269.7$, $p < 0.0001$), and each of the drug groups was significantly shifted left relative to the control value (Tukey-Kramer multiple comparisons test, control versus each of the drug groups, $p < 0.001$). There was no difference between the shifts produced by niflumic acid and phloretin ($p > 0.05$), but NS1619 produced a significantly greater leftward shift in the G/G_{max} relationship than either niflumic acid or phloretin at this single tested concentration (Tukey-Kramer, $p < 0.001$). The absolute shift in $V_{½}$ for NS1619 at 25 μM relative to control was 34.40 mV (95% confidence limit = 30.99–37.81 mV); for niflumic acid, the shift was 16.79 mV (confidence limit = 13.17–20.41 mV); and

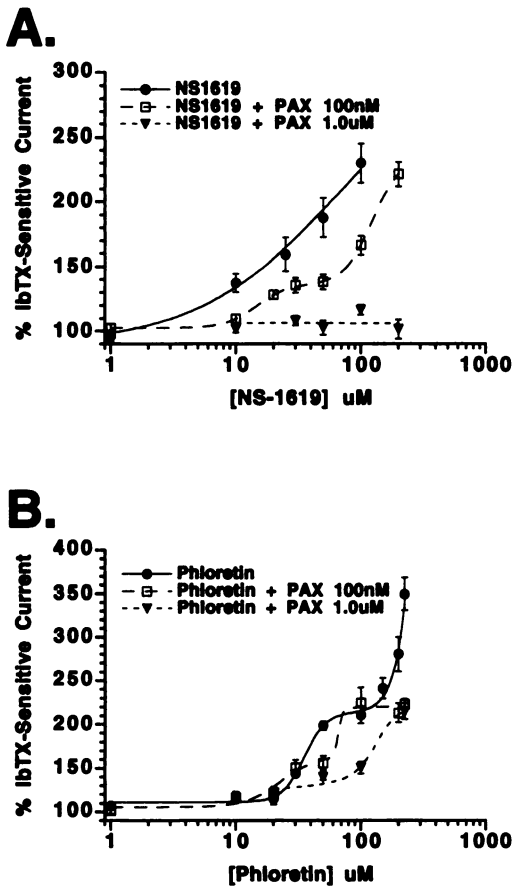

Fig. 10. The nonpeptidyl BK inhibitor paxilline, when coapplied with NS1619 (1–200 μM, A) or phloretin (1–225 μM, B) at concentrations that effectively blocked a higher affinity site (100 nM) or all of the paxilline-sensitive current (1.0 μM), produced a surprising degree of functional antagonism of the effects of these openers. A, At a lower concentration, paxilline (100 nM) competitively antagonized the effects of NS1619; note that under these conditions, NS1619 produced a clearly biphasic concentration-response relationship not observed in the absence of paxilline. At 1.0 μM, paxilline effectively blocked all effects of NS1619, making it a more useful blocker than the peptide IbTX for the functional antagonism of the effects of this class of channel activators. B, Paxilline competitively antagonized the higher affinity component of the interaction of phloretin with *hSlo* BK channels; effects on the lower affinity component could not be determined due to the upper limits of solubility for this compound at 225 μM.

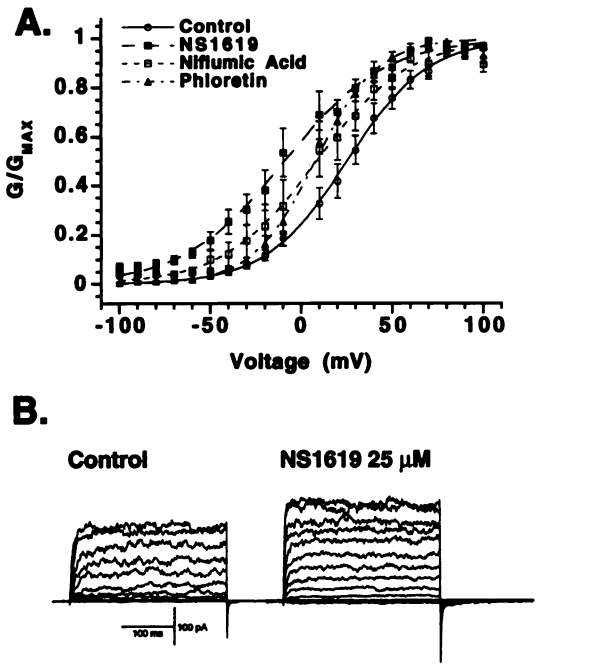

Fig. 11. A, Conductance (G/G_{MAX})-versus-voltage plots for the effects of 25 μM NS1619, 25 μM niflumic acid, and 25 μM phloretin, relative to control conductance values, on *hSlo* BK channels expressed in HEK 293 cells. All recordings were from inside-out membrane patches recorded in symmetrical 140 mM K^+, and free intracellular Ca^{2+} concentration = 4.52 μM (see text for details). All of the openers produced a significant shift to the left of the conductance-voltage relationship, with NS1619 being the most effective opener of these channels at this concentration. B, Examples of averaged currents (averages of 10 sweeps) from an inside-out membrane patch from a HEK 293 cell expressing *hSlo*; application of the benzimidazolone NS1619 produced a rapid and significant increase in BK current.

for phloretin, the shift was 17.52 mV (confidence limit = 13.90–21.14 mV).

Discussion

Our results demonstrate that expression of the mouse and human *Slowpoke* homologues *mSlo* and *hSlo* in *Xenopus* oocytes can be used for the quantitative functional assessment of the interaction of putative modulators with cloned BK channels. This is the first broad-spectrum study of the pharmacology of both openers and blockers of these important channels carried out in the same expression and assay system. The oocyte expression system coupled with two-electrode voltage-clamping is easy to perform and provided the required low level of response variability necessary to compare the effects of many compounds at multiple concentrations. In general, the effects of drugs on the currents expressed in oocytes, given the demonstrated lack of significant and interfering effects of these compounds on native currents, identified the *Slo* protein as the target of drug action rather than another protein in the native cell that was not expressed in the present experiments. Likewise, the lack of effects of some compounds, such as several of the putative BK openers tested, is strongly suggestive of an indirect action of these specific compounds on native BK channels (see below).

We also demonstrated that representatives of each class of effective opener increased *hSlo* currents in patches from HEK 293 cells and increased the apparent voltage sensitivity of these channels under conditions of constant free intracellular Ca^{2+}. The rank order of this effect (NS1619 > phloretin ~ niflumic acid) could be predicted from the oocyte experiments (see below), suggesting that both expression systems and experimental techniques validly assessed the effects of drugs on BK channels. The use of a second expression system demonstrated that the effects of these compounds on cloned BK channels was direct and did not involve mechanisms independent of the channel of interest, such as drug-dependent alteration of local Ca^{2+} concentrations.

We found that both ChTX and IbTX, peptides derived from scorpion toxins, were equally effective inhibitors of *hSlo*-mediated BK current, although IbTX was somewhat more potent. The pattern of interaction of ChTX was different, and it is not known whether the shallower concentration-response curve observed with ChTX represented multiple sites of interaction. IbTX was equally effective and potent on currents mediated by *mSlo* and *hSlo*. The alkaloid paxilline had

a complex effect on *hSlo* currents, reflecting at least two affinities. The EC_{50} for the high affinity interaction was approximately equal to the value obtained with IbTX, making this compound a very potent nonpeptidyl inhibitor of these channels, as reported previously for native channels (28). In this expression system, other reported inhibitors of native channels, including KTX, ketamine, and tetrandrine, were relatively or absolutely ineffective in reducing BK current. The rank order of inhibition determined from these experiments was paxilline (high affinity site) ~ IbTX ~ ChTX ≫ KTX.

A number of compounds, including the benzimidazolones, phloretin, and the Cl^- channel blockers, were found to be effective activators of these channels. When tested, these compounds had identical effects on *mSlo*- and *hSlo*-mediated currents, and representatives of these chemical series opened *hSlo* BK channels in excised patches. These latter findings were in agreement with previous studies demonstrating direct activation of isolated native and cloned BK channels by representatives of these classes of opener (20, 29, 38–43). The rank order of activation by these effective compounds was NS1619 ~ NS004 > phloretin ~ niflumic acid ~ flufenamic acid > NPPB. Other compounds, such as SCA-40, did not produce increases in BK-mediated outward current. These results may reflect (for both ineffective openers and blockers) an indirect effect on native BK channels (47) or the requirement of further molecular modification (via splice variation or subunit interactions) of the basic BK channel used in these studies (see below).

Coapplication studies, although not intended as an exhaustive survey of the range of interactions of these classes of opener and blocker, did provide some interesting and useful information. This was particularly true when considering the data from coapplication of IbTX and the openers. It had been our assumption, primarily based on our unpublished observations concerning the inability of radiolabeled IbTX to be displaced by NS004 or NS1619, that IbTX would completely block the effects of the openers. That it did not do so even at supramaximal concentrations suggests that the toxin may not be displaced by the opener but rather that its block of the channel is nevertheless reduced in its presence. The complete blockade of the effects of NS1619 by paxilline suggests that it may be more useful in studies in which the effects of a BK opener on a native system need to be completely antagonized.

In general, these results confirm findings from previous studies and extend the range of pharmacological tools available for the experimental manipulation of BK channels. Most importantly, the primary goal of this study was the characterization of the pharmacology of cloned mammalian (including human) BK channels and was an initial effort to define the molecular determinants of phenotypic variation in BK channels. A substantial literature has accumulated over the past decade describing the pharmacology of native BK channels, particularly focusing on the peptidyl BK channel inhibitors exemplified by ChTX (6, 9, 14, 26) and IbTX (21, 22, 27). This pioneering work resulted in the discovery and characterization of a number of useful and effective tools for studying the function of these channels, as well as revealing significant variation in the pharmacology of these channels as expressed in different cell populations. Of special interest is the previous finding that neuronal BK channels can be classified into at least two general classes based on their single-channel kinetic profile, the effects of channel phosphorylation, and, most relevant to this study, their response to the peptidyl channel inhibitors ChTX and IbTX (6).[2] Specifically, one class of neuronal BK channel (type II) seems to be relatively resistant to the blocking action of these peptides, although the second class is highly sensitive. Results of other studies suggest that there are a number of variant BK channels, based on their sensitivity to Ca^{2+} and other pharmacological agents (3). This phenotypic variation suggests several possibilities. BK channels could comprise a multimember family of channels similar to other K^+ channel families; alternative splice variation could underlie observed differences; post-translational modification of the channel protein(s) could be responsible for reported phenotypic variations; or association of the BK channel with additional proteins (receptors and/or regulatory proteins) could result in functional variants of BK channels. Combinations of these variables could further contribute to BK channel diversity.

The cloning and expression of *mSlo* (33) and its human homologue *hSlo* (34, 35) allowed us to use these cloned channels to begin to examine the molecular pharmacology of BK channels expressed in relative isolation. In addition, elucidation of the molecular biology of BK channels has given us some insight into which of the theoretical sources of phenotypic variation cited above may be the more likely candidates. For example, so far only a single family member has been cloned from mammalian (including human) genomic libraries, although the identification of a number of alternative splice variants in *dSlo*, *mSlo*, and *hSlo* strongly suggests a source of channel variation and may be responsible for some of the differences in Ca^{2+} sensitivity of native BK channels (31, 34, 35, 55). The discovery and partial characterization of a BK channel β-protein, an apparent regulatory protein or subunit, may also add another source of pharmacological variability (56, 57), and a recent report suggesting that the presence of the β-protein can affect the response to some pharmacological agents (58) is consistent with this possibility. Our results, based on experiments with *Slo* homologues that do not have the alternative splice exons and in the absence of heterologously expressed β-protein, confirm that the peptidyl BK inhibitors ChTX and IbTX and the nonpeptide paxilline interact directly with the Slo protein. The other proposed inhibitors, tetrandrine, ketamine, and (possibly) KTX, may not be acting directly on the BK channel or may only be effective when the channel is otherwise configured (by splice variation or additional subunits). We should also note, however, that we cannot rule out the possibility that a native regulatory protein may exist in the *Xenopus* oocyte, affecting the pharmacology of *mSlo* and *hSlo*.

One of the most novel and potentially interesting results of this study was the discovery of the biphasic interactions of some blockers and openers with BK channels. This was evident in the concentration-response relationship of the blocker paxilline and was most compelling for the opener phloretin, where similar biphasic relationships were observed when it was applied to oocytes expressing either *mSlo* or *hSlo*. Although this biphasic interaction was not seen with IbTX, biphasic concentration-response relationships were observed to a greater or lesser degree with all of the classes of

[2] P. Reinhart, personal communication.

opener (note the effects of NS1619 in paxilline; Fig. 10A). The nature of these two sites, whether they represent two separate sites of drug interaction (receptors) on the BK channel, different affinity states of the same site, or some form of post-translational modification remains to be determined. However, caution must be exercised in the interpretation of all oocyte data because the expression system can affect such factors as post-translational modification. It was not possible in the current study to generate extensive concentration-response data for these compounds in the HEK 293 cell patch-clamp experiments. Assuming that this phenomenon was not a unique result of the expression system, these observations could lead to a greater understanding of drug/channel interactions and to the determination of whether these sites are actually receptors for endogenous ligands that play a role in the regulation of native BK channel function.

In conclusion, in this study we presented the initial characterization of the molecular pharmacology of cloned BK channels and the first comprehensive study of BK channel pharmacology. We confirmed the direct effects of some putative and proven openers and inhibitors of these important channels and characterized their actions on BK currents. In addition, we revealed some important differences between the responses of some native channels and the cloned channels examined here, as exemplified by the relative lack of effects of the type II BK channel blocker tetrandrine and the putative BK opener SCA-40.

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